DIAGNOSTIC ASPECTS OF INFECTIONS WITH *CHLAMYDIA TRACHOMATIS, NEISSERIA GONORRHOEAE* AND HERPES SIMPLEX VIRUS

DIAGNOSTISCHE ASPECTEN VAN INFECTIES MET *CHLAMYDIA TRACHOMATIS, NEISSERIA GONORRHOEAE* EN HERPES SIMPLEX VIRUS

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M. Lit.
en volgens het besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 5 oktober 1994 om 11.45 uur

door

JOSINA VAN ULSEN

geboren op 16 januari 1951 te Diemen

Promotie commissie:

Promotor	:	Prof. Dr. E. Stolz
Co-promotor	:	Dr. J. Buitenwerf
Overige leden	:	Prof. Dr. M.F. Michel
	:	Prof. Dr. N. Masurel
	:	Prof. Dr. A. Meheus

The studies presented in this thesis were performed at the Department of Dermato-Venereology and the Department of Clinical Microbiology and Antimicrobial Therapy, University Hospital Rotterdam-Dijkzigt, Rotterdam, the Netherlands and at the Laboratory of Virology, Public Health Service of the City of Rotterdam, Rotterdam, The Netherlands.

Afbeelding omslag: Het herpes virus bekijkt de densitometrische analyse van de immunoblot van patient A.
Ets, 1989 door J. van Ulsen.

Het proefschrift werd gedrukt door drukkerij Mariënkroon te Nieuwkuyk

aan: mijn ouders en Bram

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
BCIP	5-bromo-4-chloro-3-indol phosphate-p-Toluidine Salt
CFT	Complement Fixation test
CMV	Cytomegalo virus
CNS	Central Nervous System
CPE	Cytopathogenic Effect
DIA	Dot Immunobinding Assay
DNA	Deoxyribonucleic Acid
EB	Elementary Body
EEM	Erythema Exudativum Multiforme
EIA, ELISA	Enzyme Linked Immunosorbent Assay
ESB	Electrophoresis Sampling Buffer
FITC	Fluorescein isothiocyanate
gG2	Glycoprotein G2
HIV	Human Immunodeficiency Virus
HPA	Helix Pomatia
HPV	Human Papilloma Virus
HSV	Herpes simplex virus
IF	Immunofluorescence
IFU	Inclusion Forming Units
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect Haemagglutination Assay
kD	Kilo Dalton
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
mD	Mega Dalton
MIF	Microimmunofluorescence
MoAbs	Monoclonal antibodies
MOMP	Major Outer-Membrane Protein
NC	Nitrocellulose
NBT	Nitroblue Tetrazolium Chloride
NPV	Predictive value of a negative test result
OPD	Ortho-phenylene diamine
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PID	Pelvic Inflamatory Disease
PPNG	Penicillinase producing gonococci
PPV	Predictive value of a positive test result
RB	Reticulate Body
RIA	Radio Immuno-Assay
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SEM	Skin, eyes and mouth
SDS	Sodium Dodecyl Sulphate
SOA	Sexueel overdraagbare aandoeningen
STD	Sexally transmitted diseases
TBS	Tris-buffered Saline
WBA	Western Blot Analysis
VZV	Varicella Zoster Virus

Contents

CHAPTER 1

INTRODUCTION

AND

OBJECTIVES

1.1 Introduction.

The sexually transmitted diseases (STDs) constitute a worldwide problem of major significance in terms of health, economic and social consequences [1].

The most important STDs are the bacterial infections syphilis (causative agent *Treponema pallidum* sub-species pallidum), gonorrhoea (causative agent *Neisseria gonorrhoeae, N. gonorrhoeae*), infections caused by *Chlamydia trachomatis* (*C. trachomatis*) and the viral infections herpes genitalis (causative agent herpes simplex virus, HSV), condylomata acuminata (causative agent human papilloma virus, HPV) and human immunodeficiency virus infections including AIDS. In addition, donovanosis (causative agent *Calymmatobacterium granulomatis*) and chancroid (causative agent *Haemophilus ducreyi*) are important in the developing countries [2-4].

The combat against STDs is aimed at disrupting the infectious cascade. Attempts have been made to achieve this via education (for instance: safe sex campaigns), contact tracing, the screening of high-risk groups and treatment of patients. It was expected that this would decrease the spread of STDs in the community and prevent the development of complications and sequela in the individual patient [5-8].

Correct diagnosis is of utmost importance. In addition to anamnesis and clinical presentation, the correct diagnosis of STDs relies on the detection of the various causative agents using the appropriate laboratory techniques. In symptomless infections such as gonorrhoea and *C. trachomatis* infections, such laboratory tests are the only means to trace the carriers. The treatment is of major epidemiological importance as it reduces the pool of clinically unsuspected, but infectious carriers.

Traditionally, for *N. gonorrhoeae*-, *C. trachomatis*- and HSV infections the culturing of the relevant pathogen used to be the cornerstone in the laboratory diagnosis [9-16]. For the remaining STDs, culturing of the causative agents is either very difficult or impossible, and therefore, the laboratory diagnosis is based on the detection of appropriate antibodies against the causative agents [17-19].

An advantage of the culture techniques is the availability of the microbes for additional investigations such as the testing for susceptibility to various antibiotics that are used in the treatment of that particular STD, the sub-typing of HSV, the

sero-typing of *C. trachomatis* and *N. gonorrhoeae*, the auxo-typing and screening for plasmids of *N. gonorrhoeae*. Such additional investigations may be valuable in understanding the clinical presentation and in deciding on the appropriate therapy. Moreover, the additional information can be used for epidemiological studies.

A major disadvantage of the culture technique is the time-period between the setting-up of the cultures and the availability of the results. This requires the patients to return at least a second time. This is not feasible in settings where the patients have to travel a long distance to receive treatment and in populations with poor compliance. Moreover, the time needed for culturing delays therapy and prolongs the period of infectivity [20]. A second disadvantage may be that the patient samples have to be transported to specialized laboratories. Despite the use of transportation media, the duration of transport and non-optimum temperature conditions during transport may adversely affect the results of the cultures [21-23].

Both these disadvantages of the culture techniques formed the basis for investigating the value of the Enzyme-Linked Immunosorbent Assay (ELISA) for the diagnosis of *C. trachomatis*-, *N. gonorrhoeae*- and HSV infections as a suitable alternative for the culture techniques. The ELISA is a very versatile method for detecting antigens and antibodies and offers the possibility for automation. It was hoped that the use of ELISA for detecting either antigens or antibodies may be useful in establishing the correct diagnosis of STDs. The latter type of ELISA is useful when insufficient antigen is available, as for instance, in the later stages of herpetic lesions and the late stage of herpes genitalis.

1.2 Objectives of the study

The objectives of the investigations described in this thesis were as follows.

- To compile a review on the clinical presentations of *C. trachomatis* infections, gonorrhoea and herpes genitalis and on the methods used for their detection.
- To investigate the diagnostic value of methods in which antigens are detected namely the non-marketed prototypes of ELISA kits for detecting *C. trachomatis* infections, gonorrhoea and herpes genitalis.
- To develop a serological method for distinguishing herpes simplex virus

(HSV) type 2 infections in the genitalia from previous HSV type 1 infections.

References.

1. Aral SO, Holmes KK, Epidemiology of sexual behaviour and sexually transmitted diseases. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted diseases, 2nd ed. New York: McGraw Hill 1990: 19-36.
2. Hart G. Donovanosis. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 273-8.
3. Ronald AR, Albritton V. Cancroid and *Haemophilus ducreyi.* In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 263-72.
4. Engelkens HJH, Stolz E. Genital ulcer disease. Int J Dermatol 1993: 32: 169-81.
5. Cates W Jr, Meheus A. Strategies for development of sexually transmitted diseases control program. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 1023-30.
6. Hart G, Adler MV, Stapinski A, Mroczkowski TF, Wiesner PJ. Evaluation of STD control programs in industrialized countries. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 1031-40.
7. Meheus A, Schulz KF, Cates W Jr. Development of prvention and control programs for sexually transmitted diseases in developing countries. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 1041-46.
8. Carter WB, Gayle TC, Baker S. Behavioral intervention and the individual. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 1069-74.
9. Ison CA. Laboratory methods in genitourinary medicine. Methods of diagnosing gonorrhoea. Genitourin Med 1990; 66: 453-9.
10. Hook EW III, Holmes KK. Gonococcal infections. Ann Intern Med 1985; 102: 229-43.
11. Barnes RC. Laboratory diagnosis of human chlamydial infections. Clin Microbiol Rev 1989; 2: 119-36.
12. Stamm WE, Mardh PA. *Chlamydia trachomatis.* In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 903-16.
13. Lefebvre J, Laperrière H, Rousseau H, Massé R. Comparison of three techniques for detection of *Chlamydia trachomatis* in endocervical specimens from asymptomatic women. J Clin Microbiol 1988; 26: 726-31.
14. Ashley RL. Laboratory techniques in the diagnosis of herpes simplex infection. Genito-urinary Med 1993: 69: 174-83.
15. Vestergaard BF. Laboratory diagnosis of herpes viruses. Scand J Infect Dis 1985; 47 (suppl): 22-32.
16. Corey L, Spear PG. Infections with herpes simplex viruses (second of two parts). New Eng J Med 1986; 314: 749-57.

17. Van der Sluis JJ. Laboratory techniques in the diagnosis of syphilis: A review. Genitourin Med 1992; 68: 413-9.
18. Carlson JR, Jennings MB. Isolation of human immunodeficiency virus. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 969-74.
19. Goldstein L. Immunodiagnosis of human immunodeficiency virus. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 975-87.
20. Stolz E, Tjiam KH, van Ulsen J, van Eijk RVW, van Joost Th. De waarde van sneldiagnostiek van sexueel overdraagbare aandoeningen, in het bijzonder van gonorroe, *Chlamydia trachomatis* infecties en herpes genitalis. Ned Tijdschr Geneesk 1985; 129: 2101-4.
21. Tjiam KH, van Heyst BYM, de Roo JC, et al. Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: diagnostic implications. Br J Vener Dis 1984; 60: 82-4.
22. Taylor E, Phillips I. Assessment of transport and isolation methods for gonococci. Br J Vener Dis 1980; 56: 390-3.
23. Rawls WE. Herpes simplex viruses: Types 1 and 2. In: Lennette H, ed. Laboratory diagnosis of viral infections, 2nd ed. New York: Marcel Dekker Inc 1992: 442-61.

CHAPTER 2

INFECTIONS WITH *CHLAMYDIA TRACHOMATIS, NEISSERIA GONORRHOEAE* AND HERPES SIMPLEX VIRUS

2.1 INFECTIONS WITH *CHLAMYDIA TRACHOMATIS*

2.1.1 Introduction

Chlamydiae are obligate intracellular bacteria with a unique growth cycle in which two forms of the Chlamydiae exist: the infectious Elementary Body (EB) which can survive outside the host cells and can infect new host cells and the Reticulate Body (RB) which multiplies intracellularly within a vacuole: the inclusion body. A part of the RB continues to multiply, whereas the larger part matures to EB. The infected host-cell ruptures after 48-72 hours yielding new extra-cellular EB [1].

Three species are distinguished among the Chlamydiae: *C. trachomatis, C. psittaci* and *C. pneumoniae. C. trachomatis* causes trachoma and STD and comprises 15 different serotypes: the serotypes A-K and Ba, LGV 1, 2 and 3 [1].

Trachoma is caused by serotypes A, B, Ba and C and is endemic in many developing countries, especially in densely populated areas where the hygienic conditions are poor. It is estimated that several hundreds of millions of people suffer from this disease and as a result, several millions have become permanently blind. The serotypes D-K cause sexually transmissible urogenital infections like urethritis or cervicitis and sometimes a mild conjunctivitis. The serotypes LGV 1-3 cause the STD lymphogranuloma inguinale [2]. The two latter diseases are reviewed below.

2.1.2 Infections with serotypes D-K in men

Infection of the male genitalia with *C. trachomatis* serotypes D-K may lead to urethritis with dysuria and urethral discharge or may follow an asymptomatic course. The chlamydial urethral infections are milder and follow an asymptomatic course more often than gonococcal urethritis. Epididymitis occurs in 1-2% of the cases if the infection is left untreated [3,4]. Men with chlamydia urethritis who have the major histocompatibility antigen HLA-B27 run a greater risk of developing Reiter's syndrome [5]. In homosexual men, proctitis caused by *C. trachomatis* was observed. Infections of the rectum with serotypes D-K are milder than those with one of the LGV serotypes [6].

2.1.3 Infections with serotypes D-K in women

The mucopurulent cervicitis in women is the counterpart of non-gonococcal urethritis in men [7]. Uncomplicated cervicitis mostly follows an asymptomatic course and dysuria occurs as a result of the infection spreading to the urethra. Endometritis, salpingitis and pelvic inflammatory disease (PID) occur as a result of ascending infection. Late complications are infertility or extra-uterine gravidity due to fibrosis and adhesions in the fallopian tubes. Perihepatitis (Fitz-Hugh-Curtis syndrome) is also associated with chlamydial infection [8, 9]. In men and women conjunctivitis is also possible via contact of genital discharge-contaminated finger with the eyes.

2.1.4 Infections with serotypes D-K in children

Neonates born from mothers with genital chlamydial infection may become infected during partus and may develop chlamydial conjunctivitis. The symptoms usually manifest between 1 and 3 weeks postnatal. The infection may have an asymptomatic course or may result in a purulent conjunctivitis of varying severity. Severe sequela (corneal pannus and cicatrisation of the conjunctivae) may arise. Chlamydial pneumonia may develop if nasopharynx and respiratory tract are infected. Prolonged cough and congestion are the major symptoms which appear between the third and the eleventh week [10-12].

2.1.5 Lymphogranuloma venereum

Lymphogranuloma venereum (LGV) is caused by serotypes L 1-3 and is endemic in Africa, India, South-East Asia, South America and the Caribbean. In the Western countries this disease has been imported from the endemic areas. The symptoms may consist of a primary lesion, usually an ulcus in the ano-genital region and is followed by an inflammation of the inguinal lymph node(s) which eventually may rupture. However, the disease may also follow an asymptomatic course. The disease has usually no serious sequela, but complications like proctocolitis, fistula formation and elephantiasis of the genitalia may occur [13]. The clinical and histopathological changes in early LGV proctocolitis and Crohn's disease are very similar [14,15]. In 2 of the 3 rectal biopsies from homosexual men with proctitis

caused by *C. trachomatis* of the LGV serotype, Quin et al observed histopathological changes which were consistent with those in Crohn's disease [16]. However, using the Polymerase Chain Reaction (PCR), chlamydia-DNA could not be demonstrated in the DNA extracts from the gut samples of 10 patients with Crohn's disease or in samples from 4 controls [17].

References

1. Schachter J. Biology of *Chlamydia trachomatis.* In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 167-80.
2. Stamm WE, Holmes KK. *Chlamydia trachomatis* infection of the adult. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 181-93.
3. Berger RE, Alexander ER, Monda GD, Ansell J, McCormick G, Holmes KK. *Chlamydia trachomatis* as a cause of acute idiopathic epidydimitis. N Eng J Med 1978; 298: 301-4.
4. Berger RE, Alexander ER, Harnisch JP, et al. Etiology, manifestations and therapy of acute epidydimitis: prospective study of 50 cases. J Urol 1979; 121: 750-754.
5. Martin DH, Pollock S, Kuo CC, Wang SP, Brunham RC, Holmes KK. *Chlamydia trachomatis* infections in men with Reiter's syndrome. Ann Intern Med 1984; 100: 207-13.
6. Quin TC, Stam WE. Proctitis, proctocolitis, enteritis ans esophagitis in homosexual men. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 663-83.
7. Brunham RC, Pavoonen J, Stevens CE, et al. Mucopurulent cervicitis- the ignored counterpart in women of urethritis in men. N Eng J Med 1984; 311: 1-6.
8. Henry-Suchet J. *Chlamydia trachomatis* infection and infertility in women. J of Reprod Med 1988; 33: 912-14.
9. Nettlemen MD, Jones RB. Cost-effectiveness of screening women at moderate risk for genital infections caused by *Chlamydia trachomatis.* JAMA 1988; 260: 207-13.
10. Schachter J, Lum L, Gooding CA, Ostler B. Pneumonitis following inclusion blennorrhea. J Pediatr 1975; 87: 779-80.
11. Beem MO, Saxon EM. Respiratory tract colonisation and a distinctive pneumonia syndrome in infants infected with *Chlamydia trachomatis.* N Eng J Med 1977; 296: 306-10.
12. Harrison HR, Alexander ER. Chlamydial infections in infants and children. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 811-20.
13. Perine PL, Osoba AO. Lymphogranuloma venereum. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 195-204.
14. Munday PE, Taylor-Robinson D. Chlamydial infection in proctitis and Crohn's disease. Br Med Bull 1983; 39: 155-8.
15. Swarbrick ET, Kingham JCC, Price HL, et al. Chlamydia, cytomegalovirus and yersinia in inflammatory bowel disease. Lancet 1979; 2: 11-12.
16. Quin TC, Goodell Se, Mkrtichian EC, et al. *Chlamydia trachomatis* proctitis. N Eng J Med 1981; 305: 195-200.
17. Mc Garity BH, Robertson DA, Clarke IN, Wright R. Deoxyribonucleic acid amplification and hybridization in Crohn's disease using a Chlamydial plasmid probe. Gut 1991; 32: 1011-5.

2.2 INFECTIONS WITH *NEISSERIA GONORRHOEAE*

2.2.1 Introduction

N. gonorrhoeae is a Gram-negative diplococcus which causes gonorrhoea. The gonococci can be distinguished from other Neisseriae using sugar fermentation tests and immunofluorescence techniques [1]. *N. gonorrhoeae* can be characterized according to its amino acid requirements (auxo-typing) [2,3] or via its reactivity with monoclonal antibodies against epitopes of the outer membrane protein (sero-typing) [4]. A relationship exists between the serotype on the one hand and the susceptibility to antibiotics and the localisation in a host on the other hand [5,6]. The presence of pili on the gonococcal surface has been related to the virulence [1,7]. *N. gonorrhoeae* may contain various plasmids of different sizes. The function of some plasmids is known, for instance β-plasmids of 2.9, 3.2 or 4.5 mD are responsible for the penicillin-resistance in the penicillinase-producing *N. gonorrhoeae* (PPNG) [8]. High level tetracycline-resistance has been ascribed to the acquisition of a streptococcal tetM determinant located on a 25.2 mD plasmid [9,10]. Decreased susceptibility to penicillin, tetracycline and cephalosporin is genetically determined [11]. It has been suggested that inadequate therapies result in a selection of less susceptible strains of gonococci. The prevalence of antibiotic-resistant strains is increasing and forms a major problem in the combat against infections with *N. gonorrhoeae* [1]. In the Western countries the prevalence of PPNG increased from 0 % in 1966 to 11 % (USA, 1991), 5 % (UK, 1990) and 12.7 % (Australia, 1986) [12-15]. In some developing countries, the prevalence of PPNG increased to 49 % (Gambia, 1989/1990) and 59 % (Zaire, 1988) [11,10]. In the Netherlands, the prevalence of PPNG increased from 9.6% in 1986 to 29.7% in 1990 [16-18]. The prevalence decreased to 12% in 1992. The prevalence for 1993 has been estimated to be 20% [van Klingeren, personal communication].

The properties of *N. gonorrhoeae* like auxo-type, sero-type and resistance to antibiotics may vary in time and geographical area [12]. This variability partially hampers the successful development of vaccines and diagnostic procedures. Effective therapy with cheap antibiotics such as penicillin and tetracycline is hindered in areas with a high prevalence of penicillin-resistant strains [10,19].

2.2.2 Infections in men

In men, urethritis anterior is the most frequent manifestation of infection with *N. gonorrhoeae*. Symptoms of urethral discharge and dysuria usually appear within two to five days after infection [12,20]. Asymptomatic infections occur less frequently in men than in women [21]. Complications may include urethritis posterior, epididymitis, prostatitis, seminal vesiculitis and infection of the Cowper's gland and Tyson's glands. Ano-rectal infections are mainly observed in homosexual men and are related to ano-genital sexual intercourse.

2.2.3 Infections in women

In women, the endocervical canal is the most common location for infection with *N. gonorrhoeae* [21]. The urethra, the periurethral glands (Skene glands), the Bartholin's glands, the rectum and the tonsils may also become colonized [12,21-23]. Uncomplicated infection with *N. gonorrhoeae* frequently follows an asymptomatic course. Individuals with asymptomatic infections may unknowingly spread the infection further in the community. Complications of *N. gonorrhoeae* infection in women are bartholinitis, acute proctitis and pelvic inflammatory disease (PID). The long term sequela of salpingitis are infertility and ectopic pregnancy.

2.2.4 Pharyngeal infections

Pharyngeal infections are less frequently observed in heterosexual men than in women and homosexual men. The most pharyngeal infections are asymptomatic and self-limiting. The role of pharyngeal infection in the transmission of *N. gonorrhoeae* to sex-partners is uncertain [12].

2.2.5 Disseminated gonococcal infection

Disseminated gonococcal infections are a complication of uro-genital gonorrhoea and occur more often in women than in men. The symptoms can be: arthritis, tenosynovitis, dermatitis or a combination of these symptoms. The skin lesions mostly consist of a necrotic pustule on an erythematous base, but other manifestations are possible. The syndrome is often under-diagnosed because of the asymptomatic character of the preceding uro-genital infection [12,24,25].

2.2.6 Maternal and perinatal infections

The rates for premature delivery, perinatal distress and still birth were higher In mothers infected with *N. gonorrhoeae* than in uninfected mothers [6].

Infection with *N. gonorrhoeae* in the newborn mostly results in conjunctivitis, but seldom in systemic infection [26].

References

1. Sparling PF. Biology of *Neisseria gonorrhoeae.* In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 131-47.
2. Catlin BW, Nutritional profiles of *Neisseria gonorrhoea, Neisseria meningitides* and *Neisseria lactamica* in chemically defined media en the use of growth requirements for gonococcal typing. J Infect Dis 1973; 128: 178-193.
3. Shinners EN, Catlin BW. Arginine and pyridimine biosynthetic defects in *Neisseria gonorrhoeae* strains isolated from patients. J Bacteriol 1982; 151: 295-302.
4. Knapp JS, Tam MR, Nowinsky RC, Holmes KK, Sanstrom EG. Serological classification of *Neisseria gonorrhoeae* with use of monoclonal antibodies to gonococcal outer membrane protein I. J Infect Dis 1984; 150: 44-47.
5. Woodfard N. Bindayana K, Easmon CSF, Ison CA. Associations between serotype and susceptibility to antibodies of *Neisseria gonorrhoeae.* Genitourin Med 1989; 65: 86-91.
6. Hook EW III, Judson FN, Handsfield HH, Ehret JM, Holmes KK, Knapp JS. Auxotype/serovar diversity and antimicrobial resistance of *Neisseria gonorrhoeae* in two mid-sized American cities. Sex Transm Dis 1987; 14: 141-6.
7. Pierce WA, Buchanan TM. Attachment role of gonococcal pili: Optimum conditions and quantitation of adherence of isolated pili to human cells in vitro. J Clin Invest 1987; 61: 931-43.
8. Perine PL, Thornsberry C, C, Schalla W, et al. Evidence for two distinct types of penicillinase producing *Neisseria gonorrhoea.* Lancet 1977; 2: 993-5.
10. Van Dijck E, Rossau R, Duhamel, et al. Antimicrobial susceptibility of *Neisseria gonorrhoeae* in Zaire: high level plasmid-mediated tetracycline resistance in Central Africa. Genitourin Med 1992; 68: 111-116.
11. Ison CA, Bindayana KM, Woodford N, Gill MJ, Easmon CSF. Penicillin and cephalosporin resistance in gonococci. Genitourin Med 1990; 66: 351-356.
12. Hook EW III, Handsfield HH. Gonococcal infections in the adult. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 149-63.
13. Gorwitz RJ, Nakashima AK, Moran JS, Knapp JS. Sentinel surveillance for antimicrobial resistance in *Neisseria gonorrhoeae* -United States, 1988-1991. MMWR 1993; 42: 29-39.
14. Warren C, Phillips I. Penicillinase producing *Neisseria gonorrhoeae* from St Thomas'Hospital 1976-1990-The first fifteen years. Genitourin Med 1993; 69; 201-7.
15. Australian gonococcal surveillance programme. Penicillin sensitivity of gonococci isolated in Australia, 1981-6. Genitourin Med 1988; 64: 147-51.
16. Van Klingeren B, Dessens-Kroon M, Verheuvel M. Surveillance van penicillinase vormende gonokokken in Nederland; incidentie en prevalentie in 1987. RIVM-rapport nr. 358004008, juni 1988.
17. Van Klingeren B, Dessens-Kroon M, Verheuvel M. Surveillance van penicillinase vormende gonokokken in Nederland; incidentie en prevalentie in 1989. RIVM-rapport nr 358004010, augustus 1990
18. Van Klingeren, Dessens-Kroon M, van Santen-Verheuvel M. Surveillance van resistentie bij gonokokken in Nederland; incidentie en prevalentie in 1991. RIVM-rapport nr 358004012, augustus 1993.
19. Kouri YH, González L, Pérez M, et al. Effect of penicillin and spectinomycin given for urethritis and cervicitis with *Neisseria gonorrhoeae*: high prevalence of penicillin-resistant isolates. Genitourin Med 1989; 65: 342-6.
20. Harrison WO, Hooper PJ, Wiener AF, et al. A trial of minocycline given after exposure to prevent gonorrhea. New Eng J Med 1979; 300: 1074-78.
21. Kallings LO, Moberg I. Epidemiology of Gonorrhoea. In: Skinner FA, Walker PD, Smith H, eds. Gonorrhoea epidemiology and pathogenesis. London: Academic press inc., 1977: 3-16.
22. Stolz E. Diagnostic aspects of Gonorrhoeae. Ph.D. Thesis, Erasmus University, Rotterdam, 1974.
23. Barlow D, Philips I. Gonorrhea in women: Diagnostic, clinical and laboratory aspects. Lancet

1978; 761-5.
24. Rice PA, Goldenberg DL. Clinical manifestations of disseminated infection caused by *Neisseria gonorrhoeae* are linked to differences in bactericidal reactivity of infecting strains. Ann Intern Med 1985; 95: 175
25. O'Brien JA, Goldenberg DL, Rice PA. Disseminated gonococcal infection: A prospective analysis of 49 patients and a review of pathophysiology and immune mechanisms. Medicine 1983; 2: 395-405.
26. Gutman LT, Wilfert CM. Gonococcal diseases in infants and children. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990: 803-10.

2.3 INFECTIONS WITH HERPES SIMPLEX VIRUS

2.3.1 Introduction

The herpes viridae comprise a large group of DNA viruses found in many species. Six among them have been isolated from humans. Two of these, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are sexually transmissible microorganisms. HSV-1 and HSV-2 have 50% homology in the genomes and the virions have many similar antigenic determinants.

Both virus types can cause ano-genital as well as oral, facial and pharyngeal infections, although HSV-1 is isolated more often from the latter three locations and HSV-2 is isolated more often from the ano-genital region.

HSV-1 is usually acquired during the first 10 years of life [1]. The large majority of the infections follow an asymptomatic course. About 10 % of the infections are accompanied by clinical manifestations. In a sero-epidemiological survey in the USA, the prevalence of HSV-1 antibodies was 25 % in whites and 70 % in blacks [2].

HSV-2 is usually acquired via sexual contact. In a study in the USA, antibodies against HSV-2 were demonstrated in 19.7 % of the white population, in 64.7 % of the black population and in less than 1 % of the children under the age of ten years from both populations [2]. No such data are available on the prevalence of these antibodies in Europe.

The virus remains present after asymptomatic or symptomatic infections. It is assumed that the virus retreats to the local ganglion and establishes a latent infection of neurons. Reactivation of latent virus causes recurrences which occur after nerve damage, trauma to the peripheral site receiving innervation from the latently infected ganglion or after immunosuppression [3].

It has been shown that virus may be shed during the symptomatic as well as the asymptomatic phases of the infections with HSV-1 and HSV-2 [3-5]. This release of virus particles may be important in the mode of transmission of especially HSV-1 as a cause of genital herpes [6]. However, it is assumed that the shedding of the virus from asymptomatic patients plays an important role in the transmission of genital herpes infections.

In the Western countries, HSV is the most common venereal cause of genital

ulcers [3]. In the developing countries, other venereal causes such as syphilis, chancroid, LGV and granuloma inguinale are more important in the etiology of genital ulcers [7]. Genital ulcers have been implicated as a risk-increasing factor for acquiring human immunodeficiency virus [8]. Since HSV-1 and HSV-2 infections may occur in the same individual, it is convenient to use the following definitions:
A first episode is the first period with clinical symptoms. This comprises a primary infection and an initial non-primary infection. A primary infection refers to an infection in a patient who has not suffered from previous HSV-1 or HSV-2 infections and therefore has no anti-HSV antibodies.
An initial non-primary infection is a first symptomatic episode at a given anatomical site in a seropositive individual who has not suffered from a previous symptomatic episode at that location.
A recurrence is a symptomatic episode at a given anatomical site at which a previous symptomatic episode with HSV had occurred [9].

2.3.2 Facial and oropharyngeal infections with herpes simplex virus

Facial and oropharyngeal infections with HSV are frequently caused by HSV-1, but seldom by HSV-2 [10]. Although these infections are not sexually transmitted, they are discussed here because of their role as a major source of genital HSV-1 infections [4-6].
The primary infection may progress asymptomatically or may cause gingivostomatitis and/or pharyngitis. Facial skin may also be affected. The prodromal symptoms are typical and consist of itching or tingling sensations about 5 days prior to the appearance of the characteristic group of vesicles on the erythematous skin. After a few days, the vesicles change into erosions and heal after 5-10 days forming a crust. The primary infection may be accompanied by malaise and fever. After the primary infection, the virus retreats to the trigeminal ganglion where it remains latent. After reactivation under the influence of among others, sunlight, menses, fever, emotional stress or over-fatigue, the infection recurs and is mostly located on the lips or in the perioral region. A recurrence of herpes labialis is mostly more local and has a less severe course than the first episode. However, sometimes the recurrent herpes infection may cover a major part of half of the

face. A disseminated infection (eczema herpeticum) may occur in patients with a pre-existing skin condition such as atopic eczema. Erythema exudativum multiforme (EEM) has been associated with herpes labialis. Recently, herpetic DNA was demonstrated in the skin biopsies obtained from lesions of patients with EEM [11,12]. Infection of the eye may lead to a herpetic keratoconjunctivitis which in serious cases may lead to ulceration and blindness.

Oropharyngeal herpes simplex virus infection in immunocompromised patients may lead to a persisting progressive ulcer, accompanied by necrosis, bleeding and severe pain. The infection may also spread to the oesophagus and the lower respiratory tract [13]. Oesophagitis may occur after activation and spreading via the vagus nerve. Spreading of herpetic tracheobronchitis may lead to pneumonia. A disseminated infection accompanied by hepatitis, pancreatitis and encephalitis may occur due to viraemia after oropharyngeal and genital herpes infection.

2.3.3 Genital herpes simplex virus infections

Most genital herpes simplex virus infections are caused by HSV-2. HSV-1 was isolated from about 20% of the cases with primary genital herpes infections and from about 2% of the cases with recurrent infections [14]. Higher percentages of HSV-1 have been isolated from primary genital lesions in females under 25 years of age: from 45 % in 1985 to 65 % in 1988. The author suggested that this can be explained by the delayed primary exposure to the virus, due to better hygienic conditions and due to the frequenter practice of cunnilingus relative to fellatio [6]. Fifty to 70% of the genital herpes infections follow an asymptomatic course. Asymptomatic herpes infections occur more frequently in women than in men [14] and are often caused by HSV-2 [15]. In the USA, genital herpes infections with HSV-2 are reaching endemic proportions. In urban areas 30-60% of the pregnant women from lower socioeconomic classes visiting an obstetric clinic possessed antibodies to HSV-2 [2-4,16-20].

The incubation period of a primary herpes genitalis is 3-7 days. The severity and the duration of primary genital HSV-1 or HSV-2 infections do not differ [21]. The first symptoms are mostly itching, redness and genital pain after which the vesicles appear on various parts of the genitalia. This is often accompanied by severe pain,

oedema, vaginal/urethral discharge, dysuria, urethritis and cystitis or cervicitis. The inguinal lymph nodes may be swollen and often there are general symptoms such as slackness, fever, headaches, muscular pain and dysuria [22,23]. After about 2 days, the vesicles burst and ulcers develop which form crusts and heal after 10-21 days.

The duration of a recurrence is usually shorter, the symptoms are less severe and the area involved is usually smaller as compared with a primary infection. Recurrences may continue to occur for many years and show a high individual variability of frequency and severity. More than 80 % of the patients with first episode of genital HSV-2 infection suffer from recurrences with an average of 4 times within the first 12 months. In contrast, 55 % of the patients with first episode of a genital HSV-1 infection suffer from a single recurrence during the same period [24]. A previous herpes labialis infection with HSV-1 partially protects against acquiring genital infection with HSV-1, but does not prevent it. Individuals with a first episode HSV-2 infection who had already suffered from a previous episode of HSV-1 infection had fewer systemic symptoms, fewer lesions and a shorter duration of illness than patients with a primary HSV-2 infection [21].

Complications of a genital HSV-1 and HSV-2 infection may be:

1. A self-limiting viral meningitis (particularly in primary herpes infection).
2. Extragenital lesions: buttocks, groins, thighs, fingers and HSV conjunctivitis (primary and recurrent).
3. Skin or visceral dissemination, thrombocytopenia, mono-arthritis and upper genitourinary tract infections in women. These complications are rare.
4. Invasion of the sacral ganglion after genital or rectal herpes infection. This may cause a repeating lumbosacral herpes infection: besides the skin lesions on the buttocks, the sacrum or the hips, a dysfunction of the autonomic nervous system may arise and may be accompanied by anaesthesia, tingling sensations, urine retention, obstipation, pleocytosis in the liquor and impotence in men.
5. In studies on the occurrence of HIV, the presence of genital ulcers, among others, was observed to be a risk-increasing factor for acquiring HIV

infection [25,26]. Vice versa, a HIV infection will worsen the course of a concomitant HSV infection.

2.3.4 Foetal and neonatal herpes infections

The foetus can be infected when the mother develops a disseminated infection. This infection may be lethal for the mother and her child [27]. In utero infection of the foetus due to an ascending genital herpes infection in the mother is unclear [28].

Infection of the neonate can occur during partus when the infant passes through the infected birth canal. HSV-2 has been isolated from 86% of the neonates with herpes neonatalis [29]. Postnatal infection can occur via handling of the neonate by an infected person. This infection is usually a HSV-1 infection. Postnatal infection caused by suckling from an infected breast has been reported [30].

Neonatal herpes infections can be divided into 3 categories according to severity:

1. Local infections of the skin, the eyes and the mouth (SEM).
2. Disseminated infection.
3. Central nervous system (CNS) encephalitis.

Most cases of herpes neonatalis infections can be recognized by the presence of skin, eye or oropharyngeal lesions [31].

The local infection has the best prognosis. The symptoms begin after 10-11 days. Groups of vesicles are observed at sites on the body that had been in direct contact with the infected birth canal. Keratoconjunctivitis or oropharyngitis may be present. Later on, 30% of these children develop neurological symptoms: spastic quadriplegia, microcephaly or blindness and in one study, neurological sequela developed in 20-30% of the children [32]. The course is rarely lethal, but without therapy 75% of the local infections would progress to disseminated infection [33].

The mortality of disseminated infections is high (> 80%). Early recognition is of life-saving importance [34]. The non-specific symptoms of disseminated HSV infection begin after an average of 9 days and include lethargy, poor appetite, occasional apnoea, hepatomegaly and persistent acidosis. In this group, 45% of the children were born premature. Thereafter, hepatitis, pneumonia, insults and thrombocytopenia develop. The child may die of presentations such as irreversible shock,

pneumonia or haemorrhagic diathesis. Skin, eye or mouth lesions are present in only 30% of the children with a disseminated HSV infection or HSV encephalitis [35]. High dilutions of serum, that were found to be optimally effective in antibody-dependent cellular cytotoxicity assays in the neonate or the mother, or high titres of HSV-neutralizing antibodies in the neonate could be related to the absence of disseminated HSV infections [36].
The symptoms of HSV encephalitis mostly begin 11-20 days after birth. The symptoms include insults, irritability, poor fluid intake, thermal instability, protruding fontanellae and pyramidal symptoms [37-39]. The mortality is 50 % [40], the survivors often have neurological damage [41,42]. A large majority of these children suffer from psycho-motoric retardation, microcephaly, hydranencephaly, spasticity, blindness or learning difficulties. The long-term prognosis with regards to the neurological sequela in children with HSV-1 encephalitis is better than those with HSV-2 encephalitis [40].

In view of the high mortality and the severe complications of neonatal herpes infection, one should be extra vigilant in identifying pregnant women at risk. First of all, these are the women with recurrent herpes genitalis, women with a partner with herpes genitalis and women who have suffered from an episode in the past. Forty percent of the neonates from women who had suffered an episode or who were suffering from primary herpes infection at the time of partus developed herpes neonatalis [32,43,44]. Retrospectively, it was shown that primary infections with HSV-2 in the mother were responsible for half of the cases of herpes neonatalis, whereas the other half of the cases were observed to be due to reactivation of earlier infections [45]. However, anamnestically a large proportion of the mothers who gave birth to children with herpes neonatalis, or their partners, were unaware of having suffered from a herpes episode [46,47]. Thus, anamnesis alone is insufficient for tracing women at risk. Therefore, a serological test in which HSV-2 specific antibodies can be detected would be valuable for tracing pregnant women at risk and their partners.
It has been shown that negative results of cultures from the cervix of pregnant women at risk which were performed either early in pregnancy or weekly during the

weeks 32-36 did not exclude the occurrence of herpes neonatalis [48]. In contrast, it was shown that women who had positive cervix cultures during pregnancy often appeared to have negative cultures during delivery [49,50]. These contradictory results hamper the decision on an effective strategy to prevent herpes neonatalis.
In the Netherlands, there is a consensus that pregnant women and their children at risk should be investigated. Samples for culture should be taken from the cervix and from sites of frequent recurrences from the woman "durante partu". Samples for culture from the infant should be taken from the nasopharynx. It is possible to obtain the results within the incubation period of approximately 4 days with the currently available culture techniques. As yet, there is no consensus on the approach that should be adopted in a neonate who is not yet ill, but in whom the culture is positive. One can begin treating the neonate prophylactically with Acyclovir or take a "wait and see" approach [51]. A major disadvantage of the procedures mentioned above is the difficulty in determining which women are at risk.

References

1. Corey L, Spear PG. Infections with herpes simplex virus (second of two parts). N Eng J Med 1986; 314: 749-57.
2. Johnson RE, Nahmias AJ, Magder LS, Lee FK, Brooks CA, Snowden CB. A seroepidemiologic survey of the prevalence of herpes simplex virus type 2 infection in the United States. N Eng J Med 1989; 321: 7-12.
3. Corey L, Holmes KK. Genital herpes simplex virus infections: current concepts in diagnosis, therapy and prevention. Ann Intern Med 1983; 98: 873-83.
4. Guinan ME, Wolinski SM, Reichman RC. Epidemiology of genital herpes simplex virus infection. Epidem Reviews 1985; 7: 127-46.
5. Mertz GJ, Schmidt O, Jourden JL. Frequency of acquisition of first-episode genital infection with herpes simplex virus from symptomatic and asymptomatic source contacts. Sex Transm Dis 1985; 12: 33-39.
6. Scoular A, Leask BGS, Carrington D. Changing trends in genital herpes due to herpes simplex virus type 1 in Glasgow 1985-88. Genitourin Med 1990; 66: 226.
7. Meheus A, Schulz KF, Cates W Jr. Development of prevention and control programs for sexually transmitted diseases in developing countries. In Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 771-802.
8. Holmberg SD, Stewart JA, Gerber AR, et al. Prior herpes simplex virus type 2 infection as a risk for HIV infection. JAMA 1988; 259: 1048-50.
9. Harger JH, Pazin GJ, Breinig MC. Current understanding of the natural history of genital herpes simplex infections. J Reprod Med 1986; 31: 365-73.
10. Scully C. Orofacial herpes simplex infections: current concepts in the epidemiology, pathogenesis, and treatment, and disorders in which the virus may be implicated. Oral Surg Oral Med Oral Pathol 1989; 68: 701-10.
11. Brice SL, Krzemien D, Weston WL, Huff JC. Detection of herpes simplex virus DNA in cutaneous lesions of erythema multiforme. J Invest Dermatol 1989; 93: 183-7.
12. Miura S, Smith CC, Burnett JW, Aurelius L. Detection of viral DNA within skin of healed recurrent herpes simplex infection and erythema multiforme lesions. J Invest Dermatol 1992; 98: 68-72.
13. Greenberg MS, Friedman H, Cohen SG, et al. A Comparative study of herpes simplex infections in renal transplant and leukemic patients. J Infect Dis 1987; 156: 280-7.
14. Mindel A, Coker DM, Faherty A, Williams P. Recurrent genital herpes, clinical and virological features in man and woman. Genitourin Med 1988; 64: 103-6.
15. Kawana T, Kawagoe K, Takizawa K, Chen JT, Kawaguchi T, Sakamoto S. Clinical and virological studies on female genital herpes. Obstet Gynecol 1982; 60: 456-61.
16. Becker TM, Stone KM, Cates W jr. Epidemiology of genital herpes infections in the United States. J Reprod Med 1986; 31: 359-64.
17. Chuang T-Y, Daniel Su WP, Perry HO, Ilstrup DM, Kurland LT. Incidence and trend of herpes progenitalis. Mayo Clin Proc 1983; 58: 436-41.
18. Stravraky KM, Rawls WE, Chiavetta J, Donner AP, Wanklin JM. Sexual and socioeconomic factors affecting the risk of past infections with herpes simplex virus type 2. Am J Epidemiol 1983; 118: 108-121.
19. Becker TM, Blount JH, Guinan ME. Genital herpes in private practice in the United States, 1966-1981. JAMA 1985; 253: 1601-3.
20. Kaufman RH. Clinical features of herpes genitalis. J Reprod Med 1986; 31: 379-83.
21. Corey L, Adams HG, Brown ZA, Holmes KK. Genital herpes simplex virus infections: clinical manifestations, course, and complications. Ann Intern Med 1983; 98: 959-72.
22. Mertz GJ, Coombs RW, Ashly R, et al. Transmission of genital herpes in couples with one symptomatic and one asymptomatic partner: a prospective study. J Inf Dis 1988; 157: 1169-77.
23. Bryson YJ, Dillon M, Lovett M, et al. Treatment of first episodes of genital herpes simplex virus infections with oral acyclovir: a randomized double blind controlled trial in normal subjects. N Eng J Med 1982; 308: 916-21.

24. Reeves WC, Corey L, Adams HG, Vontver LA, Holmes KK. Risk of recurrence after first episodes of genital herpes. Relation to herpes simplex virus type and antibody response. N Engl J Med 1981; 305: 315-9.
25. Simonsen JN, Cameron DW, Gakinya MN, et al. Human immunodeficiency virus infection among men with sexually transmitted diseases. N Eng J Med 1988; 319: 274-8.
26. Stamm WE, Handsfield HH, Rompalo AM, Ashley RL, Roberts PL, Corey L. The association between genital ulcer disease and acquisition of HIV infection in homosexual men. JAMA 1988; 260: 1429-33.
27. Stagno S, Whitly RJ. Herpesvirus infections of pregnancy part 2: herpes simplex virus and varicella zoster virus infections. N Eng J Med 1985; 313: 1327-9.
28. Robb JA, Benirschke K, Barmeyer R. Intrauterine latent herpes simplex virus infection: 1. Spontaneous abortion. Hum Pathol 1986; 17: 1196-1209.
29. Brunham RC, Holmes KK, Embree JE. Sexually transmitted diseases in pregnancy. In Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 771-802.
30. Sullivan-Bolay JZ, Fite KH, Jacobs RF, et al. Disseminated neonatal HSV-1 infection from a maternal breast lesion. Pediatrics 1983; 71: 455-7
31. Whitly RJ, Nahmias AJ, Visintine AM, Fleming CL, Alford CA. The natural history of herpes simplex virus infection of mother and newborn. Pediatrics 1980; 66: 489-94.
32. Arvin AM. Antiviral treatment of herpes simplex infection in neonates and pregnant women. J Am Acad Dermatol 1988; 18: 200-3.
33. Whitley RJ. Neonatal Herpes simplex virus infections. J Reprod Med 1986; 31: 426-32.
34. Whitly RJ, Corey L., Arvin A, et al. Changing presentation of herpes simplex virus infection in neonates. J. Inf Dis 1988; 158: 109-16.
35. Arvin AM, Yeager AS, Bruhn FW, Grossman M. Neonatal herpes simplex infections in the absence of mucocutaneous lesions. J Pediatr 1982; 100: 715-21.
36. Kohl S, West MS, Prober CG, Sullender WM, Loo LS, Arvin AM. Neonatal antibody dependent cellular cytotoxic antibody levels are associated with the clinical presentation of neonatal herpes simplex virus infection. J Inf Dis 1989; 160: 770-6.
37. Golden SE. Neonatal herpes simplex viraemia. Pediatr Infect Dis 1988; 7: 425-6.
38. Koskiniemi M, Happonen J-M, Jarvenpaa A-L, Pettay O, Vaheri A. Neonatal herpes simplex virus infection: a report of 43 patients. Pediatr Inf Dis 1988; 8: 30-35.
39. Brown ZA, Berry S, Vontver L. Genital herpes simplex virus infections complicating pregnancy. J Reprod Med 1986; 31: 420-425.
40. Gutman LT, Wilfert CM, Eppes S. Herpes simplex virus encephalitis in children: Analysis of cerebrospinal fluid and progressive neuro-development deterioration. J Inf Dis 1986; 154: 415-21.
41. Whitly RJ, Corey L, Arvin A, et al. Changing presentation of herpes simplex virus infection in neonates. J Inf Dis 1988; 158: 109-16.
42. Whitly RJ and the NIAID Collaborative Antiviral Study Group. Interim summary of mortality in herpes simplex encephalitis and neonatal herpes simplex virus infections: vidarabine versus acyclovir. J Antimicr Chemother 1983; 12 suppl 13: 105-12.
43. Gibbs RS. Infection control of herpes simplex virus infections in obstetrics and gynaecology. J Reprod Med 1986; 31: 395-8.
44. Feder HM. Disseminated herpes simplex infection in a neonate during prophylaxis with vidarabine. JAMA 1988; 259: 1054-5.
45. Kulhanjian JA, Soroush V, Au DS, et al. Identification of women at unsuspected risk of primary infection with herpes simplex virus type 2 during pregnancy. N Eng J Med 1992; 326: 916-20.
46. Sullivan-Bolay JS, Hull HF, Wilson C, Corey L. Neonatal herpes simplex virus infection in King County, Washington. JAMA 1983; 250: 3059-62.
47. Prober CG, Hensleigh PA, Boucher FD, Yasukawa LL, Au DS, Arvin AM. Use of routine viral cultures at delivery to identify neonates exposed to herpes simplex virus. N Eng J Med 1988; 318: 887-91.
48. Woolley PD, Monteiro E, Wilson J. Kinghorn GR. Virological screening in late pregnancy in

recurrent genital herpes. Lancet 1986; 6: 1336.
49. Hankins GDV, Cunningham F, Luby JP, Butler SL, Stroud J, Roark M. Asymptomatic genital excretion of herpes simplex virus during early labor. Am J Obstet Gynecol 1984; 150: 100-1.
50. Scher J, Bottone E, Desmond E, Simons W. The incidence and outcome of asymptomatic herpes simplex genitalis in an obstetric population. Am J Obstet Gynecol 1982; 144: 906-9.
51. Van der Meijden WI, Dumas AM. Consensus preventie van herpes neonatorum. Ned Tijdschr Geneeskd 1987; 131: 2030-4.

CHAPTER 3

LABORATORY TECHNIQUES IN THE DIAGNOSIS OF INFECTIONS WITH *CHLAMYDIA TRACHOMATIS, NEISSERIA GONORRHOEAE* AND HERPES SIMPLEX VIRUS

3.1 Introduction

In general, the laboratory diagnosis of infectious diseases is based on the demonstration of the relevant microbes or parts thereof and/or the demonstration of specific antibodies in patient samples by means of serological tests. This is also true for the STD under investigation. The procedures for demonstrating the relevant microbes or their antigenic parts in patient samples, either directly or after culture are classified under antigen detecting techniques. In addition, newer diagnostic procedures which rely on the demonstration of relevant microbial DNA or rRNA are currently available. The diagnostic techniques which rely on the demonstration of specific antibodies are classified under antibody detecting techniques. The current diagnostic procedures are not discussed here as these have been reviewed extensively elsewhere [1-8]. This Introduction will focus on the test procedures that have been recently introduced for the diagnosis of the three diseases under investigation i.e. the procedures based on the ELISA methodology and those based on the molecular biology techniques.

New diagnostic tests for diagnosis must fulfil several requirements. These have been reviewed by several authors [9-11]. One of these requirements is the evaluation of a new test by comparison with the "gold standard". As already mentioned in Chapter 1, culturing of the relevant microbes is the cornerstone of the laboratory diagnosis of infections with *C. trachomatis*, *N. gonorrhoeae* and HSV. Culturing of these microbes is currently used as the "gold standard". The latter can be described as one of the current test, that is generally accepted as being the best test available to distinguish between individuals with or without a certain characteristic feature. Two important features of diagnostic tests follow from this comparison: the sensitivity and the specificity. The sensitivity of a (new) test is the ratio between the individuals in whom this test is positive and all those in whom the "gold standard" is positive. The specificity of a (new) test is the ratio between the individuals in whom this test is negative and all those in whom the "gold standard" is negative.

An ideal test should have high sensitivity and specificity. In addition, such a test should be suitable for monitoring the effectiveness of treatment. The results of such

a test after adequate therapy should be negative to allow clear-cut diagnosis of re-infection. Such an ideal test does not exist. Therefore, it should be realized that the suitability of new tests would depend on the purpose for which it will be used. Two main purposes can be distinguished: the screening for disease in large populations and the clinical diagnosis. An important difference between both target groups is the prevalence of the disease. This is low in the population to be screened and higher in the case of clinical diagnosis. The prevalence affects the likelihood that a positive test result really indicates a truly positive individual (with disease) as well as the likelihood that a negative test result indicates a truly negative individual (without disease). Two additional parameters which indicate the probability of the correctness of a test result are the predictive value of a positive test result (PPV) and the predictive value of a negative test result (NPV). The PPV is the ratio between the number of individuals with a positive test result and the total number of individuals with the disease. The NPV is the ratio between the number of individuals with a negative test result and the total number of individuals without the disease. The PPV increases with increasing prevalence while the NPV decreases and vice versa. For screening, it is important not to miss individuals who are truly infected. Therefore, high emphasis is placed on the sensitivity of the test and on a high NPV. For clinical diagnosis, high emphasis is placed on a high specificity of the test and on a high PPV.

In addition to these theoretical considerations, technical and economic aspects also play a role. These include technical simplicity, rapidity, the ease of performing IgM-based diagnostic tests, suitability for automation and financial costs. When testing for infections with *C. trachomatis, N. gonorrhoeae* and HSV, the population to be tested (people attending the outpatient department for sexually transmitted diseases) has a high prevalence for these diseases. Here, the emphasis is placed on a high specificity and a high PPV.

3.1.1 ELISA technique

The laboratory diagnosis of infectious diseases is usually based on the demonstration of the causative agent and/or specific antibodies directed against (parts of) this agent. The latter is achieved via the reaction between the antigen and the

antibody. Such reactions require indicator systems to detect reactivity. These systems include complicated, vulnerable biological systems (i.e. the complement fixation reaction, passive haemagglutination) and fluorescent techniques in which the results have to be read using an appropriate microscope, but have the drawback that the readings are subjective. The formation of a visible precipitate in the antigen-antibody reactions which are performed in solution requires equivalent amounts of reactants. Deviations from equivalence can lead to false-negative reactions due to the absence of precipitate formation (i.e. prozone phenomenon). These problems can be circumvented by performing the antigen-antibody reaction on the surface of a solid phase to which one of the reactants has been adsorbed. The second reactant becomes attached to the solid phase via its reaction with the adsorbed reactant. After removal of the unbound material, the presence of the second reactant can be demonstrated by a labelled-detector which is specifically directed against the second reactant. A solid phase was used first in the solid phase radioimmunoassay. However, the use of radio labels required elaborate precautions, expensive appliances and appropriate measures for radioactive waste disposal. The substitution of radio-labelled compounds by enzyme-labelled reactants and chromogenic enzyme substrates led to the development of the Enzyme-Linked Immunosorbent Assay [12]. The conjugates retain their immunological and enzymatical activity after coupling of enzymes to proteins [13,14]. In this technique, there is no need for the above mentioned precautions which has undoubtedly contributed to its wide-spread use.

Several types of materials (polyvinyl, polystyrene, nitrocellulose and nylon) are suitable for use as a solid phase, because of their capacity to passively adsorb reactants. Most proteins including antibodies can be passively adsorbed to such solid phases. The nature of this binding is poorly understood. Hydrophobic and ionic interactions may be involved [15]. Antigens that have a poor binding capacity can be bound indirectly via chemical covalent binding to easily adsorbed proteins such as bovine serum albumin or high molecular weight polymers like polylysine or polyglutaraldehyde [16].

Especially, the plastics can be used in several forms such as microtitre plates, tubes, beads and micro-particles [17]. The choice of the material and form depends

on the type of the reaction, the financial costs and the possibility for automation. Membranes have a high functional surface-capacity. Nitrocellulose membranes have been used in dot-blot ELISA and nylon membranes for flow-through sample application in enzymeimmunofiltration assays. Microtitre plates and polystyrene beads are especially amenable for automation in standardized robotic systems.

Free binding sites on the solid phase that remain after coating may produce high background staining by non-specific binding of one or more of the reactants. Such free sites must be blocked with an indifferent protein. Bovine serum albumin, casein, gelatin and non-fat dried milk have been used.

The final signal in the ELISA is produced by an enzyme-substrate combination. The choice of a proper enzyme-substrate combination is dictated by the possibility to visualize the reaction via a change in colour. Frequently used enzyme-substrate combinations are alkaline phosphatase with *p*-nitrophenyl phosphate or horse radish peroxidase with *o*-phenylenediamine. More recently, several signal-amplifying alternatives such as the biotin-strepavidin system have been developed [15]. The colour change can be read by the naked eye or measured using a spectrophotometer. The intensity of the colour is a measure for the amount of antibodies present in the patient sample.

The possibility to passively adsorb antibodies and antigens to the solid phase and a high flexibility in the manner of performance renders the ELISA technique suitable for application in the field of infectious diseases.

Three types of ELISAs have been used for the detection of antibodies in patients.

In the indirect or sandwich ELISA, antigen is coated onto the solid phase. The antibodies in the patient sample binds to the coated antigen. Anti-human antibodies that are labelled with an enzyme are added and allowed to bind to the antibodies of the patient. Finally, the substrate for the enzyme is added and the enzyme reaction after having proceeded during a fixed time is stopped and the intensity of the colour developed measured. This ELISA principle can also be used for detecting antibodies in the sera from patients, using antigens which have been blotted or spotted onto nitrocellulose strips. However, here the soluble colourless substrate (NBT/BCIP) is converted into a purple precipitate resulting in a purple dot. The reaction is read by the naked eye or measured using a densitometer.

In the competitive ELISA, enzyme-labelled antibodies specific for the antigen are incubated with the patient sample in wells of a microtitre plate coated with the antigen. The antibodies in the patient sample, if present, will compete with the enzyme-labelled antibodies for binding to the antigen. This results in a reduction of the colour in comparison with a negative control sample. The reduction in colour is a measure of the amount of antibodies present in the samples of the patients.

The antibody capture ELISA can be used to identify antibodies of different immunoglobulin classes, for instance IgM. In this ELISA, the solid phase is coated with affinity purified antibodies specific for a class of human immunoglobulins. These immunoglobulins are captured from the added patient sample by the coated antibodies. After the removal of unbound material, antigen is added and will be bound by the captured antibodies if they are present. The enzyme which produces the final signal may be bound directly to the antigen or to additional antibodies specific for the antigen [15].

The ELISA methods for detecting antigen comprise the capture ELISA, the dot-spot ELISA and the competition ELISA.

In the capture ELISA, the antigen to be detected is captured from the patient sample added to wells of a microtitre plate or polystyrene beads coated with polyclonal or monoclonal antibodies. After washing, a second antibody specific for the antigen and labelled with an enzyme is added. The developed colour after incubation with the substrate can be measured .

In the dot-spot ELISA, the patient sample is passively adsorbed onto a nitrocellulose membrane. In the direct version of this test, the antigen, if present, is detected by overlaying the spot with a specific enzyme-labelled antibody. In the indirect version the spots are first overlaid with unlabelled antibody specifically directed against the antigen, followed by an incubation with enzyme-labelled antibody, specifically directed against the first antibody. The reading of the reaction is the same as mentioned above for the dot-spot ELISA for the detection of antibodies.

In the competition ELISA, antigen-enzyme complexes compete with the antigen in the patient sample for the binding by specific antibodies coated onto the solid phase. This results in a decrease in the absorbance as compared with negative controls, measured using a spectrophotometer. In this method, a large amount of

purified antigen is required and therefore it is not routinely used for testing for STDs. However, the production of large amounts of antigen is possible with the currently available recombinant DNA technology.

3.1.2 Molecular biology techniques

New molecular biology techniques have been used in the diagnosis of STDs. Strands of DNA of interest, together with a cloning vector DNA are cleaved with the same restriction endonuclease. This enzyme produces complementary single stranded ends on the foreign DNA and the vector DNA. These ends anneal and are ligated with a DNA ligase. The recombinant molecule is inserted into the appropriate host usually E. coli strain K 12, by transfection or micro-injection. The vector should have a selectable marker gene (for instance tetracycline resistance) to distinguish between transformed and untransformed host cells. The host cell should preferably be easy to culture and rapidly growing for a rapid multiplication of the DNA of interest.

The newly synthesized DNA can be used as probes for the hybridization technique and for sequence analysis. The knowledge of the sequence can be used for the synthesis of primers and probes for use in the polymerase chain reaction (PCR).

Large quantities of the protein is produced when the corresponding gene is expressed in the host cell. This is of special interest for the production of antigens of microbes such as *T. pallidum* and human papilloma virus (HPV), which cannot be cultured on artificial media, antigens from microbes such as *M. tuberculosis* with very low growth rate or when the purification of the antigen is very laborious or has a very low yield. For instance, Glycoprotein G for detection of herpes simplex virus type-specific antibodies is expressed in recombinant-baculovirus-infected insect cells or in *E. coli* [18,19].

Hybridization technique

In 1977, the HPV genome was cloned in bacterial plasmid vector and available for mapping and typing of HPV from various anatomical sites using hybridization [20]. This is done with homogenized tissue biopsies using Southern blotting or dot blotting. In Southern blotting, the purified DNA from patient samples is first

electrophoresed in a gel and then blotted onto nitrocellulose. In the dot blot technique, the DNA is directly spotted onto a nylon or nitrocellulose filter. The DNA on the filter is probed with ^{32}P-labelled cloned HPV-DNA. The results are visualized by autoradiography [21]. In the in situ hybridization technique, viral DNA or RNA can be detected in specific cells in tissue sections by using labelled DNA-probes [22-24]. Since then, these techniques have also been used for diagnosis of other STDs and are described further in the appropriate sections of this chapter.

The polymerase chain reaction

The PCR is used for amplification of selected DNA sequences from selected material to enable detection with probes. Using this technique, small amounts of specific DNA can be detected. The method requires a knowledge of the nucleotide sequences flanking the target DNA in order to generate oligonucleotide primers which mediate cycles of DNA synthesis. After denaturation of the target DNA and annealing of the primers, template-directed incorporation of deoxynucleoside triphosphate occurs after the addition of the heat-stable DNA polymerase, Taq 1. The newly synthesized DNA strands may serve as templates themselves and repeated cycles of denaturation, primer annealing, and extension result in many million-fold increase in the number of copies of the region flanked by the primers. Generally 25-40 cycles are performed within three to four hours using an automated thermal cycling device. The amplified DNA in its single stranded form is detected by hybridization with the appropriately labelled probes [25].

A critical feature of the PCR is the specific annealing of the oligonucleotide primers to their complementary target DNA sequence and their subsequent extension. The design of primers of appropriate sequence is therefore, of paramount importance for successful amplification. The specificity may be a problem when the target DNA is present in a sample that also contains many other closely related DNA sequences. This may be overcome using "nested" primers in a second reaction tube. These match sequences just inside the two original primer sites. Thus "nested" amplification of a product in the PCR depends on the accurate recognition by the four independent oligonucleotides [26].

The PCR also has some other disadvantages. One problem is the ease with which

spurious amplification products may arise due to contamination from extraneous sources. This may be overcome by the use of closed systems. In addition, the number of pathogens cannot be quantified accurately using PCR. For instance, for diagnosis of an acute infection caused by Cytomegalovirus (CMV) or Epstein Barr virus, accurate quantification is desirable because the virus is present in small amounts in sero-positive but asymptomatic individuals. This is in contrast to the PCR for HIV in which the detection of even one viral particle is important [27].

References

1. Barnes RC. Laboratory Diagnosis of human chlamydial infections. Clin Microbiol Rev 1989; 2:119-36.
2. Taylor-Robinson D, Thomas BJ. Laboratory techniques for the diagnosis of chlamydial infections. Genitourin Med 1991; 67:256-66.
3. Mårdh PA, Danielsson D. Neisseria gonorrhoeae. In Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 903-16.
4. Fife KH, Corey L. Herpes simplex virus. In Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill 1990: 941-52.
5. Corey L, Holmes KK, Genital herpes simplex infections: current concepts in diagnosis, therapy and prevention. Ann Intern Med 1983; 98: 973-83.
6. Vestergaard BF. Laboratory diagnosis of herpes simplex viruses. Scand J Infect Dis 1985 suppl; 47: 22-32.
7. Ashley RL. Laboratory techniques in the diagnosis of herpes simplex infection. Genitourin Med 1993; 69: 174-83.
8. Ison CA. Laboratory methods in genitourinary medicine. Methods of diagnosing gonorrhoea. Genitourin Med 1990; 66: 453-9.
9. Stolz E, Tjiam KH, van Ulsen J, van Eyk RVW and van Joost T. De waarde van sneldiagnostiek van sexueel overdraagbare aandoeningen, in het bijzonder van gonorroe, Chlamydia trachomatis-infecties en herpes genitalis. Ned Tijdschr Geneesk 1985; 129: 2101-04.
10. Griffiths DAT, Ruitenberg EJ. Preventive screening of adults: an evaluation of methods and programmes. Report of the select committee of experts of the council of Europe. Maisonneuve, Paris 1988.
11. IJsselmuiden OE. Development and evaluation of modern enzyme immunoassays for comprehensive syphilis serology. Ph.D. Thesis, Erasmus University, Rotterdam, 1989.
12. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 1971; 8: 871-4.
13. Avrameas S. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry 1969; 6: 43-52.
14. Nakane PK, Kawaoi A. Peroxidase-labelled antibody. A new method of conjugation. J Histochem Cytochem 1974; 22: 1084-91.
15. Herbrink P, Noorduyn A, van Dijk WC. Introduction to enzyme immuno assay. In Bullock G, Warhol M, van Velzen D, Herbrink P, eds. Techniques in Diagnostic Pathology, vol 2: ELISA techniques. Academic Press London 1991; 1-21.
16. Avrameas S, Ternijnck T. The cros-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 1969; 6: 53-66.
17. Tarcha PJ. The chemical properties of solid phases and their interaction with proteins. In Butler JE, ed. Immunochemistry of solid-phase immunoassay. Boca Raton. CRC Press Inc 1991:27-46.
18. Parkes DL, Smith CM, Rose JM, Brandis J, Coates SR. Sero-reactive recombinant herpes simplex virus type 2-specific Glycoprotein G. J Clin Microbiol 1991; 29: 778-81.
19. Sánchez-Martínez D, Pellett PE. Expression of HSV-1 and HSV-2 glycoprotein G in insect cells by using a novel baculovirus expression vector. Virology 1991; 1991: 229-38.
20. Gissman L, Pfister H, zur Hausen H. Human papilloma virus (HPV): characterization of 4 different isolates. Virology 1977; 76: 569-580.
21. De Villiers EM. Laboratory techniques in the investigation of human papilloma virus infection. Genitourin Med 1992; 68: 50-54.
22. Grussendorf EI, zur Hausen H. Localisation of viral DNA-replication in sections of human warts by nucleic acid hybridization with complementary RNA of human papilloma virus type I. Arch Dermatol Res 1979; 264: 55-63.
23. Grussendorf-Conen EI, Ikenberg H, Gissman L. Demonstration of HPV-16 genomes in the nuclei of cervix carcinoma cells. Dermatologica 1985; 170: 199-201.
24. Beckman AM, Myerson D, Daling JR, Kiviat N, Fenoglio CM, Mc Dougall JK. Detection and

localisation of human papilloma virus DNA in human genital condylomas by in situ hybridization with biotinylated probes. J Med Virol 1985; 16: 265-273.
25. Rapley R, Theophilus BDM, Bevan IS, Walker MR. Fundamentals of the polymerase chain reaction: a future in clinical diagnostics? Med Lab Sciences 1992; 49: 119-128.
26. Markham AF. The polymerase chain reaction: a tool for molecular medicine. Brit Med J 1993; 306: 441-6.
27. Shibati D. PCR diagnostics of herpes virus group infections. Ann Med 1992; 24: 221-4.

3.2 LABORATORY TECHNIQUES IN THE DIAGNOSIS OF INFECTIONS WITH *CHLAMYDIA TRACHOMATIS*

3.2.1 Antigen detecting techniques

Direct identification of Chlamydia in infected secretions

Direct identification is performed microscopically using stained smears of patient material on microscopic slides [1]. Initially, the smears, which usually contained low numbers of inclusion bodies and large numbers of elementary bodies (EBs), were observed after staining for inclusion bodies with iodine or Giemsa stain. However, the sensitivity of the method was very low because of the low number of inclusion bodies. Evaluation of the smears by an indirect immunofluorescent technique using polyclonal antibodies was unsatisfactory because of the high background staining. The use of monoclonal antibodies (MoAbs) prevents the background staining and allows a reliable identification of EBs in smears of infected secretions. Two MoAbs have been used thus far. The MoAb against the genus-specific lipopolysaccharide (LPS) epitope also reacts with *Staphylococcus aureus* protein A and with antigenic determinants of Gram-negative bacteria [2,3]. The Moab against the species-specific major outer-membrane protein (MOMP) reacts with the EBs. The anti-MOMP MoAbs may cross-react with other bacteria. This is a serious drawback for investigating samples obtained from the rectum. However, samples obtained from other parts of the body are less contaminated and the method can be used successfully [4-6].

The results of 24 studies in which the anti-MOMP MoAbs were used were analyzed in a review [7]. The cut-off point for a positive test result varied from 1 to 10 EBs per preparation. The specificity decreased when the cut-off point was low, but the sensitivity did not necessarily increase. However, adequate sampling is very important: for cervical samples, containing up to 5 endocervical cells, the sensitivity was 70 %, for cervical samples containing more than 5 endocervical cells the sensitivity was 92 %. The overall sensitivity observed for endocervical samples varied from 56 % to 100 %. The overall sensitivity for samples obtained from urethra of men varied from 49 % to 100 %.

The advantages of this method are that the quality of sampling can be evaluated, the sensitivity is not reduced by transportation and the test is easy to perform.

However, the microscopic evaluation of the preparations requires a certain amount of experience and the reading of a large number of preparations is tedious.

Culture of *Chlamydia trachomatis*

In 1957, *C. trachomatis* was cultured for the first time on the yolk-sacs of chick embryos using samples from the eyes of a patient with trachoma [8]. In 1965, Gordon and Quan were the first to describe a method for culturing Chlamydia on monolayers of continuous cell lines [9]. Nowadays, the most commonly used cell lines are the McCoy and HeLa 229 cell lines for culturing Chlamydia using samples from urethra, cervix, rectum, oropharynx and the eye. The presence of Chlamydia in the inoculum is determined by the formation of inclusion bodies within the cultured cells. Their formation is stimulated by centrifuging the inoculum onto the monolayers [10], by including anti-metabolites (cycloheximide, iododeoxyuridine and cytochalasine B) to promote the formation of giant cells and by pretreating cells with a polycation (DEAE dextran) to reduce their negative surface charge. The efficiency of culturing can be improved further by adding an initially infected, but disrupted monolayer to a fresh monolayer (blind passage). The presence of inclusion bodies is read microscopically after staining the fixed monolayers with iodine or Giemsa. The sensitivity of culturing is increased even more by staining the infected monolayers with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody [11]. Culturing *C. trachomatis* is cumbersome and time-consuming (72 h). The exact sensitivity of culturing on cell lines is unknown. The sensitivity was estimated to be ± 77% after repeatedly culturing a few samples from the cervix [12]. In 1984, Schachter estimated the sensitivity of culturing a single specimen to be between 70% and 80% in women with cervical infection and to be 90% in men with urethritis [13]. The specificity of culturing is high because of the characteristic morphology of the inclusion bodies in the infected cells. Efficient culturing of Chlamydia is hampered because of loss of infectious material when samples from the patients have to be transported under non-optimum conditions [14].

Antigen detection by ELISA methods

From the various commercially available kits, two have been extensively evaluated.

In the Chlamydiazyme test kit (Abbott Diagnostics, USA), samples from the patients are treated with detergents to extract the chlamydial genus-specific LPS antigen. Polystyrene beads are added to the extract which results in the binding of the LPS to the beads. After rinsing, the beads are incubated with a polyvalent rabbit anti-LPS antiserum. The beads are incubated with a peroxidase labelled anti-rabbit immunoglobulin conjugate after washing. Subsequently, after rinsing, the beads are incubated with the appropriate substrate for the peroxidase and the absorbance is read spectrophotometrically.

In the IDEIA (Boots-Celltech Diagnostics, UK), the wells of a 96-well polystyrene microtitre plate are coated with anti-LPS monoclonal antibody. The LPS-antigen extracted from the samples of the patients as described for the previous test is added to the wells. The antigen, if present, is captured by the coated anti-LPS antibodies. The presence of antigen is detected in a manner similar to that described for the previous test.

A third test, the Test Pack Chlamydia (Abbott laboratories, USA) is a commercially available ELISA test based on the capture principle. Polyclonal antibodies against chlamydial antigens are coated onto the solid phase and are used to capture chlamydial antigens from the samples of the patients. The antigen, if present is detected with a detector system in which polyclonal antibodies are used. This test is simpler to execute than all the other tests. The results are obtained in 30 min and its execution does not require any special expensive equipment (e.g. spectrophotometer) which makes it suitable for use in small laboratories. Although in an initial study [15], a sensitivity of 76% and a specificity of 99.5% were observed, the test still requires further evaluation.

In Chapters 5 and 6 the performance of the ELISA methods is evaluated further.

3.2.2 Nucleic acid detecting methods

Recently, a non-radioactive nucleic acid hybridization assay (PACE 2 assay, Gen-Probe, San Diego, USA) has appeared on the market. In this assay, *C. trachomatis* rRNA from patient samples is solubilized in a special buffer and is subsequently detected using the chemiluminescence-labelled probe. The results in women were comparable with those of cultures on monolayers. In two studies, the sensitivity and

the specificity of the modified version (PACE 2) were observed to be 95.2% and 98.2% [16] and 93% and 98% respectively [17]. In one study, the sensitivity in men was observed to be 77.2%. It was suggested that this lower sensitivity could have been due to a more cumbersome sampling technique in men [16].

In the polymerase chain reaction (PCR), a primer that is common for *C. trachomatis* plasmid [18-21] or common for rRNA [19,22] are mostly used. Initially, there was concern regarding the specificity because of the risk of contamination with DNA from the laboratory [23]. This was overcome by using a closed system. In 228 clinical samples, Ostergaard observed that in comparison with culture, the PCR had a sensitivity of 100% and a specificity of 93 %. By comparing the PCR results with the combined results of culture and IDEIA (Boots-celltech, UK) to identify the *C. trachomatis* infected patients, the PCR had a specificity of 99% [20]. In 216 men with non-gonococcal urethritis, Ratti observed a sensitivity of 100% and a specificity of 96.8% as compared with culture. The sensitivity of culture in comparison with the PCR was 92.4% [18]. Bobo used an enzyme immunoassay (PCR-EIA) to demonstrate amplified DNA. One hundred and four cervical samples were investigated with PCR-EIA and culture. Forty-six of these 104 cervical samples were positive both in PCR-EIA and in culture. An additional 2 samples were observed to be positive in the PCR-EIA [22]. From the 168 samples from the patients (62 men and 106 women), Näher observed 30 samples to be positive both in the PCR and in culture. An additional 16 samples were observed to be positive in the PCR. On dilutions of suspensions of *C. trachomatis* laboratory strains, the PCR appeared to be a hundred times more sensitive than culture [21]. In the studies mentioned above, the sensitivity of the PCR was invariably 100 %. The specificity of the PCR in these studies was lower than the specificity of the culture. This indicates that the culture technique is not able to detect all individuals carrying *C. trachomatis.* This is supported by the results of the additional tests that were performed. This challenges the role of culture as the "gold standard". The question still remains whether such a sensitive test would not detect residual DNA from dead microorganisms and would not remain positive for prolonged periods after the patient has received adequate antibiotic treatment. However, in follow-up studies, Claas et al [19] and Ostergaard et al [24] observed no residual chlamydia-DNA

using PCR after adequate antibiotic treatment. This demonstrated that the PCR was suitable for monitoring the effectiveness of therapy.
In conclusion, PCR is more sensitive than culture and also has a high specificity when appropriate precautions to exclude contamination are taken.

3.2.3 Antibody detecting techniques

Three test have been developed for the detection of anti-Chlamydia antibodies. These are Complement Fixation Test (CFT), Immunofluorescence Test (IF) and Enzyme-linked Immunosorbent Assay (ELISA).

Complement Fixation Test

Genus-specific chlamydial LPS antigen that is produced in embryonated hen eggs is used as an antigen in the complement fixation test. The test is of little value for detecting uncomplicated *C. trachomatis* infections because of its low sensitivity of 15 % in uncomplicated urethral infections in men and 40 % in uncomplicated cervical infections [13]. The test is valuable in the diagnosis of psittacosis and lymphogranuloma venereum (LGV). The results of the test were positive (titre >1:10) in more than 80% of the cases with LGV [25].

Immunofluorescent Tests

In the Inclusion Fluorescent Antibody Assay (IF test), inclusion bodies from a single serotype in cell cultures on slides is used as the antigen. Antibodies against Chlamydia are detected by overlaying the slide with the serum of a patient. Antibodies, if present, are detected by a fluorescent conjugate. The test has a higher sensitivity than the complement fixation test. The sensitivity was 82 % in men and 95 % in women as compared with culture. However, 50.2 % of the female controls and 27 % of male blood donors were positive in this IF test [26]. Later, it was suggested that the high rate of positive results in the controls was due to the thus far underestimated frequency of infections with *C. pneumoniae* [27]. In this test a distinction between the antibodies against *C. psittaci* and those against *C. trachomatis* is not possible and in this respect, the IF test offers no additional advantages over the complement fixation test [26].

In the type-specific micro immunofluorescence test (MIF), the elementary bodies of *C. trachomatis* serotypes A-K and LGV types 1-3 harvested from infected egg yolk sacs are fixed to glass slides and used individually as antigens [28]. This is a very laborious test. Therefore, it was attempted to simplify the test by pooling the antigens [29,30]. A further simplification consisted of the use of purified EBs of one serotype, namely the rapidly growing and broadly cross-reacting LGV-2 as an antigen. Peterson et al compared this Single-Antigen Microimmunofluorescence Assay with the Inclusion Fluorescent-Antibody Assay with serotype LGV-2 as the antigen. The correlation between these two assays was poor [31]. In the Chlamydia spot-IF test (Bio Merieux) which is commercially available, the elementary bodies of *C. trachomatis* serotype LGV-2 and *C. psittaci* are coated separately onto a microscope slide. It was claimed that using this test it was possible to distinguish between *C. trachomatis* and *C. psittaci* infections. However, Mannion compared the results of this test with those of the MIF. He observed that the test as supplied by the manufacturer was not suitable for distinguishing both types of antibodies [32]. Taken together, the simplified test procedures appeared unsatisfactory in diagnosing uncomplicated chlamydial infections and in distinguishing between infections caused by *C. trachomatis* and *C. psittaci*. In complicated infections also they are of limited value.

Therefore, the MIF in its original form is the only useful test, but is used only at a few research laboratories. It is useful for detecting IgM, IgG and local secretory antibodies by using immunoglobulin class-specific fluorescein-labelled conjugates. Antibodies of the IgG class against *C. trachomatis* may persist for many years and they were detected in 25-45 % of the normal adults, in 20-25 % of the asymptomatic sexually active males, in 50-70 % of the asymptomatic sexually active females and in more than 85 % of the prostitutes attending a STD clinic [13]. For this reason, MIF performed only once is not useful for diagnosing recent *C. trachomatis* infection in patients attending STD clinics. Only a seroconversion or a fourfold or higher increase in the titre of IgG in consecutive serum samples or the presence of IgM antibodies indicate a recent infection [33]. In patients with pelvic inflammatory disease, this strategy does not apply because most patients have suffered from a chlamydial cervicitis or salpingitis [34].

The MIF test has been successfully used for the diagnosis of trachoma and for the diagnosis of *C.trachomatis* infection in infants [35-37].

Antibody detection by ELISA methods

The lack of sensitivity of the CFT and the difficulty in performing the MIF test has prompted the search for new serologic tests for detecting chlamydial antibodies.

Lewis et al. used intact Chlamydia of a laboratory strain of *C. psittaci* in an ELISA and showed a diagnostic rise in antibody titre in all of the 14 patients with clinically diagnosed psittacosis during an outbreak of this disease. The mean titres were higher for ELISA (419) then for CFT (94), indicating a higher sensitivity for the ELISA. Sera of 6 individuals with clinical LGV were also positive in the ELISA indicating that the assay with the antigen used in the study is genus-specific rather than species-specific [38].

Jones et al. used ELISAs with reticulate bodies of type C and elementary bodies of type L2 as antigens in 42 men with NGU. Although there was a good correlation between the results of both ELISAs and the MIF, there was no correlation in this group of patients between the presence of anti-chlamydial antibodies and the results of culture [39]. Saiku used reticulate bodies of serotype L2 as an antigen in the ELISA and also found a good correlation between positive results in this ELISA and the MIF [40]. In the studies of Duc-Goiran et al. and Mahony et al. solubilized elementary bodies of serotype D and L2 respectively were used as an antigen. In both studies a good correlation was observed between the results of ELISA and MIF [41,42]. In all the studies mentioned above, the ELISA was found to be rapid, more easy to perform than the MIF and was as sensitive as the MIF. A serious drawback of these tests is that genus-specific antibodies are detected. Therefore, antibodies against *C. trachomatis, C psittaci* and *C. pneumoniae* cannot be distinguished from each other. Some authors suggested that this test should be used as a sensitive first step screening test and if positive, should be followed by a specific test to detect chlamydial antigen [27]. However, this option is of little practical value in a population with a high background prevalence of anti-chlamydial antibodies.

Enzyme-Linked Fluorescent Assay

Theunissen et al. developed an enzyme-linked fluorescent assay (ELFA) with non-fixed elementary bodies of *C. trachomatis* serotypes E, F, H, I, J and LGV 2 as the antigens. A good correlation (0.88) was observed between the sensitivity of the ELFA and MIF. In contrast to the results of ELISA, cross-reactions between *C. trachomatis* and *C. pneumonia* were not observed in the ELFA. A major technical advantage of this test over the MIF is that the results can be objectively read using a Fluoroskan. The use of this test has been advised for epidemiological studies and in cases of suspected complicated chlamydial infections such as pelvic inflammatory disease (PID) [43].

Western Blot Assay

The Western Blot Assay is mainly of scientific importance for investigating the appearance and the persistence of antibodies directed against various chlamydia antigens. In this test, sera of high IgG and IgA titres in the peroxidase assay reacted with more peptides than those which showed low titres [44]. Wagar et al investigated the reaction of sera from women with PID using two 60 kD antigens of *C. trachomatis*. All sera reacted with 57-60 kD M cysteine-rich structural protein of the outer membrane complex "omp 2". The other protein, a Triton X-100 soluble protein was characterized as the Chlamydia equivalent of a heat shock protein "gro EL". Antibodies against this protein were observed in 31% of the women with PID and a positive MIF test and in 81% of the women with ectopic pregnancy and a positive MIF test. The author suggested that women with PID who have antibodies against "gro EL" are the ones who are likely to develop chronic sequela [45].

References

1. Stam WE. *Chlamydia trachomatis.* In Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases 2nd ed. New York: McGraw Hill 1990: 917-25.
2. Saikku P, Puolakkainen M, Leinonen M, Nurminen M, Nissinen A. Cross reactivity between Chlamydiazyme and Acinetobacter strains. N Eng J Med 1986; 314: 922-3.
3. Taylor Robinson D, Thomas BJ, Osborn MF. Evaluation of enzyme immunoassay (Chlamydiazyme) for detecting *Chlamydia trachomatis* in genital tract specimens. J Clin Pathol 1987; 40: 184-9.
4. Rompalo AM, Suchland RJ, Price CB, Stam WE. Rapid diagnosis of *Chlamydia trachomatis* rectal infection by direct immunofluorescence staining. J Infect Dis 1987; 155: 1075-6.
5. Stamm WE. Diagnosis of *Chlamydia trachomatis* genitourinary tract infections. Ann Intern Med 1988; 108: 710-7.
6. Cles LD, Bruch K, Stamm, WE. Staining characteristics of six commercially available monoclonal immunofluorescence reagents for direct diagnosis of Chlamydia trachomatis infections. J Clin Microbiol 1988; 26: 1735-7.
7. Barnes RC. Laboratory diagnosis of human chlamydial infections. Clin Microbiol Rev 1989; 2: 119-36.
8. Tang FF, Chang HL, Huang YT, Wang KC. Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. Chin Med J 1957; 75: 429-42. Cited by Barnes RC. Laboratory diagnosis of human chlamydial infections. Clin Microbiol Rev 1989; 2: 119-36.
9. Gordon F, Quan AL. Isolation of trachoma agent in cell culture. Proc Soc Expt Biol Med 1965; 118: 354-9.
10. Darougar S, Cubitt S, Jones BR. Effect of high speed centrifugation on the sensitivity of irradiated McCoy cell culture for the isolation of Chlamydia. Brit J Vener Dis 1974; 50: 308-12.
11. Bell TA, Kuo CC, Stamm WE, et al. Direct fluorescent monoclonal antibody stain for rapid detection of infant *Chlamydia trachomatis* infections. Pediatrics 1984; 74: 224-8.
12. Lefebvre J, La Perrière H, Rousseau H, Massé R. Comparison of three techniques for detection of *Chlamydia trachomatis* in endocervical specimens from asymptomatic women. J Clin Microbiol 1988; 26: 726-31.
13. Schachter J, Biology of *Chlamydia trachomatis.* In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases. 1st ed. New York: McGraw Hill, 1984: 243-257.
14. Tjiam KH, van Heyst BYM, de Roo JC, et al. Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: diagnostic implications. Brit J Vener Dis 1984; 60: 92-4.
15. Coleman P, Varitec V, Mushahlewar IK, et al. Test Pack Chlamydia a new rapid assay for the direct detection of *Chlamydia trachomatis.* J Clin Microbiol 1989; 27: 2811-4.
16. Kluytmans JAJW, Niesters HGM, Mouton JW, et al. Performance of a nonisotopic DNA probe for detection of *Chlamydia trachomatis* in urogenital specimens. J Clin Microbiol 1991; 29: 2685-9.
17. Iwen PCT, Blair MM, Wood GL. Comparison of the Gen-Probe PACE-2 system, direct fluorescent antibody, and cell culture for detecting *Chlamydia trachomatis* in cervical specimens. Am J Clin Pathol 1991; 95: 578-82.
18. Ratti G, Moroni A, Cevenini R. Detection of *Chlamydia trachomatis* DNA in patients with non-gonococcal urethritis using the polymerase chain reaction. J Clin Pathol 1991; 44: 564-8.
19. Claas HCJ, Wagenvoort JHT, Niesters HGM, Tio TT, van Rijsoort-Vos, Quint WGV. Diagnostic value of the polymerase chain reaction for Chlamydia detection as determinant in a follow-up study. J Clin Microbiol 1991; 29: 42-5.
20. Ostergaard I, Birkeland S, Christiansen G. Use of polymerase chain reaction for detection of *Chlamydia trachomatis.* J Clin Microbiol 1990; 28: 1254-60.
21. Näher H, Drzonek H, Wolf J, von Knebel Doeberitz M, Petzoldt D. Detection of *Chlamydia*

trachomatis in urogenital specimens by polymerase chain reaction. Genitourin Med 1991; 67: 211-4.

22. Bobo L, Coutlee F, Yolken RH, Quinn T, Viscidi RP. Diagnosis of *Chlamydia trachomatis* cervical infection by detection of amplified DNA with an enzyme immunoassay. J Clin Microbiol 1990; 28: 1968-73.
23. Taylor-Robinson D, Thomas BJ. Laboratory techniques for the diagnosis of chlamydial infections. Genitourin Med 1991; 67: 256-66.
24. Ostergaard L, Traulsen J, Birkeland S, Christiansen G. Evaluation of urogenital *Chlamydia trachomatis* infections by cell culture and the PCR using a closed system. Eur J Clin Microbiol Infect Dis 1991; 10: 1057-61.
25. Schachter J, Smith DE, Dawson CR, et al. Lymfogranuloma venereum. I. Comparison of the Frei test, complement fixation test, and isolation of the agent. J Infect Dis 1969; 120: 372-5.
26. Saikku P, Paavonen J. Single-antigen immunofluorescence test for chlamydial antibodies. J Clin Microbiol 1978; 8: 119-22.
27. Barnes RC. Laboratory diagnoses of human chlamydial infections. Clinical Microbiology Reviews 1989; 2: 119-36.
28. Wang SP, Grayston JT, Kuo CC, Alexander ER, Holmes KK. Serodiagnosis of Chlamydia trachomatis infection with the micro immunofluorescence test. In: Hobson O, Holmes KK, eds. Non-gonococcal urethritis and related infections. Washington D.C.: American Society for Microbiology 1977: 237-48.
29. Wang SP, Grayston JT, Alexander ER, Holmes KK. Simplified micro immunofluorescence test with trachoma-lymphogranuloma venereum (Chlamydia trachomatis) antigens for use as a screening test for antibody. J Clin Microbiol 1975; 1: 250-5.
30. Treharne JD, Darougar S, Jones BR. Modification of the microimmunofluorescence test to provide a routine serodiagnostic test for chlamydial infection. J Clin Pathol 1977; 30: 510-17.
31. Peterson EM, Oda R, Tse P, Gastaldi C, Stone SC, de la Maza LM. Comparison of a single-antigen micro immunofluorescence assay and inclusion fluorescent-antibody assay for detecting chlamydial antibodies and correlation of the result with neutralizing ability. J Clin Microbiol 1989; 27: 350-52.
32. Mannion PT, Mallinson H, Treharne JD. Serological diagnosis with the Chlamydia Spot-IF test. J Med Microbiol 1991; 35: 244-48.
33. Harrison MR, Magder LS, Boyce WT, et al. Acute *Chlamydia trachomatis* respiratory infection in childhood. Am J Dis Child 1986; 140: 1068-71.
34. Brunham RC, Peeling R, Maclean I, Kosseim MR, Paraskevas M. *Chlamydia trachomatis*-associated ectopic pregnancy: serologic and histologic correlates. J Infect Dis 1992; 165: 1076-81.
35. Stamm WE, Mardh PA. *Chlamydia trachomatis.* In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases. 2nd ed. New York: McGraw Hill, 1990: 917-26.
36. Swensson L, Weström L, Mardh PA. Acute salpingitis with *Chlamydia trachomatis* isolated from the fallopian tubes: clinical, cultural and serologic findings. Sex Trans Dis 1981; 8: 51-4.
37. Wang SP, Grayston JT. Micro-immunofluorescence serology of Chlamydia trachomatis. In: de la Maza LM and Peterson EM, eds. Medical Virology III. New York: Elsevier, 1984, 87-118.
38. Lewis VJ, Thacker WL, Mitchell SH. Enzyme-linked immunosorbent assay for Chlamydia antibodies. J Clin Microbiol 1977; 6: 507-10.
39. Jones RB, Bruins SC, Newhall WJ. Comparison of reticulate and elementary body antigens in detection of antibodies against *Chlamydia trachomatis* by an Enzyme-linked Immunosorbent assay. J Clin Microbiol 1983; 17: 466-71.
40. Saikku P, Paavonen J, Väänänen P, Vaheri A. Solid-phase enzyme immunoassay for chlamydial antibodies. J Clin Microbiol 1983; 17: 22-27.

41. Duc-Goiran P, Raymond J, Léauté JB, Orfila J. Use of the Enzyme-linked immunosorbent assay for detection of antibodies to *Chlamydia trachomatis*. Eur J Clin Microbiol Infect Dis 1983; 2: 32-38.
42. Mahony JB, Schachter J, Chernesky MA. Detection of antichlamydial immunoglobulin G and M antibodies by enzyme-linked immunosorbent assay. J Clin Microbiol 1983; 18: 270-5.
43. Theunissen HJW. Ph.D. Thesis, Erasmus University, Rotterdam, 1993.
44. Wagar EA, Schachter J, Baroil P, Stephens RS. Differential human serologic response to two 60.000 molecular weight *Chlamydia trachomatis* antigens. J Infect Dis 1990; 162: 922-27.

3.3 LABORATORY TECHNIQUES IN THE DIAGNOSIS OF INFECTIONS WITH NEISSERIA GONORRHOEAE

3.3.1 Antigen detecting techniques

Direct identification of *N. gonorrhoeae*

The conventional diagnosis of infection with *N. gonorrhoeae* is based on the presence of intracellular Gram-negative diplococci in smears and in culture.

Gram-stained preparations, provided they are evaluated by an experienced technician have high sensitivity (83%-96%) and specificity (95%-99%) in men with symptoms of urethritis [1,2]. If evaluated by inexperienced technicians, the sensitivity can decrease from 95% to 54% [1]. The results of Gram-stained preparations in women were poorer [1-4] with a maximum sensitivity of 65% [1]. This difference could be possibly due to the presence of a larger diversity of the vaginal or cervical bacterial flora in which the Gram-negative diplococci are difficult to distinguish.

Culture of *Neisseria gonorrhoeae*

Culturing of *N. gonorrhoeae* can be performed using selective and non-selective culture media. Culturing of *N. gonorrhoeae* using non-selective media is usually difficult to assess because of the overgrowth by other bacteria, especially Proteus species. *N. gonorrhoeae* is mostly cultured using selective culture media although the best results are obtained by using a combination of cultures on selective and non-selective media [5]. The original Thayer-Martin selective medium contains vancomycin, colistinemethate and nystatin. *N. gonorrhoeae* grows slowly in this medium and the growth of Proteus species is insufficiently suppressed. In the modified Thayer-Martin medium more agar and glucose are added and it also contains trimethoprim to inhibit the growth of Proteus species [6]. Vancomycin in the culture medium is less preferable in regions where many vancomycin-susceptible gonococci occur (sometimes up to 30%) [7]. Other selective media are the Martin-Lewis medium [8] and the New York City medium [9]. Neisseria colonies can be selected by their morphology and colour. The recognition of *N. gonorrhoea* needs further evaluation by sub-culturing and subsequent use of an identification test. Conventional sugar fermentation tests and the Rapid Carbohydrate Utilization test can be used for identification. Various biochemical identification systems are

commercially available [10]. In addition, immunological identification methods incorporating either polyclonal or monoclonal antibodies are also commercially available [11].

The culture of gonococci, including the identification procedures takes 2 to 4 days. The advantage of culturing is that the bacterium is available for auxo-typing and for determining susceptibility to antibiotics whereby screening for penicillinase producing gonococci (PPNG) and plasmid-induced resistance of gonococci to tetracyclines are important. These determinations together with those establishing the sort and the size of the plasmids play a very important role in epidemiological studies.

Culture has the best results when the sample from the patient is either directly inoculated or is inoculated within 6 hours after having been transported in a non-nutrient transport-medium. The sensitivity decreases considerably upon storage for longer periods depending on the transport-medium and the temperature. This decrease ranged from 4.5% for Stuart medium at 4°C to 79% for Stuart agar at room temperature [12].

Immunofluorescence

Direct immunofluorescence on smears of vaginal, cervical and urethral discharge using polyclonal antibodies was observed to be expensive, laborious and less sensitive than the culture of *N. gonorrhoeae*. The same was also true for the indirect immunofluorescence technique [10,13,14]. The use of monoclonal antibodies against epitopes of protein I in a direct IF test led to a considerable improvement in the sensitivity, particularly when used on smears, obtained from women. Testing of duplicate smears prepared at the same time increased the sensitivity from 85% to 89% in men and from 72% to 88% in women as compared with culture [15].

Antigen detection by ELISA methods

A solid phase enzyme immunoassay which can be used to detect gonococcal antigen in cervical and urethral samples from patients is commercially available (Gonozyme, Abbott Diagnostics, USA). Polyclonal antibodies produced by injecting

different strains of *N. gonorrhoeae* in rabbits are used in this test. Specimen obtained from the urethra or endocervix of the patients are applied to the wells of a reaction tray. A pretreated polystyrene bead is added to facilitate adsorption of *N. gonorrhoeae*. The anti-gonococcal antibodies are added after removal of the unbound antigen. After subsequent washing, horse radish peroxidase-labelled sheep anti-rabbit antibodies are added to react with the antigen-antibody complex. Following the last washing, each bead is transferred to a 5 ml cuvette and the substrate solution containing o-phenylenediamine and H_2O_2 is added. The extinction is measured against a substrate blank at 492 nm using a spectrophotometer. The Gonozyme test is further evaluated in Chapter 6.

3.3.2 DNA probes and Polymerase Chain Reaction (PCR)

In 1983, Totten et al reported a DNA hybridization technique for demonstrating gonococcal DNA in samples obtained from patients using radio-labelled 2.6 mega dalton (mD) gonococcal cryptic plasmid as a probe. Using this technique, the results were positive in 63 of the 71 (89%) culture-positive men, whereas none of the 42 culture-negative men were positive. Most of the false negative results were observed with gonococcal strains of a unique auxotype in which the cryptic plasmid was absent [16]. In a refined version of this test, both the 2.6 mD and the 4.4 mD cryptic plasmids were used as probes. This allowed not only the detection of gonococci, but also the identification of the PPNG [17]. Panke et al evaluated a modified DNA probe assay (PACE 2, Gen-Probe, San Diego, USA) which required only 2 hours to investigate 469 cervical samples. In that assay, an acridinium ester-labelled single-stranded DNA probe complementary to rRNA of *N. gonorrhoeae* was used. The results in this study were compared with those of culture using samples obtained from 290 asymptomatic pregnant women (gonorrhoeal prevalence of 2%) and 179 symptomatic women (gonorrhoeal prevalence of 11.7%). In the 469 patients, the DNA probe test as compared with culture had a sensitivity of 88.9% and a specificity of 100%. The test appeared to be a suitable alternative for culture, especially in situations where the problems of transport may possibly result in the loss of viable *N. gonorrhoeae*. Another advantage is that a single sample can be tested both for *N. gonorrhoeae* antigen and for *C. tracho-*

matis antigen. The test has not yet been evaluated for examining pharyngeal and rectal samples or samples from children [18]. Cano et al evaluated an assay in which a fluorescent oligonucleotide probe from a sequence from the plasmid encoded gene cppB was used for detecting *N. gonorrhoeae*. A total of 119 reference strains of *N. gonorrhoeae* including 8 without plasmids were investigated. The results were positive in all strains. One hundred samples from urethra and vagina of patients attending the clinic for STD were tested. From these, 31 samples were positive and 69 were negative in culture. All the 31 culture-positive samples were also positive in the fluorescent DNA hybridization assay (sensitivity of 100%). Three of the 69 culture-negative samples were positive in the fluorescent DNA hybridization assay (specificity of 95.8%) [19].

To date, few reports have been published on the use of the PCR for demonstrating *N. gonorrhoeae*. In the report by Ho et al, PCR was compared with culture for investigating urethral and cervical samples from men and women. Thirty-four out of the 52 samples were positive in culture. The 34 culture-positive samples were also positive in PCR. An additional 2 samples were observed to be positive in PCR. These 2 samples were observed to be positive using the confirmatory Gonozyme test [20].

3.3.3 Antibody detecting techniques

To date several serological tests have been developed for detecting gonorrhoea. They comprise the presently obsolete complement fixation test and tests in which pili are used as the antigen.

Complement Fixation Test

The Complement Fixation Test (CFT) was the first serological test to be used. In this test, crude antigens prepared from whole cells of one or several gonococcal strains were initially used. The test was modified later and was generally used until ten years ago [21]. The sensitivity of this test to detect an ongoing infection was low: 2-5% for patients with uncomplicated infection and 16-30% for patients with complicated infection. Therefore, the test was no longer considered to be of clinical value [22].

Tests in which pili are used as antigen

The most successful newer tests are the Indirect Haemagglutination Assay (IHA), the Enzyme-Linked Immunosorbent Assay (ELISA) and the Radioimmuno Assay (RIA) in which gonococcal pili are used as the antigen.

Oranje et al compared 3 serological tests for the detection of antibodies against gonococci. In the ELISA and the IHA, gonococcal pili were used as the antigen, whereas in the complement fixation test (CFT), whole gonococci were used as the antigen. In the sera from reconvalescent patients with a culture-proven gonococcal infection, the ELISA and the IHA had a sensitivity of 53 % and 54 % respectively in women and 41 % and 50 % respectively in men. In these groups the CFT had a sensitivity of 22 % and 14 % respectively. The sensitivity of these tests was the highest in patients with oropharyngeal gonorrhoea (96 % and 75 %) or with complicated gonococcal infections (89 % and 100 %) [23].

The indirect haemagglutination test in which gonococcal pili were used as the antigen was extensively evaluated by Reiman et al. Among others, they investigated 1376 patients for gonorrhoea (from these, 386 were observed to suffer from gonorrhoea), 1384 healthy individuals and 54 patients with meningococcal infections. The indirect haemagglutination test was observed to have a higher

sensitivity than the CFT. Using the CFT, 8 % (men) and 11 % (women) of the patients with an ongoing culture-proven gonorrhoea were observed to be seropositive, whereas 51% (men) and 88% (women) were observed to be positive using the indirect haemagglutination test. They concluded that in the indirect haemagglutination test, a non-specific positive test result was rare. The test result remained positive in 50% of the culture-negative patients who had suffered from gonorrhoea in the past [22]. The same test was used by Duncan et al in a sero-epidemiological study of gonorrhoea in Ethiopian women. They observed gonococcal antibodies in 54% of the women with no clinical evidence of gonococcal infection [24].

Miettinen et al. modified the ELISA in which gonococcal pili was used as the antigen to investigate the distribution of IgM, IgG and IgA antibodies. The determination of the class of antibodies in this test was accompanied by a severe loss in sensitivity. Therefore, this did not lead to an improvement of this test for its use in clinical diagnosis because of the low overall detection rate [25]. Robertson et al used the ELISA with gonococcal pili as the antigen to investigate whether infertile women with tuba obstruction had a higher incidence of antibodies against gonococcal pili. A very low percentage (2-5%) of the sera from both the test group and the control group were observed to be positive for antibodies against gonococcal pili indicating that gonococci did not play a major role in the etiology of tuba obstruction [26]. In 1988, the same authors reported positive results with the same test in 32 % of women with ectopic pregnancy which was significantly higher than 4 % in women with intrauterine pregnancy. According to the authors the relationship between ectopic pregnancy and anti-gonococcal antibodies indicated a role of gonococcal infection in the etiology of ectopic pregnancy [27].

Western Blot Assay

The Western Blot Assay has been used thus far mainly to investigate the antibody response to gonococcal antigens in an attempt to delineate those antigens which may be suitable for developing a vaccine.

Aoun et al noted the absence of antibodies against a 70 kD group-specific antigen of Neisseria in the sera of patients with a primary or a recurrent gonococcal

infection and the presence of these antibodies in the sera of healthy controls who never experienced a gonococcal infection. Meningococcal carriage often induces anti-70 kD antibodies in children. It was shown that people with first or repeated gonococcal infections frequently lack anti-70 kD antibodies. The authors proposed the hypothesis that anti-70 kD antibodies could offer protection against *N. gonorrhoeae* [28]. Demarco de Hormache et al described a surface oligosaccharide antigen, the so-called epitope C which was the target antigen in bactericidal reactions of *N. gonorrhoeae* by monoclonal antibodies and complement. This epitope was present in fresh isolates of *N. gonorrhoeae*, but absent in strains which were repeatedly cultured in vitro. In the western blot, it was observed that antibodies against epitope C were present in 80% of the 51 patients with gonococcal infections. None of the sera from 271 healthy controls contained antibodies against epitope C, although they contained antibodies to other lipopolysaccharide (LPS) determinants. The authors concluded that epitope C is expressed in vivo in humans and that it is antigenic. The efficacy of epitope C as a protective immunogen as suggested by the authors has to be investigated further [29].

References

1. Goodhart ME, Oyden J, Zaidi AA, Kraus SJ. Factors affecting the performance of smear and culture tests for the detection of *Neisseria gonorrhoeae.* Sex Trans Dis 1981; 9: 63-9.
2. Rothenberg RB, Simon R, Chipperfield E, et al. Efficacy of selected diagnostic tests for sexually transmitted diseases. JAMA 1976; 235: 49-51.
3. Wald ER. Gonorrhea diagnosis by gram stain in the female adolescent. Am J Dis Child 1977; 131: 1094-6.
4. Lossick JG, Smeltzer MP, Curan JW. The value of the cervical gram stain in the diagnosis and treatment of gonorrhea in women in a veneral disease clinic. Sex Trans Dis 1982; 9: 124-7.
5. Reyn A, Bentzon MW. Comparison of a selective and a non-selective medium in the diagnosis of gonorrhea to ascertain the sensitivity of *Neisseria gonorrhoeae* to vancomycin. Brit J Vener Dis 1972; 48: 363-8.
6. Thayer JD, Martin JE. Improved medium selective for cultivation of *N. gonorrhoeae* and *N. meningitidis.* Pub Health Rep 1966; 81: 559-62.
7. Windall JJ, Hall MM, Washington JA, et al. Inhibitory effects of Vancomycin in *Neisseria gonorrhoeae* in Thayer Martin medium. J Infect Dis 1980; 142: 142-75.
8. Martin JE, Lewis JS. Anisomycin: Improved antimycotic activity in modified Thayer Martin medium. Public Health Lab 1977; 35: 53-62.
9. Faur YC, Weisburd MH, Wilson ME, May PS. A new medium for the isolation of pathogenic Neisseria (NYC medium) 1. Formulation and comparisons with standard media. Health Lab Sci 1973; 10: 44-45.
10. Mårdh PA, Danielsson D. *Neisseria gonorrhoeae.* In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ, eds. Sexually transmitted diseases, 2nd ed. New York: McGraw Hill 1990: 903-16.
11. Laughon BE, Ehret JM, Tanino TT, et al. Fluorescent monoclonal antibody for confirmation of *Neisseria gonorrhoeae* cultures. J Clin Microbiol 1987; 25: 2388-90.
12. Taylor E, Philips I. Assessment of transport and isolation methods for gonococci. Brit J Vener Dis 1980; 56: 390-3.
13. Hare MJ. Comparative assessment of microbiological methods for the diagnosis of gonorrhea in women. Brit J Vener Dis 1974; 50: 437-41.
14. Danielsson D, Forsum U. Diagnosis of Neisseria infections by defined immunofluorescence. Methodologic aspects and applications. Ann N Y Acad Sci 1975; 254: 334-49.
15. Ison CA, McLean K, Gedney J, et al. Immunofluorescence for the rapid diagnosis of gonorrhoeae. 6th International Meeting of the International Society for STD Research, Brighton, England 1985: Abstract 95.
16. Totten PA, Holmes KK, Handsfield HH, Knapp JS, Perine PL, Falkow S. DNA hybridization technique for the detection of *Neisseria gonorrhoeae* in men with urethritis. J Inf Dis 1983; 148: 462-71.
17. Perine PL, Totten PA, Holmes KK, et al. Evaluation of a DNA-hybridization method for detection of African and Asian strains of *Neisseria gonorrhoeae* in men with urethritis. J Inf Dis 1985; 152: 59-63.
18. Panke ES, Yang LI, Leist PA, Magevney P, Fry RJ, Lee RF. Comparison of Gen Probe DNA probe test and culture for the detection of *Neisseria gonorrhoeae* in endocervical specimens. J Clin Microbiol 1991; 29: 883-8.
19. Cano RJ, Palomares JC, Torres MJ, Klem RE. Evaluation of a fluorescent DNA hybridization Assay for the detection of *Neisseria gonorrhoeae.* Eur J Clin Microbiol Infect Dis 1992; 11: 602-9.
20. Ho BSW, Feng WG, Wong BKC, Egglestone SI. Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples. J Clin Pathol 1992; 45: 439-442.
21. Donegan EA. Serological tests to diagnose gonococcal infections. In: Brooks GF and Donegan EA, eds. Gonococcal Infection. London: Edward Arnold, 1985: 168-77.

22. Reimann K, Odum L, Larsen SO, Lind I. Indirect haemagglutination test using gonococcal pilus antigen: how useful to diagnose gonorrhoea? Genitourin Med 1987; 63: 250-55.
23. Oranje AP, Reimann K, van Eijk RW, Schouten HJA, de Roo A, Tideman GJ, Stolz E, Michel MF. Gonococcal serology, a comparison of three different tests. Brit J Vener Dis 1983; 59: 47-52.
24. Duncan ME, Reimann K, Tibaux G, Pelzer A, Letebirhan M, Lind I. Seroepidemiological study of gonorrhoea in Ethiopian women. 1. Prevalence and clinical significance. Genitourin Med 1991; 67: 465-92.
25. Miettinen A, Hakkarainen K, Grönroos P, et al. Class specific antibody response to gonococcal infection. J Clin Pathol 1989; 42: 72-6.
26. Robertson JN, Ward ME, Conway D, Caul EO. Chlamydial and gonococcal antibodies in sera of infertile women with tubal obstruction. J Clin Pathol 1987; 40: 377-83.
27. Robertson JN, Hogston P, Ward ME. Gonococcal and chlamydial antibodies in ectopic and intrauterine pregnancy. Brit J Obstet Gyn 1988; 95: 711-6.
28. Aoun L, Cremieux AC, Casin I, Morel P, Martin PMW. Serum antibody response to the 70,000-molecular-weight neisserial common antigens in humans infected by *Neisseria gonorrhoeae*. J Clin Microbiol 1988; 26: 1898-1900.
29. Demarco de Hormache R, Jessop H, Bundell C. Antibodies to the C epitope of *Neisseria gonorrhoeae* are present in patients with gonorrhoea and absent in normal sera. J Gen Microbiol 1988; 134: 1289-97.

3.4 LABORATORY TECHNIQUES IN THE DIAGNOSIS OF INFECTIONS WITH HERPES SIMPLEX VIRUS

3.4.1 Antigen detecting techniques

Direct identification in patient material

An infection with herpes simplex virus (HSV) induces several changes in the cell: ballooning degeneration, fusion of infected cells into multinucleated giant cells, and the formation of intranuclear inclusions. These changes can be visualised in patient material after cytological staining as in the Tzanck test. In this test, scrapings from the base of vesicles, pustules or erosions are smeared onto a glass slide and air-dried. The material is fixed in methanol and stained with a cytological stain. The staining of the smear is simple and rapid (within minutes) but experience is needed for the reading. The test can be used for diagnosis of varicella zostervirus (VZV) or HSV, but the two viruses cannot be distinguished from each other. The test can be very helpful in situations requiring a rapid answer whether a lesion is herpetic or not [1,2].

Electron and immuno-electron microscopy can be used for a rapid (within hours) identification of herpes virus in suspected lesions. These techniques can be used in life-threatening situations as in immuno-compromised patients, neonatal HSV infections or neonatal varicella. Using negative staining a sensitivity of 80 % was reached. Using colloidal gold immuno-electron microscopy on concentrated virus preparations a sensitivity of 95 % was observed [3]. The latter technique allows a distinction between HSV and VZV.

Culture of HSV

The presence of HSV can be demonstrated by culture on monolayers of cells. Various types of cells (i.e. human embryonic lung fibroblasts (Hel), Vero cells and Hep-2 cells) have been used to culture HSV. Herpes simplex virus causes a characteristic cytopathogenic effect (CPE): swelling and rounding of infected cells and formation of polynuclear giant cells (syncitia). The cells die and become detached from the surface. These phenomena are characteristic for HSV and usually start in several areas of the cell monolayer. The CPE indicates the presence of HSV. The speed with which CPE occurs depends on the type of the

virus and the type of the host cells that are used. Samples obtained from a herpes lesion at an early stage (blisters or ulcers that are less than 4 days old) contain more virus particles and are positive more often than samples obtained from lesions (ulcers and crustae) that are older than 4 days. With virus-containing samples, CPE usually occurs between 2 and 6 days [4].

The sensitivity of the culture method and the speed of demonstration of HSV were improved considerably by centrifuging the samples onto monolayers prior to culture and staining the infected cells with labelled-MoAbs against HSV. Using this procedure, 65-100% of the positive samples can be identified within 48 hours [5-7]. Gleaves et al reported that 22 samples out of 98 fresh samples showed CPE after 6 days using the conventional culture method. With the centrifugation and MoAb staining technique, 23 out of the 98 samples were observed to be positive within 16 hours after inoculation. This showed that the time required to detect the virus was shortened considerably without any reduction in the sensitivity [5]. At present, this technique is commonly used. The virus can be typed by staining one-half of the cover slip with anti-HSV-1 MoAb and the other half with anti-HSV-2 MoAb.

Other confirmatory methods used to establish the presence of HSV in cultures were an ELISA-technique [8] or a fluorescent detection technique with fluorescein-labelled Helix pomatia lectin [9].

ELISA methods for the detection of HSV

One of the first ELISA system that was used to detect HSV antigen in patient samples was developed by Land et al. They used commercially available rabbit antibodies to HSV-1 (RIgα1) and HSV-2 (RIgα2) in a capture ELISA. The antibodies were adsorbed to separate wells of a tray. Samples of patients were added. Then, peroxidase-conjugated rabbit antibodies to HSV types 1 and 2 were added. After adding the substrate the optical density was measured at 490 nm. The assay could be completed within 5 hours. According to the authors, this ELISA method allowed the simultaneous detection and typing of HSV antigen [10]. The capture principle was used in several commercially available ELISA kits: the Dakopatt ELISA (Dako, Denmark) [11,12], the Ortho HSV ELISA (Ortho, USA) [12-14] and the HERPCHEK ELISA (Du Pont Co., USA) [15]. Slightly different ELISA

systems are the IDEIA (Novo Norddisk) [16,17] and the one developed by Clayton [18]. It is not possible to distinguish between HSV-1 and HSV-2 infections with the latter kits.

In the Abbott ELISA for HSV detection (Abbott, USA) pretreated polystyrene beads are used as the solid phase. This test is evaluated and discussed in Chapter 8.

3.4.2 DNA detecting methods

In 1989, Cao et al demonstrated HSV-DNA among DNA extracted from paraffin embedded skin biopsies from 7 patients with HSV infections using the polymerase chain reaction (PCR). No such DNA was detected among DNA extracted from 3 normal skin biopsies and 1 herpes zoster lesion [19].

The PCR was also tested using samples obtained from the genitalia. Hardy et al observed positive results in all 8 samples from which HSV had also been isolated. The results of the PCR were also positive in all 4 patients with herpes labialis from whom HSV-1 had been isolated [20]. Cone et al investigated a total of 4 relapsing episodes of herpes genitalis infections in 3 women. During each episode in these patients, daily samples during a period of 10 days were obtained for the PCR and the culture. The culture remained positive for an average of 2.6 days, whereas the PCR remained positive for an average of 6.8 days. Fifteen out of the 17 samples of HSV lesions at the ulcer stage were positive in the PCR, whereas 3 out of the 17 samples were positive in the culture. From the results of that study, it appeared that the PCR was superior to the culture for detecting HSV in the lesions at an advanced stage [21]. Rogers et al investigated a large number of patients with herpes genitalis. A total of 79 samples from women undergoing routine examination for HSV were tested. The results of both the PCR and the culture were positive in 32 samples, whereas the results of both were negative in 40 samples. Six samples were positive in the PCR, and negative in the culture. One sample was positive in the culture, but negative in the PCR. There was no satisfactory explanation for the latter observation. However, the results of the PCR using the supernatant from the culture of the same sample were positive [22].

Piiparinen et al developed a method for detecting and typing HSV simultaneously by using a primer pair from the HSV-DNA polymerase coding genes. Using this

method, 30 samples out of a total of 31 samples were typed correctly [23].
Brice et al demonstrated HSV-DNA in 9 out of the 13 skin biopsies obtained from patients with herpes-associated Erythema Exsudativum Multiforme (EEM) and in 6 out of the 9 patients with idiopathic EEM [24]. Using the PCR technique, Miura et al demonstrated HSV-DNA in 5 biopsies from skin lesions in patients with relapsed HSV infections. In that study, HSV was demonstrated in 5 out of the 8 biopsies obtained from patients with HSV-associated EEM [25].
The PCR was also used in the "non-invasive" diagnosis of HSV-encephalitis to detect HSV-DNA in the liquor. Positive results were observed as early as 2 days after the onset of neurological symptoms in 9 out of the 10 patients with confirmed HSV-encephalitis. The results in all the 12 controls were negative [26]. Similar results were also reported in two other studies [27,28]. Aurelius et al used PCR for extensive investigations into the presence of HSV-DNA in 151 liquors obtained from 43 patients. The results were positive in 42 patients, whereas the results in all 60 liquors from controls were negative. The only patient in whom the results were negative had received prior therapy. The results in 270 controls, in which liquor was replaced by buffer and which were investigated to check for internal contamination were negative [29]. Kimura et al used the PCR to detect HSV-DNA in samples obtained from the mouth, the skin and liquor of 7 neonates with HSV infection. Three of the infants had disseminated infections, 2 had infections in the central nervous system and 2 had local skin, eye and mouth (SEM) infections. HSV-1 was isolated from all but one patient, but in whom like the others, the PCR result was positive. No false- positive results were observed in the controls [30].
At present, commercial kits for routine screening are either unavailable or have not yet been sufficiently evaluated. However, it is expected that such kits would become available in the near future. By using a closed system, it appears that the initial problems of contamination in the PCR have been satisfactorily resolved.

3.4.3 Antibody detecting methods

Traditionally, serological techniques have occupied a valuable place in the diagnosis of HSV infections. The initial test for recognizing anti-HSV antibodies was the complement fixation test. Other tests are neutralization tests and immunoas-

says like indirect immunofluorescence. Newer tests are the Western Blot Analysis (WBA) and the Dot Immunobinding Assay (DIA). However, serological tests are mainly used to complement other tests such as virus cultures. In cases in which the isolation of the virus is not or no longer possible, HSV infection can only be established by means of antibody determination [31-33]. This offers the possibility to distinguish between a primary and a recurrent infection, but a distinction between HSV-1 or HSV-2 infection is not possible with the traditional serological tests for HSV. The distinction between HSV-1 and HSV-2 infection is desirable for the following reasons:

1. For the prognosis: HSV-2 infections often recur genitally, whereas HSV-1 infections rarely recur genitally [34].
2. For epidemiological purposes.
3. For the screening of HSV-2-positive pregnant women or their partners to identify neonates at risk of herpes neonatalis.

Since there is 50% homology between the genomes of HSV-1 and HSV-2, the virions have many determinants which could cause cross-reactions. This hampers the development of type-specific immunoassays. Therefore, the possibilities to demonstrate HSV type-specific antibodies are limited. The most widely used methods are based on demonstrating a difference in the neutralizing antibodies against HSV-1 and HSV-2. Such tests have low sensitivity, particularly if antibodies against both HSV types are present.

Complement Fixation Test

The complement fixation test (CFT) is a non-standardized test in which self-prepared crude antigens are mostly used. In the commercially available CFT, the Simplex-2 test (Gene Link, Australia) HSV-2 AG-4 early antigen is used. Under optimum laboratory conditions, the sensitivity was 87% and the specificity was 90-94% for sera collected between 14 and 28 days after the onset of the symptoms from patients with a culture-proven primary or recurrent infection [35]. However, in routine use, the sensitivity and specificity are presumably much lower, but precise

data are not available. The CFT is used for demonstrating an increase in HSV antibodies, but the test cannot be used to detect type-specific antibodies [36].

Neutralization test

Neutralizing antibodies are able to inhibit the growth of virus in cultured cells in vitro. Such antibodies may be detected by the plaque reduction test or the micro-neutralization test in which a constant quantity of virus and various concentrations of serum are used. The highest dilution of serum which results in 50% reduction of the cytopathogenic effect is the serum titre in the neutralization assay.

In the plaque reduction test the monolayer is covered with agar and the virus can only spread from cell to cell producing the plaques. The monolayers are stained and the plaques are counted macroscopically. The titre is the highest serum dilution which produces a 50% reduction in the plaques. This test has been scarcely used for HSV-infections. This test has been mainly used to distinguish between HSV-1 and HSV-2 infections by incubating HSV-1 and HSV-2 cultures with serial dilutions of serum from the patients. The titres of the neutralizing antibodies to HSV-1 and HSV-2 are used to calculate the Rawl's index which indicates the type of the infecting virus [37]. Before the introduction of type-specific tests such as the Western blot or tests with glycoprotein G, the neutralizing test was used as the standard to evaluate other tests. Of the 220 sera with HSV-2 antibodies in Western blot, only 106 (48%) were sero-typed correctly using the micro-neutralizing assay [38]. Virus neutralization tests are very laborious and time-consuming.

Immunoassays

Immunofluorescent tests are performed on HSV-infected cells fixed to glass slides. Antibodies which bind to viral antigens after overlaying the monolayer with serum from patients are detected with an anti-IgG (or anti-IgM) conjugate. Several tests are commercially available. Advantages are the possibility to detect low levels of anti-HSV antibodies and the possibility to distinguish between IgM and IgG antibodies by the proper choice of the conjugate. The latter possibility allows to distinguish between early and recurrent infections. The titres obtained with the immunofluorescent tests are similar to those obtained by neutralizing tests [31]. In

immunofluorescence tests, both type-common and type-specific antibodies are detected. Therefore, these tests cannot be used to distinguish between HSV-1 and HSV-2 infections [39].

Crude viral antigen has been used in a radioimmunoassay (RIA) and a solid phase enzyme immunoassay (EIA) [40,41]. Although these tests are more sensitive than the micro-neutralization test, they have not been evaluated further. Both tests are useless for identifying type-specific antibodies [42].

Western Blot Assay

Extracts of cell cultures which have been infected with HSV-1 or HSV-2 are loaded on a polyacrylamide gel. After electrophoresis (SDS-PAGE), the (glyco)proteins are transferred to a nitrocellulose membrane (blotting). The membrane is cut into strips, which are incubated with the serum of a patient suffering from an HSV infection. Antibodies from this serum bind to the antigen(s) of the virus on the nitrocellulose strip and are made visible by staining, usually after incubation with an enzyme-conjugate and the substrate for the enzyme. The serum antibodies react with both the structural viral proteins and with virus-induced cellular proteins [43,44]. The band pattern that is obtained can be analyzed further using a densitometer [45]. For HSV-2, a few type-specific bands can be detected. These bands correspond to the polypeptides of glycoprotein G2 (gG2) or its precursors [46-49]. Similar polypeptides of glycoprotein G1 (gG1) have been reported for HSV-1 using monoclonal antibodies (MoAbs) [50]. The western blot analysis for the detection of antibodies to HSV is described in detail in Chapter 8.

Glycoprotein G

The HSV-2 type-specific gG2 (92kD) can be purified by affinity chromatography using either Helix pomatia lectin columns or columns loaded with anti-gG2 MoAb. The HSV-1 type-specific gG1 can be purified only with gG1 MoAb-loaded columns [50]. Recently gG1 and gG2 were expressed in recombinant-baculovirus-infected insect cells [51] and *E.coli* [52]. The type specific glycoproteins G1 and G2 have been be used as antigens in various test systems such as EIA or DIA [46,50]. These tests are specific, but antibodies against gG appear late in infection. A

disadvantage is that the best results are observed with sera obtained from patients with recurrent infection or sera taken later than two weeks after the onset of clinical symptoms. Tests based on the use of gG2 are described in detail in Chapter 8.

References

1. Barr RJ, Herten J, Graham JH. Rapid method for Tzanck preparation. JAMA 1977; 237: 119-20.
2. Folkers E, Vreeswijk J, Oranje AP, Duivenvoorden JN. Rapid diagnosis in varicella and herpes zoster: re-evaluation of direct smear (Tzanck test) and electron microscopy including colloidal gold immuno-electron microscopy in comparison with virus isolation. Brit J Dermatol 1989; 121: 287-96.
3. Folkers E, Vreeswijk J, Oranje AP, Wagenaar F, Duivenvoorden JN. Improved detection of HSV by electron microscopy in clinical specimens using ultracentrifugation and colloidal gold immunoelectron microscopy: comparison with viral culture and cytodiagnosis. J Virol Methods 1991; 34: 273-89.
4. Fife KH, Corey L. Herpes simplex virus. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990, 941-2.
5. Gleaves CA, Wilson DJ, Wold AD, Smith TF. Detection and serotyping of herpes simplex virus in MRC-5 cells by use of centrifugation and monoclonal antibodies 16h post-inoculation. J Clin Microbiol 1985; 21: 29-31.
6. Pruneda RC, Almanza I. Centrifugation-shell vial technique for rapid detection of herpes simplex virus cytopathic effect in Vero cells. J Clin Microbiol 1987; 25: 423-4.
7. Woods GL, Mills RD, Conventional tube cell culture compared with centrifugal inoculation of MRC-5 cells and staining with monoclonal antibodies for detection of HSV in clinical specimens. J Clin Microbiol 1988; 26: 570-2.
8. Michalski F, Shaik M, Sahraie F, Desais S, Verano L, Vallabhaneni J. Enzyme linked immunosorbent assay spin amplification technique for herpes virus antigen detection. J Clin Microbiol 1986; 24: 310-1.
9. Slifkin M, Cumbi R. Rapid detection of herpes simplex virus with fluorescein-labelled Helix pomatia lectin. J Clin Microbiol 1989; 27: 1036-9.
10. Land SA, Skurrie IJ, Gilbert GL. Rapid diagnosis of herpes simplex virus infections by enzyme-linked immunosorbent assay. J Clin Microbiol 1984; 19: 865-9.
11. Alexander I, Ashley CR, Smith KJ, Harbour J, Roome APCH, Darville JM. Comparison of ELISA with virus isolation for the diagnosis of genital herpes. J Clin Pathol 1985; 38: 554-7.
12. Warford AL, Levy RA, Rekrut KA. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of herpes simplex virus antigen. J Clin Microbiol 1984; 20: 490-3.
13. Warford AL, Levy RA, Rekrut KA, Steinberg E. Herpes simplex virus testing of an obstetric population with an antigen enzyme-linked immunosorbent assay. Am J Obstet Gynecol 1986; 154: 21-8.
14. Sewell DL, Horn SA. Evaluation of a commercial enzyme-linked immunosorbent assay for the detection of herpes simplex virus. J Clin Microbiol 1985; 21: 457-8.
15. Baker DA, Gonik B, Mitch PO, Berkowitz A, Lipson S, Verma K. Clinical evaluation of a new herpes simplex virus ELISA: a rapid diagnostic test for HSV. Obstet Gynecol 1989; 73: 322-5.
16. Kudesia G, van Hegan A, Wake S, van Hegan RJ, Kinghorn GR. Comparison of cell culture with an amplified enzyme immunoassay for diagnosing genital herpes simplex infection. J Clin Pathol 1991; 44: 778-80.
17. Silles M. Clinical evaluation of enzyme immunoassay in rapid diagnosis of herpes simplex virus infections. J Clin Pathol 1992; 45: 165-7.
18. Clayton AL, Roberts C, Godley M, Best JM, Chantler SM. Herpes simplex virus detection by ELISA: effect of enzyme amplification, nature of lesion sampled and specimen treatment. J Med Virol 1986; 20: 89-97.
19. Cao M, Xiao X, Egbert B, Carragh T, Yen TSB. Rapid detection of cutaneous herpes simplex virus infection with the polymerase chain reaction. J Invest Dermatol 1989; 82: 381-2.
20. Hardy DA, Arvin AM, Yasukawa LL, et al. Use of polymerase chain reaction for successful identification of asymptomatic genital infection with herpes simplex virus in pregnant women at delivery. J Infect Dis 1990; 162: 1031-5.
21. Cone RW, Hobson AC, Palmer J, Remington M, Corey L. Extended duration of HSV DNA in

genital lesions detected by the polymerase chain reaction. J Infect Dis 1991; 164: 757-60.
22. Rogers BB, Josephson SL, Mah SK, Sweeney PJ. Polymerase chain reaction amplification of herpes simplex virus DNA from clinical samples. Obstetrics gynecol 1992; 79: 464-9.
23. Piiparinen H, Vaheri A. Genotyping of herpes simplex viruses by polymerase chain reaction. Arch Virol 1991; 119: 275-83.
24. Brice SL, Krzemien D, Weston WL, Huff JC. Detection of herpes simplex virus DNA in cutaneous lesions of erythema multiforme. J Invest Dermatol 1989; 93: 183-7.
25. Miura S, Smith CC, Burnett JW, Aurelius L. Detection of viral DNA within skin of healed recurrent herpes simplex infection and erythema multiforme. J Invest Dermatol 1992; 98: 68-72.
26. Klapper PE, Cleator GM, Dennett C, Lewis AG. Diagnosis of herpes encephalitis via Southern blotting of CSF DNA amplified by polymerase chain reaction. J Med Virol 1990; 32: 261-4.
27. Puchammer-Stockel E, Popow Kaupp T, Heinz FX, Mandel CW, Kunz C. Establishment of PCR for the early diagnosis of herpes simplex encephalitis. J Med Virol 1990; 32: 77-82.
28. Rowley AH, Whitley RJ, Lakeman FD, Wolinski SM. Rapid detection of herpes-simplex-virus DNA in cerebrospinal fluid of patients with herpes simplex encephalitis. Lancet 1990; 33: 440-1.
29. Aurelius E, Johansson B, Sköldenberg B, Staland Å, Forsgren M. Rapid diagnosis of herpes simplex encephalitis by nested PCR assay of cerebrospinal fluid. Lancet 1991; 337: 189-92.
30. Kimura H, Futamura M, Mideyuki K, et al. Detection of viral DNA in neonatal herpes simplex virus infections: frequent and prolonged presence in serum and cerebrospinal fluid. J Infect Dis 1991; 164: 289-93.
31. Fife KH, Corey L. Herpes simplex virus. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, eds. Sexually Transmitted Diseases, 2nd ed. New York: McGraw Hill, 1990, 941-2.
32. Koutski LA, Ashley RL, Holmes KK, et al. The frequency of unrecognized type 2 herpes simplex virus among women. Sex Transm Dis 1990; 17: 90-4.
33. Koutski LA, Stevens CE, Holmes KK, et al. Underdiagnosis of genital herpes by current clinical and viral-isolation procedures. N Engl J Med 1992; 326: 1533-9.
34. Corey L, Holmes KK. Genital herpes simplex virus infections: current concepts in diagnosis, therapy, and prevention. Ann Intern Med 1983; 98: 973-83.
35. Field PR, Ho WT, Irving WL, Isaacs D, Cunningham AL. The reliability of serological tests for the diagnosis of genital herpes: a critique. Pathology 1993; 25: 175-9.
36. Sherlock CH, Ashley RL, Shutleff MLK, et al. Type specificity of complement fixing antibody against herpes simplex virus type 2 AG-4 early antigen in patients with asymptomatic infection. J Clin Microbiol 1986; 24: 1093-7.
37. Rawls WE, Iwamoto K, Melnick JL. Measurements of antibodies to herpes virus types 1 and 2 in human sera. J Immunol 1970; 104: 599-606.
38. Ashley RL, Dalessio J, Dragavon J, et al. Underestimation of HSV-2 seroprevalence in a high-risk population by microneutralization assay. Sex Transm Dis 1993; 20: 230-5.
39. Plummer G. A review of the identification and titration of antibodies to herpes simplex viruses type 1 and type 2 in human sera. Cancer Res 1973; 33: 1469-76.
40. Vestergaard BF. Laboratory diagnosis of herpes viruses. Scand J Infect Dis 1985; Suppl 47: 22-32.
41. Ashley R, Benedetti J, Corey L. Humoral immune response to HSV-1 and HSV-2 viral proteins in patients with primary genital herpes. J Med Virol 1985; 17: 153-66.
42. Ashley R, Cent A, Maggs V, Nahmias A, Corey L. Inability of enzyme immunoassays to discriminate between infections with herpes simplex virus types 1 and 2. Ann Intern Med 1991; 3: 520-6.
43. Bernstein DI, Garraty E, Lovett MA, Bryson YL. Comparison of western blot analyses to microneutralization for the detection of type specific herpes simplex virus antibodies. J Med Virol 1985; 15: 223-230.
44. Bernstein DI, Lovett MA, Bryson YL. The effects of acyclovir on antibody response to herpes simplex virus in primary genital herpetic infections. J Inf Dis 1984; 150: 7-13.

45. Ashley RL, Militoni J. Use of densitometric analysis for interpreting HSV serologies based on western blot. J Virol Methods 1987; 18: 159-68.
46. Lee FK, Coleman M, Pereira L. Bailey PD, Tatsuno M, Nahmias AJ. Detection of herpes simplex virus type 2-specific antibody with glycoprotein G. J Clin Microbiol 1985; 22: 641-4.
47. Marsden HS, Buckmaster A, Palfreyman JW, Hope RG, Minson AC. Characterization of the 92,000-dalton glycoprotein induced by herpes simplex virus type 2. J Virol 1984; 50: 547-54.
48. Roizman B, Norrild B, Chan C, Pereira L. Identification and preliminary mapping with monoclonal antibodies of a herpes simplex virus 2 glycoprotein lacking a known type 1 counterpart. Virology 1984; 133: 242-7.
49. Olofssen S, Lundstrom M, Marsden H, Jeansson S, Vahlne A. Characterization of a herpes simplex virus type 2-specified glycoprotein with affinity for N-acetylgalactosamine-specific lectins and its identification as g92 K or gG. J Gen Virol 1986; 7: 737-44.
50. Lee FK, Pereira L, Griffin C, Reid E, Nahmias A. A novel glycoprotein for detection of herpes simplex virus type 1-specific antibodies. J Virol Methods 1986; 14: 111-8.
51. Sánchez-Martínez D, Pellett PE. Expression of HSV-2 glycoprotein G in insect cells by using a novel baculovirus expression vector. Virology 1991; 182: 229-38.
52. Parkes DL, Smith CM, Rose JM, Brandis J, Coates SR. Seroreactive recombinant herpes simplex virus type 2-specific glycoprotein G. J Clin Microbiol 1991; 29: 778-81.

CHAPTER 4

SOLID-PHASE ENZYME IMMUNOASSAY FOR DETECTION OF *CHLAMYDIA TRACHOMATIS*

Abstract

The prototype of a solid-phase enzyme immunoassay for the detection of *Chlamydia trachomatis* antigen was tested in 403 men and 135 women attending a venereal disease clinic. Culture on HeLa 229 cells was used as reference method. In men the overall sensitivity and specifity of the enzyme immunoassay was 70% and 98.5% respectively. In the 158 men with non-gonococcal urethritis the sensitivity and specifity was 73% and 96.5% respectively, in the 31 men with post-gonococcal urethritis 82% and 100%, in the 48 men with gonococcal urethritis 67% and 100%, in the 125 men with discharge but normal urinary sediment 54% and 99.1% and in the 41 men with no signs or symptoms 50% and 100%.

In women the sensitivity and specifity of the assay was 70% and 92% respectively. On account of the low degree of sensitivity in both men and women and the low degree of specifity in women the prototype of the enzyme immunoassay is not yet considered suitable for clinical use. An improved model is being developed.

Introduction

Although genital infections causes by *Chlamydia trachomatis* seem to occur no less frequently than genital gonococcal infections and to give rise to complications as frequently, considerably less attention is paid to them. This can partly be attributed to the expensive and time-consuming methods required for culture of *Chlamydia trachomatis* [1]. Recently, two rapid methods based on antigen detection were developed for the diagnosis of *Chlamydia trachomatis* infection. One method uses fluorescein-labelled monoclonal antibodies against *Chlamydia trachomatis.* The other method, a solid-phase enzyme immunoassay, is similar to an enzyme immunoassay developed for detection of gonococcal antigen [2-5] but in contrast to the latter assay has not yet been evaluated extensively. In a recent study an enzyme immunoassay was used to detect *Chlamydia trachomatis* infection in men and women attending a venereal disease clinic [6].

This study presents the results obtained with the prototype of a solid-phase enzyme immunoassay for the detection of non-complicated urethral infections in men and cervical infections in women caused by *Chlamydia trachomatis*, using conventional cell culture as reference method.

Materials and Methods

Patients. Of the 403 men examined 158 were suffering fom non-gonococcal urethritis, 31 from post-gonococcal urethritis and 48 from gonococcal urethrisi. The criterion for diagnosis of urethritis was the presence of more than 10 leucocytes/field (magnification 250 x) in the sediment of the first 10-15 ml of urine collected when the patient had not voided for 4h or longer. The presence of discharge in 125 men without this increase in leucocyte count was not regarded as urethritis, however no early morning smears were performed in these men. Forty-one men had no signs or symptoms at all. The 135 women examined were screened regularly for sexually transmissible diseases at an outpatient clinic.

Samples. In men the samples were taken from the urethra and in women from the endocervix. All samples were collected between November 1983 and June 1984. Samples were taken for the following: culture for *Neisseria gonorrhoeae*, gramstained smear (if possible), culture for *Chlamydia trachomatis*, and the enzyme immumnoassay. The samples for the culture were taken with the aid of a metal-shafted cottonwool-tipped swab moistened in 0.2 M sucrose phosphate buffer containing 25 µg/ml gentamicin and 2.5 µg/ml amphotericin. The swab was inserted into the urethra in men and the endocervix in women, rotated for a few seconds then replaced in 2 sucrose phosphate (2 SP) transport medium. The samples were stored in liquid nitrogen until processed. Plastic-shafted cottonwool-tipped swabs were used for collection of samples for the enzyme immunoassay, as recommended by the manufacturer. The swabs were placed in a tube with 100 µl of storage buffer (Abbott). The tubes were kept at 4^0 C and processed within three days.

Culture. Chlamydiae were cultured on monolayers of HeLa 229 cells seeded on coverslips in flat-bottom tubes. The full-grown monolayer was prerinsed with 30 µg/ml DEAE dextran in Hank's balanced salt solution. Then, 250 ml of the carefully vortexed sample was added to two flat-bottom tubes and centrifuged for 1 h at 2700 X *g*. After drawing off the sample material the tubes were incubated for three days at 37^0 C with complete glucose medium containing 25 µg/ml gentamicin, 25 µg/ml vancomycin and 2.5 µg/ml amphotericin. After fixation and Giemsa staining the coverslips were screened for inclusion bodies using darkfield illumination at a

magnification of 250 times. The flat-bottom tubes for subpassage were subsequently sonicated at a frequency of 10 kHz for 10 sec and 250 µl of the materials was transferred to a fresh monolayer. Cultures were considered positive if one or more inclusions were observed in primary or passaged monolayers.

Solid-Phase Enzyme immunoassay. The assay was a non-marketed research prototype for detection of *Chlamydia trachomatis* antigen. One ml of dilution buffer (Abbott) was added to the tube with the sample. After incubation for 10 min at room temperatuse the contents of the tubes were carefully mixed on the Vortex in three cycles of 15 sec. The liquid was expressed from the swabs by rotating and pressing the tip of the swab against the wall of the tube. Sample material (200 µl) was introduced into the wells of plastic plates. One positive and three negative controls were tested simultaneously. A bead, pretreated to permit absorption of *Chlamydia trachomatis* antigen on the surface, was added to each well. The wells were covered with adhesive seals, and the plates incubated for 45 min in an 37^0 C water bath. After a washing procedure which consisted of three rinsings with distilled water using a pentawasher (Abbott), 200 µl of antibody preparation (rabbit) and then 200 µl of conjugate (goat horseradish peroxidase conjugated antibody to rabbit immunoglobulin G) were added. Each step was followed by incubation and the washing procedure described abbove. The beads were then transferred to 5 ml cuvettes, 300 µl of substrate (orthopenylene diamine with H_20_2) added, and the cuvettes incubated for 30 min at room temperature. The reaction was stopped by adding 1 ml of N H_2S0_4. This resulted in a yellow colour change in samples containing *Chlamydia trachomatis* antigen. After mixing, the colour intensity was read at 492 nm on a Quantum spectrophotometric analyzer against a substrate blank. Test results with an extinction value of 0.100 OD plus the average of negative controls were regarded as positive.

Statistical Analysis. The sensitivity, specifity and predictive value of a positive and negative test result were calculated by the method of Vecchio [7]. The results of the enzyme immunoassay were compared with the combined results of culture and subculture.

Results.

The results of the enzyme immunoassay and culture are shown in Table 1. In men both tests were positive in 53 of 403 patients; culture only was positive in 23 patients and enzyme immunoassay only in five patients. When the men were divided into groups according to symptoms and signs, the number of positive results obtained by culture was consistently higher than that obtained by enzyme immunoassay, although in some categories too few specimens were tested to make firm conclusions. In women both tests were positive in 16 of 135

Table 1: Detection of *Chlamydia trachomatis* by enzyme immunoassay and culture in various patient groups.

Patient group	N	Assay + Culture +	Assay - Culture +	Assay + Culture -	Assay - Culture -
Men					
Non-gonococcal urethritis	158	32	12	4	110
Post-gonococcal urethritis	31	9	2	0	20
Gonococcal urethritis	48	4	2	0	42
Discharge, no abnormalities in sediment	125	7	6	1	111
No signs or symptoms	41	1	1	0	39
Total men	403	53	23	5	322
Women	135	16	7	9	103

cervical samples; culture only was positive in seven women and enzyme immunoassay only in nine.

The prevalence of a positive *Chlamydia trachomatis* culture, the sensitivity, specifity and predictive value of a positive and negative result of the enzyme immunoassay versus culture and subculture are given in Table 2. The sensitivity of the enzyme immunoassay was 70% in both men and women, and ranged from 50% to 82% in the various groups of men, with large 95% confidence

Table 2: Prevalence of a positive *Chlamydia trachomatis* culture, sensitivity, specifity and predictive value of a positive and negative result of the enzyme immunoassay versus culture and subculture in various patient groups (95% confidence limits in parenthesis).

Patient group	N	Prev. pos. cult. (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Men						
Non-gonococcal urethritis	158	27.9 (21.0-35.5)	73 (57.2-85.0)	96.5 (91.3-99.0)	89 (73.9-96.6)	88.7 (83.5-94.8)
Post-gonococcal urethritis	31	35.5 (19.2-54.6)	82 (48.2-97.7)	100 (83.2-100)	100 (66.4-100)	91 (70.8-98.9)
Gonococcal urethritis	48	12.5 (4.7-25.3)	67 (22.3-95.7)	100 (91.6-100)	100 (39.8-100)	96 (84.5-99.4)
Discharge, no abnormalities in sediment	125	10.4 (5.7-17.1)	54 (25.1-80.8)	99.1 (95.3-100)	88 (48.2-99.7)	94.4 (89.2-98.1)
No signs or symptoms	41	4.9 (0.6-16.5)	50 (1.4-98.7)	100 (91.0-100)	100 (0-100)	98 (87.3-99.9)
Total men	403	18.9 (15.2-23.0)	70 (58.1-79.8)	98.5 (96.6-99.5	91 (81.0-97.1)	93.6 (90.4-95.9)
Women	135	17.0 (11.1-24.5)	70 (47.4-86.8)	92.1 (85.3-96.3)	64 (42.5-82.0)	93.6 (87.3-97.4)

intervals. The highest sensitivity was reached in groups with the highest prevalence of positive cultures.

The overall specifity was 98.5% in men and 92% in women. In men the specifity in the various groups varied from 96.5% to 100%. The lowest value was observed in men with non-specific urethritis. The predictive value of a positive enzyme immunoassay result was notably low in women.

Discussion

The sensitivity of the enzyme immunoassay in the various groups of men with signs and symptoms of urethritis varied from 67% to 82%. The specifity in these groups varied from 96.5% to 100%. In women the sensitivity and specifity was 70% and 92.1% respectively. Using the same test Jones et al. [6] found a sensitivity of 81% and a specifity of 98% in a group of men and women with urethritis and cervicitis.

The ranges of the 95% confidence limit in Table 2 are large. To narrow these ranges more positive patients would have to be examined. The prevalence of a positive *Chlamydia trachomatis* culture in male patients suffering from non-specific urethritis, gonococcal urethritis and in particular post-gonococcal urethritis was rather low (Table 2) but does not differ from previous findings in similar patients (unpublished data).

The sensitivity of the enzyme immunoassay for detection of *Chlamydia trachomatis* was lower than that of an enzyme immunoassay for detection of gonococcal antigen [2-5] which is already used routinely. In women the specifity of the enzyme immunoassay for *Chlamydia trachomatis* was much lower than the specifity of the enzyme immunoassay for *Neisseria gonorrheae*. Apart from an enzyme immunoassay which detects antigen, immunofluorescence tests with labelled monoclonal antibodies can also be used for rapid diagnosis of chlamydial infections. With the latter method Tam et al. [8] obtained a sensitivity of 93% and a specifity of 96% using culture on McCoy cells as reference method. In our experience, however, this test involves highly monotonous work for laboratory staff in a dark room. Provided an enzyme immunoassay with a higher sensitivity and - notably for women- a higher specifity can be developed, it would be preferable to an immunofluorescence test as it permits the screening of large numbers of samples.

An improved model of the enzyme immunoassay is at present under evaluation.

References

1. Wingerson L. Two new tests for chlamydia get quick results without culture. JAMA 1983; 250: 2257-2259.
2. Aardom HA, de Hoop D, Iserief COA, Michel MF, Stolz E. Detection of *Neisseria gonorrhoeae* antigen by a solid-phase enzyme immunoassay. Brit J Vener Dis 1982; 58: 359-362.
3. Danielsson D, Moi H, Forslin L. Diagnosis of urogenital gonorrhoea by detecting gonococcal antigen with a solid-phase enzyme immunoassay (Gonozyme TM). J Clin Pathol 1983; 36: 674-677.
4. Schachter J, McCormack WM, Smith RF, Parks RM, Bailey R, Ohlin A. Enzyme immunoassay for diagnosis of gonorrhea. J Clin Microbiol 1984; 19: 57-59.
5. Stamm WE, Cole B, Fenneli C, et al. Antigen detection for the diagnosis of gonorrhea. J Clin Microbiol 1984; 19: 399-403.
6. Jones MF, Smith TF, Houglum AJ, Herrmann JE. Detection of *Chlamydia trachomatis* in genital specimens by the Chlamydiazyme test. J Clin Microbiol 1984; 20: 465-467.
7. Vecchio TJ. Predictive value of a single diagnostic test in unselected population. N Engl J Med 1966; 274: 1171-1173.
8. Tam MR, Stamm WE, Hunter Handsfield H, et al. Culture-independent diagnosis of *Chlamydia trachomatis* using monoclonal antibodies. N Engl J Med 1984; 310: 1146-1150.

CHAPTER 5

EXPERIENCE WITH A SOLID PHASE ENZYME IMMUNOASSAY FOR THE DETECTION OF NON-COMPLICATED CERVICAL INFECTION IN WOMEN CAUSED BY *CHLAMYDIA TRACHOMATIS.*

Abstract

To evaluate the usefulness of a solid phase enzyme immmunoassay for diagnosis of cervical chlamydial infections, samples of 246 women were tested using the assay and cell cultures. The overall sensitivity of the assay was 67% and specifity was 92.4%.

When the patients were classified into groups according to the semiquantitative leucocyte score (0-4) the highest prevalence of Chlamydia-positive culture (21.3%) and the highest sensitivity (77%) were found in patients with score 3-4. The mean absorbance in the assay was higher, and a higher percentage strong positive cultures was found in culture positive samples of patients with leucocyte score 3-4. The sensitivity of the immunoassay was 54% in samples with chlamydial culture with less than 20 inclusions per slide. Therefore this enzyme immunoassay failed to detect chlamydial infections in nearly the half of the patients with a low load of chlamydial antigen.

Introduction

During the past decade more attention has been paid to genital infections caused by *Chlamydia trachomatis*. In women the uncomplicated chlamydial genital infections can occur without any symptoms but the sequelae may be serious; endometritis, acute or chronic salpangitis, and perihepatitis. Salpingitis is associated with an increased risk of infertility and ectopic pregnancy due to post-infections damage to the fallopian tubes [1].

Genital infections due to *Chlamydia trachomatis* can cause major diagnostic problems. Most physicians have no facilities to culture *Chlamydia trachomatis*. The culture methods also have some serious disadvantages such as being laborious, expensive and time consuming. Technical problems in transportation and in culture methods can lead to false negative results. Men with symptoms of non-specific urethritis can be treated effectively with antibiotics against *Chlamydia trachomatis*. But in women usually with none of only subtle clinical manifestations, the diagnosis of chlamydial infection is more difficult [2,3].

Rapid diagnostic test which detect chlamydial antigens without the need to culture the organism may provide an effective tool for the early detection and subsequent

control of genital infections caused by *Chlamydia trachomatis* and to prevent complications by this microorganism. In the past, a rapid method using fluoresceine labelled monoclonal antibodies was introduced to identify *Chlamydia trachomatis* elementary bodies in genital smears [4-6]. A second rapid method for detection *Chlamydia trachomatis* antigen is a solid phase enzyme immunoassay [7-11]. In this study 246 women attending the University Hospital's venereology outpatient department were screened for *Chlamydia trachomatis* infection using two different methods. The results obtained using a rapid solid phase enzyme immunoassay [7-11] were compared to those obtained using a conventional cell culture technique.

Materials and Methods

Patients. Two hundred and forty six women, visiting the outpatient department for sexually transmissible diseases were examined. Cervical samples were collected from symptomatic as well as asymptomatic patients and from female contacts of males with non-specific urethritis. Patients were classified in various groups as follows: a. the semiquantative leucocyte number in the Gramstained cervical smear; b. age, and c. the outcome of the gonococcal culture.

Samples. In addition to the samples from the cervix for *Neisseria gonorrhoeae* culture and a Gram-stain smear, two separate samples were collected. The first was used to culture *Chlamydia trachomatis* and the second was used to perform the solid-phase enzyme immunoassay. The samples for the culture were taken after cleaning the portio from superfluous mucous with a metal shafted cottonwool-tipped swab. The swab was rotated in the endocervix for several seconds and then placed in a vial containing 2.0 ml of 0.2 M sucrose phosphate buffer (2 SP) containing 25 µg/ml gentamicin and 2.5 µg/ml amphotericin. The samples were stored in liquid nitrogen until processed. Plastic shaft swabs as recommended by the supplier (Abbott, USA) were used to obtain samples for the solid-phase enzyme immunoassay using the same technique and placed in a tube containing 0.1 ml specimen storage reagent (Abbott). All specimen were stored at 4^0 C and processed within 72 hours.

Cell-culture. Samples for culture were mixed on a Vortex mixer and 250 µl of the diluted sample was inoculated into duplicate flat bottom tubes containing DEAE

dextran pretreated full grown HeLa 229 monolayers on coverslips. The tubes were centrifuged for one hour at 2700 x g. After three days incubation at 37^0 C one coverslip was fixed and stained according to Giemsa. The whole coverslip was examined for inclusion bodies using darkfield illumination. Coverslips containing at least one inclusion were considerd to be positive. If more than 20 inclusions were observed in the coverlips of the first passage, the culture was considered to be strongly positive. To perform a second passage the duplicate culture was sonicated and 250 µl of the diluted sample was inoculated onto a fresh monolayer.

Solid Phase Enzyme Immmunoassay. The assay was an experimental research prototype for the detection of *Chlamydia trachomatis* antigen (Abbott). Prior to the assay 1 ml Chlamydiazyme specimen dilution buffer (Abbott) was added to each tube containing a swab with patient material. After incubation for 10 minutes at room temperature the tube was mixed on a Vortex mixer for three cycles of 15 seconds. The swab was rotated and pressed against the wall of the tube to squeeze out the excess fluid which was discarded. Two hundred microliter of sample material, positive and negative controls, were plated into the wells of a plastic tray. To each well, a bead was then added and the tray was incubated for 45 minutes in a waterbath at 37^0C. After rinsing 200 µl of an polyclonal rabbit anti-chlamydia-antibody preparation was added to each well. After incubation for 45 minutes at 37^0C and rinsing, 200 µl of goat horseradish peroxidase-conjugated antibody immunoglobulin G was added. After incubation for 45 minutes at 37^0C followed by rinsing, the beads were transferred to 5 ml cuvettes. The reaction was developed by the addition of 300 µl of the substrate (Orthophenylene diamine with H_2O_2). After incubation at room temperature for 30 minutes, the reaction was stopped by the additon of 1ml of N H_2SO_4. The absorbance was read at 492 nm on a Quantum spectrophotometer (Abbott). The cut-off point of a positive test was set at an optical density (OD) of 0.100 plus the mean absorbance observed in the three negative controls.

Statistical analysis. The Fisher's exact test and the Mann Whitney U test are used to determine statistical significance.

Results

When the patients were classified into groups according to the semi-quantative leucocyte score (0-4) in the Gram stained cervical smear, the concordant and discordant results between enzyme immunoassay and culture are shown in Table 1. The results of both detection methods were positive in 24 of the 246 patients. Culture was exclusively positive in 12 patients, enzyme immunoassay in 16 patients.

Table 1. The detection of *Chlamydia trachomatis* by enzyme immunoassay and cell culture and results of gonococcal culture in relation to leucocyte score.

		C. trachomatis				*N.gonorrh.*
Leucocyte score*	N	Assay pos. Culture pos.	Assay pos. Culture neg.	Assay neg. Culture pos.	Assay neg. Culture neg.	Culture pos.
0	26	2	3	2	19	0
1	82	5	5	3	69	3
2	77	7	3	4	63	8
3	48	6	4	3	35	11
4	13	4	1	0	8	3
Overall results	246	24	16	12	194	25

* 0 = no leucocytes per field
1 = 1-5 leucocytes per field
2 = 6-10 leucocytes per field
3 = 11-20 leucocytes per field
4 = >20 leucocytes per field

The prevalences of positive chlamydial and gonococcal cultures are shown in Table 2. For these subgroups the sensitivity and the specifity of the enzyme immunoassay versus culture and subculture expressed in percentages are also shown in Table 2.

In patients with a relatively low leucocyte number (0-2) in the Gram stained smear, the prevalence of a chlamydia-positive culture was 12.4% and the prevalence of a positive gonococcal culture was 6%. In this group the sensitivity

Table 2. The prevalence of positive cultures for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, and the sensitivity and the specifity of the enzyme immunoassay as compared to cell culture of *Chlamydia trachomatis*.

	Prevalence pos. culture		EIA *C. trachomatis*	
Leucocyte score	*C. trachomatis* (%)	*N. gonorrhoeae* (%)	sensitivity (%)	specifity (%)
0	15.4	0	50	86.4
1	9.8	3.7	62.5	93.2
2	14.3	10.4	63.6	95.5
3	18.8	22.9	66.7	89.7
4	30.8	23.1	100	88.9
Overall result	14.6	10.2	67	92.4

of the enzyme immunoassay as compared to culture was 61%.

In culture-positive patients with a leucocyte score of 0-2, the percentage of strongly positive cultures was 21.7. The mean absorbance in the enzyme immunoassay was 0.344.

In patients with a relatively high leucocyte number (3-4), the prevalence of a chlamydial positive culture was 21.3% (not significant). The prevalence of a positive gonococcal culture was 23% (significant, P = 0.004). The sensitivity of the assay was 77% (not significant) for this group.

In culture-positive patients with a leucocyte score of 3-4, the percentage of strongly positive cultures was 38.5% (not significant). The mean absorbance in the enzyme immunoassay was 1.144 (significant, P = 0.183).

In patients who were positive for *Neisseria gonorrhoeae* culture, the prevalence of a positive chlamydial culture was 24% (Table 3). In this group the sensitivity of the enzyme immunoassay as compared to cell culture was 50% and the specifity was 84.2%.

Table 3. Results of culture for *C.trachomatis* and sensitivity and specifity of the enzyme immunoassay versus culture in patients with positive of negative culture for *N.gonorrhoeae.*

Culture for N. gonorrhoeae	*C. trachomatis*		
	results of culture prevalence % (no.)	results of assay sensitivity % (no.)	specifity % (no.)
Positive	24 (6/25)	50 (3/6)	84.2 (16/18)
Negative	13.6 (30/221)	70 (21/30)	93.2 (178/191)

In patients with a negative culture for *Neisseria gonorrhoeae* the prevalence of a chlamydia-positive culture was 13.6%, the sensitivity was 70% and the specifity was 93.2%.

In Table 4 the relationship between age and the prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* is shown. In the younger age group (≤ 25 years) the prevalence of *Chlamydia trachomatis* was much higher (24.2%) as compared to the prevalence of *Neisseria gonorrhoeae* (13.7%). For both *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, the highest prevalences were observed in the younger age groups. There was little difference in the sensitivity and specifity of the enzyme immunoassay in the two age groups.

Table 4. Prevalence of positive cultures for *C.trachomatis* and *N.gonorrhoeae* and results of the enzyme immunoassay in two age groups.

Age group (years)	N	Prevalence		Results assay versus culture	
		N.gonorrhoeae % (no.)	*C.trachomatis* % (no.)	sensitivity % (no.)	specifity % (no.)
<25	95	13.7 (13/95)	24.2 (23/95)	69.6 (16/23)	91.7 (66/72)
>25	151	7.9 (12/151)	8.6 (13/151)	61.5 (8/13)	92.8 (128/138)

In six patients a mucopurulent cervical discharge was observed. Of these patients

one was positive both for chlamydial and gonococcal culture.

Of the nine known contacts of males with nonspecific urethritis two (22%) were positive for culture of *Neisseria gonorrhoeae* and three (33%) for *Chlamydia trachomatis*.

Twenty-six patients had less than 20 inclusions in the first slide of the *Chlamydia trachomatis*-culture. Fourteen of these 26 (54%) were positive in the enzyme immunoassay. Ten patients had a strong positive culture for *Chlamydia trachomatis* (more than 20 inclusions in the coverslip of the first passage). All of them (100%) were positive in the solid phase immunoassay. This difference was statistically significant ($P = 0.02$).

Discussion

In this study the overall sensitivity of the solid phase enzyme immunoassay as compared to cell culture was 67% and specifiticy was 92.4% with a prevalence of 14.6%.

Using the same immunoassay Jones et al [7] observed a sensitivity of 81% and a specifity of 98% in a group of men and women with urethritis and cervicitis.

Moi [9] observed a sensitivity of 86.3% and a specifity of 94.7% for the Chlamydiazyme in males. Pathier [1]) observed in 417 specimens from females with a low disease prevalence (8.1%) a sensitivity of 91.2% and a specifity of 96.6% as compared to cell culture. Cultures in all three studies were stained with iodine. In a previous study [11] the highest prevalences (12.5 - 27.9%) and the highest sensitivities (67-82%) for this enzyme immunoassay were observed in men with urethritis (more than 10 leucocytes in the sediment of the first voided urine). In men without urethritis, the prevalence varied from 4.9 to 10.4% and the sensitivities from 50 to 54%.

When the results of this study were classified into the semiquantative leucocyte score in the Gram-stained cervical smears, the highest prevalences (18.8% - 30.8%) and the highest sensitivities (66.7 - 100%) were observed in women with a leucocyte score of 3 to 4. These groups also showed the highest numbers of strong positive cultures (38%) and the highest mean absorbance (1.144) in the immunoassay. These results suggest that the antigen load in patients with

leucocyte score of 3 to 4 is higher than that in patients with leucocyte score of 0 to 2.

When the patients were classified in subgroups according to the outcome of the gonococcal culture, or according to their age, variations in the prevalence of Chlamydia positive culture were found but no clear relationship between the prevalence and the sensitivity was observed.

In the patients with strongly positive chlamydial cultures (≥ 20 inclusions per slide), the sensitivity of the enzyme immunoassay was 100%. In patients with chlamydial cultures with less than 20 inclusions per slide, the sensitivity of the enzyme immunoassay was 54%. Therefore the enzyme immunoassay failed to detect chlamydial infections in nearly half of the patients with a low load of chlamydial antigen.

For the evaluation of a new diagnostic technique it is extremely important to have also samples of patients with less than severe chlamydial infection. Selection criteria of patients can have influence on the sensitivity of this enzyme immunoassay. This variation ranged from 50% to 100% in the present study. Modification in enzyme immunoassay of cell culture can further influence the outcome of these comparative studies.

Acknowledgement

The authors thank Mrs. M. de Ridder-Goetjaer for preparation of the manuscript.

References

1. Weström L, Mårdh PA. Salpingitis. In: Holmes KK, Mårdh PA, Sparling PF, Wiesner PJ eds. Sexually Transmitted Diseases. 1st ed. New York: McGraw Hill 1984: 615-632.
2. Brunham RC, Paavonen J, Stevens CE, et al. Mucopurulent cervicitis: the ignored counterpart of urethritis in men. N Engl J Med 1984; 311: 1-6.
3. Hare MJ, Toone E, Taylor-Robinson D, et al. Follicular cervicitiscolposcopic appearances and association with *Chlamydia trachomatis*. Brit J Obstet Gyn 1981; 88: 174-180.
4. Tam MR, Stamm WE, Handsfield HH, et al. Culture-independent diagnosis of *Chlamydia trachomatis* using monoclonal antibodies. N Engl J Med 1984; 310: 1146-1150.
5. Uyeda CT, Welborn P, Ellison-Birang N, Shunk K, Tsaouse B. Rapid diagnosis of chlamydial infections with the Microtrak direct test. J Clin Microbiol 1984; 20: 948-950.
6. Wingerson L. Two new tests for Chlamydia get quick results without culture. JAMA 1983; 250: 2257-2259.
7. Jones MF, Smith TF, Houglum AJ, Herrmann JE. Detection of *Chlamydia trachomatis* in genital specimens by the Chlamydiazyme test. J Clinl Microbiol 1984; 20: 465-467.
8. Tjiam KH, van Heijst BYM, van Zuuren A, et al. Evaluation of an enzyme immunoassay for the diagnosis of chlamydial infections in urogenital specimens. J Clin Microbiol 1986; 23: 752-754.
9. Moi N, Danielsson D. Diagnosis of genital *Chlamydia trachomatis* infection in males by cell culture and antigen detection test. Eur J Clin Microbiol Infect Dis 1986; 5: 563-568.
10. Pothier P, Kazmierczak A. Comparison of cell culture with two direct Chlamydia tests using Immunofluorescence of enzyme-linked immunosorbent assay. Eur J Clin Microbiol 1986; 5: 569-572.
11. Van Ulsen J, van Zuuren A, Tjiam KH, van Heijst BYM, Stolz E. Solid phase enzyme immunoassay for detection of *Chlamydia trachomatis*. Eur J Clin Microbiol Infect Dis 1985; 4: 397-399.

CHAPTER 6

EXPERIENCE WITH A MODIFIED SOLID-PHASE ENZYME IMMUNOASSAY FOR DETECTION OF GONORRHOEA IN PROSTITUTES

Abstract

The modified Gonozyme Test (Abbott Laboratories; Chicago, IL), a new enzyme immunoassay for detection of *Neisseria gonorrhoeae*, was evaluated in testing of 266 prostitutes. When the enzyme immunoassay was used with material from the cervix, the prevalence of gonorrhoea was 8.4%. As compared with culture results, this rate represents a sensitivity of 88% and a specifity of 99.2%. A sensitivity of 71% and a specifity 98.9% were registered for tests of urethral material. In a comparison of the two tests without accepting either one as standard, the sensitivity of the enzyme immunoassay was 90% for material from the cervix and the specifity was 100%. There are several possible causes for discordance between results of culture and of enzyme immunoassay. The high predictive value of a negative enzyme immunoassay in screening urogenital samples from prostitutes makes this test very suitable for rapid elimination of gonorrhoea from this pool of patients.

Introduction

Women are normally screened for gonorrhoea by making a Gram stain of material from the cervix and urethra and a culture of material from the cervix, urethra, rectum and tonsils. The Gram stain has the advantage that the outcome is available very quickly. It is also specific, but its sensitivity, at best, is 65% [1]. A culture is much more sensitive and more specific but has the disadvantage that the result is not available for at least 48 hr; this incubation time means a fairly long interval between the first visit and treatment of the patient. For prostitutes in particular, there is a need for rapid, sensitive, and specific test for gonorrhoea.

A previous publication [2] reported on the use of a solid-phase enzyme immunoassay (EIA) in a study of the prevalence of *Neisseria gonorrhoeae* antigen in various groups of patients attending a sexually transmitted diseases (STD) clinic. When the test was used with a group of 102 prostitutes, its sensitivity and specifity appeared to be satisfactory. Due to the limited number of positive results, however, the range of the 95% confidence interval was rather large. In order to determine more precisely, the diagnostic value of both the EIA and culture was studied in a larger group of prostitutes. The importance of the EIA as an aid in detecting

sources of gonorrhoea among prostitutes is discussed on the basis of the results obtained.

Patients and Methods

Patients examined

A group of 266 prostitutes attending the outpatient clinic submitted to examinations every two weeks. A total of 572 examinations were performed during a three month period in 1983. The birthdate of all patients was recorded.

Collection of Specimens

Specimens were collected from the urethra and endocervix. Sampling material from the cervix was done with two cotton wool-tipped plastic swabs. One swab was used for EIA and one for culture. To obtain cervical material, mucus was removed from the cervix, and the swabs were rotated for several seconds in the endocervix. The swabs were then removed, and care was taken to avoid contact with the vaginal wall. Sampling of material from the urethra was done by use of two cotton wool-tipped plastic swabs, one for the EIA and one for culture. The sampling sequence of urethral and clinical specimens for EIA and culture was in accord with a list of random numbers.

The swabs used for EIA were immersed in 0.2 ml of specimen storage agent (Abbott Diagnostic Products GmbH, Wiesbaden, West Germany) and stored at 4 ^{0}C for a period not exceeding five days. According to the manufacturer, results of the EIA are still reliable if the test is performed within this period.

Culture Procedure

The swab used for obtaining the culture specimen was immediately inoculated onto modified Thayer-Martin medium and onto chocolate agar. The plates were incubated at 37 ^{0}C for two days in an atmosphere containing 3-5% CO_2. Gonococci were identified by gram-staining and by sugar fermentation tests.

Assay Procedure-

The Gonozyme diagnostic kit contains the following reagents: polystyrene beads pretreated to facilitate adsorption of *N.gonorrhoeae* antigen; antibodies to *N.gonorrhoeae* obtained by injecting gonococcal strains into rabbits, the diversity of the antibodies having been extended since the previous study [2] by injecting also

antigens from gonococcal strains isolated from patients with false-negative results in the Gonozyme test, the first prototype of this test; a goat anti-rabbit IgG horseradish conjugate; a positive and a negative control; specimen storage solution; and specimen dilution buffer (Abbott). The substrate in the enzyme immunoassay was *o*-phenylenediamine, plus H_2O_2.

Before assay 1ml of dilution buffer (Abbott) was added to the tube containing the swab with the clinical material. After 2-5 min the swab was shaken vigorously on a vortex mixer for 15 sec, expressed, rotated against the wall of the tube in order to remove excess fluid, and discarded. The beads were immersed in 0.2 ml of the suspension in the wells of a tray. After immersion of the beads the tray was covered with an adhesive seal and incubated for 45 min in a waterbath (37 ^{0}C). For removal of unadsorbed material, the beads were then washed three times in a total volume of 15 ml of distilled water with an Abbott Pentawasher II. Next, 0.2 ml of antibody to *N.gonorrhoeae* and 0.2 ml of antibody-enzyme conjugate were added consecutively to each well. Each step was followed by incubation of the tray and washing as indicated. After the last washing each bead was transferred to a 5 ml cuvette, and 0.3 ml of freshly prepared substrate was added. After incubation for 30 min at room temperature (~22 ^{0}C), the enzyme reaction was stopped by addition of 1 ml of 1 N HCl; the extinction was measured against a substrate blank on a Quantum spectrophotometric analyzer (at 492 nm). The cut-off point of a positive test was set at an OD of 0.19 plus the mean extinction observed for three negative controls.

Statistical Method

The X^2 method for independent samples was used for determination of the level of significance. Sensitivities, specifities, and predictive values were calculated according to the method of Vecchio [3]. In addition, the approach described by Hui and Walter [4] was applied for estimation of the sensitivities and specifities of both tests (EIA and culture) simultaneously without accepting either as standard. This method was applied to cervical samples only. It required division of the subjects into two subgroups according to objective criteria (age).

Results

Concordant and discordant results between culture and EIA are shown in Table 1. Discordant results between culture and EIA were observed more often among urethral than among cervical samples.

Table 1. Numbers of specimens with concordant and discordant results between culture and Enzyme Immunoassay (EIA) for cervical, urethral, and cervical and/or urethral specimens.

Result	Cervix	Urethra	Cervix and/or Urethra
Culture +, EIA +	44	25	47
Culture - , EIA +	4	6	6
Culture +, EIA -	6	10	6
Culture - , EIA -	518	531	513

The prevalence of positive cultures as well as the sensitivities, specifities, and the predictive values of the EIA as compared with culture for specimens of various origin are shown in Table 2. The prevalence of positive cultures was lower for the urethral (6.1%) than for the cervical specimens (8.7%). The sensitivity of the

Table 2. Prevalences of positive cultures, sensitivities en specifities of Enzyme Immunoassay (EIA) vs. culture, and predictive values of positive and negative EIA.

	Percentage (95 % Confidence Limits)		
Measurement	Cervix	Urethra	Cervix/Urethra
Prevalence of positive culture	8.7 (6.6-11.4)	6.1 (4.3-8.4)	9.3 (7.0-11.9)
Sensitivity of EIA	88 (76-95)	71 (54-85)	89 (77-96)
Specificity of EIA	99.2 (98.1-99.8)	98.9 (97.6-99.6)	98.8 (97.5-99.6)
Predictive value of positive EIA	92 (80-98)	81 (63-93)	89 (77-96)
Predictive value of negative EIA	98.9 (97.5-99.6)	98.2 (96.6-99.1)	98.8 (97.5-99.6)

EIA for cervical samples was 88% (95% confidence limits, 76-95%); the specifity was 99.2% (95% confidence limits, 98.1-99.8%). The corresponding figures for

urethral samples were 71% (54-85%) and 98.9% (97.6-99.6%). The figures obtained when the results for cervical and urethral samples are combined hardly differ fom the results with cervical specimens alone.

For assessment of the sensitivities and specifities of EIA and culture without accepting either as standard [4], women were divided into two groups on the basis of age; one group was born between 1928 and 1955 (group 1), and the other was born between 1956 and 1967 (group 2). Concordant and discordant results between culture and EIA for cervical specimens for women of these age groups are shown in Table 3.

Table 3. Concordant and discordant results between culture and Enzyme Immunoassay (EIA) of cervical specimens from two age groups.

	No. of specimens	
Result	Group 1 [1]	Group 2 [2]
Culture +, EIA +	13	31
Culture - , EIA +	1	3
Culture +, EIA -	2	4
Culture - , EIA -	254	264

[1]Women born between 1928 and 1955
[2]Women born between 1956 and 1967

As shown in Table 4, the prevalence of a positive culture among patients from group 1 (5.6%) was about half the prevalence among patients from group 2 (11.6%). No significant differences in sensitivity and specifity of culture (vs. EIA) and EIA (vs. culture) were observed between groups 1 and 2.

The difference in prevalence and the agreement of sensitivities and specifities between the two groups are essential conditons for the application of the statistical approach described by Hui and Walter [4]. If neither culture nor EIA is used as the standard, sensitivities of both diagnostic methods equal 90% and the equivalent specifities approach 100% (Table 5).

Table 4. Prevalence of positive culture and sensitivities and specifities of culture and Enzyme Immunoassay (EIA): analysis of cervical specimens for two age groups, with culture or EIA as standard.

	Percentage with Indicated Result	
Measurement	Group 1	Group 2
Prevalence of positive culture	5.6	11.6
Sensitivity* of positive culture	93	91
Specifity* of culture	99.2	98.5
Sensitivity† of EIA	87	89
Specifity† of EIA	99.6	98.8

*With EIA as standard.
†With culture as standard.

Table 5. Prevalence of gonorrhoea and sensitivities and specifitities of culture and Enzyme Immunoassay (EIA), with neither culture nor EIA as standard.

Measurement	Percentage (95% Confidence Limits)
Prevalence of gonorrhoea group 1	5.9 (1.7-10.3)
Prevalence of gonorrhoea group 2	12.7 (7.7-17.7)
Sensitivity of culture	90 (70-100)
Specifity of culture	99.8 (97.4-100)
Sensitivity of EIA	90 (66-100)
Specifity of EIA	100 (98.3-100)

The influence of the sampling order of cervical and urethral specimens on results of EIA and culture is shown in Table 6. For cervical specimens the sensitivity of the EIA was 91% (95% confidence limits, 72-99%) if the sample was taken first, and only 85% (66-96%) if the sample was taken second. For urethral specimens the sensitivity of the EIA was 88% (64-99%) if the sample was taken first, and only 56% (31-78%) if the sample was taken second. For cervical specimens the specifity

of the EIA was 99.3% (95% confidence limits, 97.3-99.9%) if the sample was taken first, and 99.2% (97.2-99.9%) if the sample was taken second. For urethral specimens the specifity of the EIA was 98.9% (96.9-99.8%) if the sample was taken first, and 98.9% (96.7-99.8%) if the sample was taken second. For both the cervix and the urethra, use of the X^2 test showed that the differences observed were not significant.

Table 6. Effect of sampling order of cervical and urethral specimens on the results of culture and Enzyme Immunoassay (EIA).

Sample	Cult. + EIA +	Cult. - EIA +	Cult. + EIA -	Cult. - EIA -
Cervix				
EIA sample first	21*	2	2	264
Culture sample first	23	2	4	425
Urethra				
EIA sample first	15	3	2	269
Culture sample first	10	3	8	262

*Number of specimens giving indicated result.

Cervical samples from four patients were positive in the EIA but gave negative cultures. The urethral sample from one of these patients was positive in both EIA and culture. Another patient who was suspected of having gonoccoccal adnexitis sought treatment elsewhere, and thus evaluation was impossible. Urethral samples from six patients were positive in the EIA only. Cervical samples from three of these patients were positive in both EIA and culture. The remaining three patients had cervical samples negative in both EIA and culture. Cervical specimens from six patients gave positive cultures but negative EIA results. One of these women had a positive result for both EIA and culture of the urethral specimen.

Discussion

On the basis of cervical cultures, the prevalence of gonorrhoea among the prostitutes screened in this study was 8.7% (Table 2). In view of the regular check-ups ondergone by these women, this prevalence is rather high.

For cervical samples the EIA showed a sensitivity of 88% and a specifity of 99.2% (Table 2). The high predictive value of a negative EIA (98.9%, Table 2) for cervical samples makes this test very suitable for gonorrhoea screening of a group of prostitutes with a fairly high prevalence of gonorrhoea.

In the analysis of a new diagnostic test for gonorrhoea, culture for *N. gonorrhoeae* is usually accepted as the standard; its sensitivity and specifity are taken to be 100%. By dividing the patients according to age into two populations with different prevalences of positive cultures (Tables 3 and 4), it became possible to apply a statistical model in which neither test served as standard [4]. According to this model the sensitivity of culture was 90% (95% confidence limits, 70-100%), while the sensitivity of the EIA was also 90% (95% confidence limits, 66-100%; Table 5). In our study EIA was very sensitive and specific in comparison with culture (Table 5).

Aardoom et al. [2] used the EIA among a group of 102 prostitutes and obtained a sensitivity of 92% (95% confidence limits, 62-100%) and a specifity of 97% (95% confidence limits, 91-99%). Danielson et al.[5] found that the EIA had a sensitivity of 91% and a specifity of 100% among a group of 150 women attending a STD clinic. These results were better than those observed by others [6-8].

In our study we observed that, when sample material for the EIA was taken before that for culture, the sensitivity of the EIA was higher. This observation applied more to urethral than to cervical samples (Table 6). However, the differences observed were not statistically significant (X^2 test).

In a number of cases there was discordance between results of the EIA and results of culture. Some patients had a positive EIA and a negative culture. This finding could be the result of the presence of dead gonococci killed by previous use of antibiotics or disinfectants. Or, the discrepancy might be attributable to cross-reactions with other microorganisms or increased sensitivity of gonococci to antibiotics contained in selective nutrient media. A negative EIA and positive culture

might result from a deficiency in the spectrum of the antibodies contained in the EIA kit. On the other hand, it is conceivable that gonococcal antigen is masked by the presence of local antibodies of the IgA type.

A major disadvantage of performing the EIA without culture is the lack of insight into the resistance of *N.gonorrhoeae* to antibiotics, in particular a lack of insight into the prevalence of *ß*-lactamase-producing gonococci. However, rapid tests like the EIA can be very important aids in counteracting dissemination of *N.gonorrhoeae* because early identification of infected persons permits early treatment. This importance is even greater when such tests are used for identification of promiscuous carriers of gonococci, e.g., sex-club women and prostitutes. Within the latter group women who are positive could be informed of the result on the day of the test and be asked to refrain from work and to report next day for culture and treatment. Indeed, in Rotterdam the EIA is used in such a way. Enzyme immunoassay-positive patients are tested again before treatment, and gonococcal cultures are performed during this investigation.

References

1. Young H. Advances in routine procedures for the diagnosis of gonorrhoea. In: Harris, LRW, ed. Recent advances in sexually transmitted diseases. Edinburgh: Chirchill Livingstone, 1981: 59.
2. Aardoom HA, De Hoop D, Iserief COA, Michiel MF, Stolz E. Detection of *Neisseria gonorrhoeae* antigen by a solid-phase enzyme immunoassay. Br J Vener Dis 1982; 58:359-62.
3. Vecchio TJ. Predictive value of a single diagnostic test in unselected populations. N Engl J Med 1966; 274: 1171-3.
4. Hui SL, Walter SD. Estimating the error rates of diagnostictests. Biometrics 1980; 36: 167-71.
5. Danielsson D, Moi H, Forslin L. Diagnosis of urogenital gonorrhoea by detecting gonococcal antigen with a solid phase enzyme immunoassay (Gonozyme TM). J Clin Pathol 1983; 36: 674-7.
6. Stamm WE, Cole B, Fennell C, et al. Antigen detection for the diagnosis of gonorrhea. J Clin Microbiol 1984; 19: 399-403.
7. Papasian CJ, Bartholomew WR, Amsterdam D. Validity of an enzyme immunoassay for detection of *Neisseria gonorrhoeae* antigens. J Clin Microbiol 1984; 19: 347-50.
8. Schachter J, McCormack WM, Smith RF, Parks RM, Bailey R,Ohlin AC. Enzyme immunoassay for diagnosis of gonorrhoea. J Clin Microbiol 1984; 19: 57-9.

CHAPTER 7

EVALUATION OF AN ENZYME IMMUNOASSAY FOR DETECTION OF HERPES SIMPLEX VIRUS ANTIGEN IN GENITAL LESIONS

Abstract

To evaluate a non-marketed research prototype of a solid-phase enzyme immunoassay for detection of herpes simplex virus in genital lesions, 154 clinical specimens were collected from 127 men and 27 women with symptoms suggestive of herpes simplex virus infection (erythema, vesicles, ulcers and crustae). The samples were tested using the assay and cultures on four monolayers of human embryonic lung fibroblasts and Vero cells. When the culture was used as reference method, sensitivity was 76.9% and specifity 100% (prevalence 42.4%). Comparison of results by patient group showed that sensitivity was highest in material from patients with vesicles and ulcers. The highest sensitivity was obtained in specimens which developed a cytopathological effect within 48 h and in specimens with three or four positive cell cultures. These findings suggest that the assay is more successful in specimens with high virus titres. The enzyme immunoassay was found to be a rapid, moderately sensitive, highly specific test for detection of herpes simplex virus from genital lesions, but the usefulness of the assay is limited and culture methods should be preferred.

Introduction

The conventional culture method has so far been the most sensitive and specific technique of detecting herpes simplex virus (HSV). In combination with immunofluorescence using type specific monoclonal antibodies the technique can be used to distinguish between HSV-1 and HSV-2. However, at least one day is required before the cytopathogenic effect (CPE) becomes visible; in some cases the culture only proves to be positive after as many as ten days due to the low amount of virus in the inoculum. Thus there is a need for a rapid, sensitive and specific diagnostic method. Immunofluorescence is very satisfactory for confirming culture results and for typing, however its sensitivity is too low to warrant its use as a direct test [1]. Several ELISA systems for direct HSV antigen detection have been evaluated [2-6]. We evaluated a non-marketed research prototype of a sandwich method EIA in patients with genital lesions suggestive of HSV infection.

Materials and Methods

Patients. Clinical specimens were collected from genital lesions of 127 men and 27 women with symptoms suggestive of HSV infection (erythema, ulcers, vesicles, crustae) and symptoms less suggestive of HSV (wounds, erosions).

Samples. The samples were taken with the aid of a dry cottonwool-tipped plastic-shafted swab by firmly rubbing the base of the lesion. Two samples were collected from each patient. The first sample (A) was placed in a tube containing 1 ml transport medium (Abbott, USA) and used for EIA and culture. The second sample (B) was placed in a transport medium of the Regional Virological Laboratory, Rotterdam (3.5 ml Hanks salt solution supplemented with 0.5% gelatin, gentamicin and nystatin) and used for a second culture. The major difference between the two transport media was that the Abbott medium did not contain nystatin. All samples were stored at 4 ^{0}C for no longer than three days until processed.

Culture. HSV was cultured on monolayers of human embryonic lung fibroblasts (HEL) and Vero cells (Flow Laboratories, UK). After removal of the medium fluid from the tubes 200 µl of the patient samples was inoculated for 2 h at 35^{0} C. The inoculum was then replaced by 1.5 ml maintenance medium (MEM-Hanks, Gibco) and gentamicin, supplemented with 5% fetal bovine serum and 1% newborn bovine serum for HEL and Vero-cells respectively, and the tubes were incubated at 35 ^{0}C. They were examined after overnight incubation (or after two days on the weekend) and then every two days for 14 days. In the case of a positive culture the virus was subtyped by indirect immunofluorescence using type-specific monoclonal antibodies (Orthodiagnostics, Belgium).

Enzyme immunoassay. The EIA was a non-marketed research prototype (Abbott) for detection of HSV antigen. The tubes containing the clinical samples were shaken on the Vortex for 15 s. The excess fluid from the swab was removed by pressing and rotating the swab against the side of the tube, whereupon the swab was discarded. A polystyrene bead pretreated to permit adsorption of HSV was added to each well of a plastic plate and 200 µl of sample material was then introduced into the wells. Three negative controls and a panel of four positive controls with the different antigen titres in duplicate were tested simultaneously. The wells were covered with adhesive seals and the plates were incu-

bated for 120 min in a water bath at 37 ^{0}C. After a washing procedure three rinsings with distilled water using a pentawasher (Abbott), 200 µl antibody preparation (rabbit) and then 200 µl conjugate (goat horseradish peroxidase-conjugated antibody to rabbit immunoglobulin G) were added. Each step was followed by incubation for 60 min in a water bath at 37 ^{0}C and the washing procedure described above. The beads were then transferred to 5 ml cuvettes; 300 µl substrate (orthophenylene diamine with H_20_2) was added and the cuvettes were incubated for 30 min at room temperature. The reaction was stopped by adding 1ml N H_2SO_4. This resulted in a yellow colour change in samples containing HSV antigen. After mixing the colour intensity was read at 492 nm on a Quantum spectrophotometric analyser (Abbott) against a substrate blank. Test results with an absorbance value of 0.100 OD plus the average of negative controls were regarded as positive.

Statistical Analysis. The sensitivity, specifity and predictive value of a positive and a negative test result were calculated by the method of Vecchio [7]. The EIA results were compared with the culture results for samples A and B and with the combined results.

Results

Comparing the EIA of sample A with the tissue culture results of samples A and B together, 50 of the total of 154 samples were positive both in the EIA and in cell culture, and 15 were positive only in cell culture (Table 1). Not a single sample was positive in the EIA and negative in combined cell culture methods. For the entire group this implied a sensitivity of 76.9% (90% confidence limits 66.71-85.1) and a specifity of 100% (95% confidence limits 96.69-100.00) (Table 2). The sensitivity of the EIA in the 127 men was 79.6% (39/49) and that in the 27 women was 68.8% (11/16). In the patient groups with various symptoms the sensitivity varied from 57.1% to 87.1%. The specifity was again 100% for all groups.

Table 1: Detection of herpes simplex virus by enzyme immunoassay and culture in 154 patients with various symptoms.

Patient group	N	Assay pos. Culture pos.	Assay neg. Culture pos.	Assay neg. Culture neg.
Men	127	39	10	78
Women	27	11	5	11
Erythema	41	11	7	23
Vesicles	12	7	2	3
Ulcers	65	27	4	34
Crustae	8	0	0	8
Other symptoms	28	5	2	21

Table 2: Prevalence of a positive herpes simplex virus culture, sensitivity, specifity and predictive values of a positive and negative result of the enzyme immunoassay versus culture in various patient groups.

Patient group	N	Prevalence pos. culture	Sensitivity	Specifity	PPV	NPV
Men	127	49/127	39/49	78/78	39/39	78/88
Women	27	16/27	11/16	11/11	11/11	11/16
Erythema	41	18/41	11/18	23/23	11/11	23/30
Vesicles	12	9/12	7/9	3/3	7/7	3/5
Ulcers	65	31/65	27/31	34/34	27/27	34/38
Crustae	8	0/8	0/0	8/8	0/0	8/8
Other	28	7/28	5/7	21/21	5/5	21/23

The results with samples A and B are listed in Table 3. Of the sample A cultures 51 proved positive; 8 of these were missed by the EIA (sensitivity 84.3%). Four samples were false positive in the EIA (specificity 95.9%), however, these proved to be positive in the sample B cultures from the same patients.

Table 3: EIA results compared with cell culture results in two separate samples transported in a commercial medium not containing nystatin (Sample A) and a medium of the Regional Virological Laboratory, Rotterdam (Sample B).

	N	EIA+ Cult+	EIA- Cult+	EIA+ Cult-	EIA- Cult-	Preva-lence	Sensi-tivity	Specifity
A	149	43	8	4	94	34.2% (51/149)	84.3% (43/51)	95.5% (94/98)
B	150	46	12	0	92	38.7% (58/150)	79.3% (46/58)	100% (92/92)

On comparison with culture results for sample B, the sensitivity of the EIA was 79.3% and the specifity 100%.

HSV-1 was isolated in tissue culture from 11 of the total 65 positive cultures and HSV- 2 from 54. The EIA was positive for HSV-1 in 10 (sensitivity 91%) and for HSV-2 in 40 instances (sensitivity 74%). The tissue cultures were studied for CPE over a period of 14 days. After two days 41.5% of the positive cultures showed a CPE, after three days 65%, after four days 85% and after five days 92%. HSV-1 was cultured for an average of 4.6 days before a CPE occurred, and HSV-2 for an average of 2.9 days. In Table 4 the sensitivity of the EIA is related to the number of days required for the culture to show a CPE.

For HSV-2 , 24 cultures were positive after two days. Of these, two were missed by the EIA (sensitivity 91.7%). In 30 positive cultures three or more

Table 4: Relationship between number of days until cytopathological effect (CPE) develops in tissue culture and EIA sensitivity for HSV types 1 and 2.

HSV type	Number of days for CPE	EIA pos Cult pos	EIA neg Cult pos	Sensitivity
Type 1	1st + 2nd day	3	0	3/3 (100%)
	≥ 3 days	7	1	7/8 (88%)
Type 2	1st + 2nd day	22	2	22/24 (92%)
	≥ 3 days	19	11	19/30 (63%)

Table 5: Relationship between number of positive cell cultures of the four cell cultures started per patient sample and the EIA results.

Number positive cultures	Number	% of total	EIA pos Cult neg	EIA neg Cult pos	Sensitivity EIA
1	5	8	3	2	60%
2	20	31	11	9	55%
3	8	14	6	2	75%
4	32	48	30	2	94%

days were required before a CPE was observed; 11 were missed by the EIA (sensitivity 63.3%).

In Table 5 the sensitivity of the EIA is related to the number of positive cultures per patient. For samples with 1 and 2 positive cultures the sensitivity was 60% and 55% respectively, while for samples with 3 and 4 positive cultures the sensitivity was 75% and 93% respectively. For samples from vesicles on average 3.6 of the 4 cultures were positive; the corresponding figure samples from ulcers and erythema was 3.0 and 2.6 respectively.

Discussion

Other investigators [2-6] have already evaluated several ELISA systems for direct detection of HSV antigen and the first results were very promising. Vestergaard [2] reported 97% agreement with culture results. Land [3], using commercially available antibodies against HSV in a personnally developed ELISA system, attained 94% sensitivity and 93% specifity in 457 samples from patients.

Using a commercial ELISA system (Orthodiagnostics), Morgan [4] attained 72.3% sensitivity and 100% specifity in 263 samples from symptomatic patients. Using the same test, Warford [5] obtained poor results with cervical samples from asymptomatic women attending an obstetric out-patient clinic. Sensitivity in this study was 40-44% and many false-positive test results were obtained. Warford's results with skin lesions were better. Using the same ELISA system, Sewell [6] attained 69.7% sensitivity and 93.3% specifity in 136 samples from patients with suspected HSV infections. In this last study the EIA (Orthodiagnostics) was found

to be more sensitive in clinical samples which rapidly developed a CPE in the culture. In our study using a non-marketed research prototype of an ELISA for HSV antigen, the sensitivity of the EIA was 76.9% in relation to the combined culture results with samples A and B, and the specifity 100%. In routine works, however, an additional sample is rarely used. The first swab, which was placed in Abbott's transport medium, was found to yield fewer positive results than the medium of the Regional Virological Laboratory. The number of women in our study was too small to warrant conclusions on the sensitivity of the EIA in this group. The same applies for the groups with vesicles and crustae and the group with other symptoms. A striking fact is that HSV was cultured from 7 of the 21 patients with less obvious lesions. The sensitivity of the EIA was highest in patients with ulcers (87%). As ulcers may pose more diagnostic problems than vesicles, a rapid diagnostic test could be useful in such cases. HSV-1 was found in 11 patients, however to determine the sensitivity of the test for type 1, a larger group of patients with HSV-1 would have to be studied.

In this study we used two criteria to estimate roughly the amount of virus antigen: the first was the speed with which the cell cultures developed a CPE, and the second was the number out of a total of four tissue cultures showing a CPE. These variables are however influenced by virus characteristics, such as rate of growth and preference for a particular cell type. In the samples containing more virus particles according to the above criteria, the EIA gave distinctly better test results. New diagnostic tests should also be evaluated in patient samples containing only little antigen. A disadvantage of the EIA method is that it is not possible to perform further tests to differentiate between HSV-1 and HSV-2 since the virus is not isolated. The lower sensitivity in comparison with the culture method is another disadvantage which would be of particular importance in patients suffering from serious herpetic infections. Although not applicable to the non-commercial prototype studied here, the advantages of the EIA method in general are the low cost in comparison with culture, the speed of the test in which results can be known within six hours, and the fact that less quallified laboratory personnel can perform the test. In conclusion, the usefulness of the non-commercial prototype we tested would appear to be limited and culture methods are still to be preferred.

References

1. Moseley RC, Corey L, Benjamin D, Winter C, Remington ML. Comparison of viral isolation, direct immunofluorescence and indirect immunoperoxidasetechniques for detection of genital herpes simplex virus infection. J Clin Microbiol 1981; 13: 913-918.
2. Vestergaard BF, Jensen O. Diagnosis and typing of herpes simplex virus in clinical specimens by the enzymne-linked immunosorbent assay. In: Nahmias AJ, ed.): The human herpes virusses: an interdisciplinary perspective. New York: Elsevier 1981: 391-394.
3. Land SA, Skurrie IJ, Gilbert GL. Rapid diagnosis of herpes simplex virus infections by enzyme-linked immunosorbent assay. J Clin Microbiol 1984; 19: 865-869.
4. Morgan MA, Smith TF. Evaluation of an enzyme-linked immunosorbent assay for the detection of herpes simplex virus antigen. J Clin Microbiol 1984; 19: 730-732.
5. Warford AL, Levy RA, Rekrut KA. Evaluation of a commercial enzyme-linked immmunosorbent assay for detection of herpes simplex virus antigen. J Clin Microbiol 1984, 20: 490-493.
6. Sewell DL, Horn SA. Evaluation of a commercial enzyme-linked immunosorbent assay for the detection of herpes simplex virus. J Clin Microbiol 1985; 21: 457-458.
7. Vecchio TJ. Predictive value of a single diagnostic test in unselected populations. N Engl J Med 1966; 274: 1171-1173.

CHAPTER 8

EVALUATION OF A DOT IMMUNOBINDING ASSAY FOR DETECTION OF HERPES SIMPLEX VIRUS ANTIBODIES

Abstract.

To evaluate the value of a dot immunobinding assay (DIA) using glycoprotein G from herpes simplex virus type 2 (HSV-2) for detection of anti-HSV-2 antibodies in sera of 219 patients, the sera were tested using the DIA and Western Blot Analyses (WBA). The highest number of positive results were observed in patients with recurrent episodes. From 44 patients with recurrent HSV-2 infection, 39 (88%) were positive in the DIA and 41 (93%) were positive in WBA. In 82 sera of patients with HSV-2 infections 1-5 weeks after onset, the sensitivities of both assays were much lower: 40% for WBA and 20% for DIA. Fifty sera from schoolchildren reacted neither in WBA nor in DIA to detect HSV-2 antibodies. In 47 patients with early genital or extragenital HSV-1 infections, six were positive for HSV-2 antibodies in WBA and three in DIA. The majority (4/6) of the discordant results occurred in patients with genital lesions. In 19 patients with recurrent HSV-1 infection, one serum reacted in DIA and WBA. From 13 patients with recurrent herpes labialis two sera reacted in DIA and in WBA. In conclusion the DIA using glycoprotein G of HSV-2 is very useful for rapidly testing large numbers of sera for HSV-2 antibodies. In both assays, the best results were obtained in patients with recurrent episodes.

Introduction

Herpes simplex virus (HSV) can cause lesions at many different sites. In 80% of the cases, genital lesions are caused by herpes simplex virus type 2 (HSV-2), whereas lesions located elsewhere on the body are usually caused by herpes simplex virus type 1 (HSV-1). However, the nature and the location of the lesions provide insufficient information for distinguishing between the two types which requires isolation and typing of the virus. In order to establish whether a patient has ever been infected the detection of type-specific antibodies would be useful. The two viruses have a 50% homology in their genome so that they have similar antigenic determinants. For this reason, it is difficult to develop immunological assays which can be used to distinguish circulating antibodies against HSV-1 from circulating antibodies against HSV-2. The methods most commonly used to distinguish the two types of antibodies are based on the difference in titers of neutralizing antibodies against the two types (Rawl's index) [1]. Virus neutralization

tests are very laborious. The sensitivity and the specifity of such tests are low, especially when antibodies against both virus types are present in the same patient. Neither the complement-fixation test (CF) nor the immunofluorescence test can be used to detect type-specific antibodies. However, the complement-fixation test in combination with immunofluorescence test for IgM antibodies is very useful for distinguishing between early and recurrent infections. Western Blot analysis has been discribed by several authors [2-7], and is more useful for demonstrating antibodies to HSV-2. In 1984, a previously described HSV-2 induced glycoprotein G (glycoprotein G2) with a molecular weigth of 92 KD [8] was identified as a HSV-2-specific protein [9, 10]. This protein, purified using immuno-affinity columns or columns containing helix pomatia lectin can be used in various serologic assays [6,7,11-13]. In the present report, our results with the WBA and the DIA using helix pomatia (HPA)-lectin column purified glycoprotein G2 as antigen are described. Sera from patients in whom cultures for HSV-1 or HSV-2 were positive and sera from controls were tested in these two assays.

Patients and Methods

Patients. If possible acute phase sera and reconvalescence sera were obtained from patients with culture positive HSV-1 and HSV-2 infections. The patients with HSV-2 infections were divided in two groups. Group A consisted of 41 patients with early infections. A patient was recorded as having an early infection if a seroconversion was either measured in the complement-fixation test for HSV, or no seroconversion was measured but IgM antibodies were present. Group B consisted of 44 patients with late infections. A patient was recorded as having a late infection if the patient either had a history of recurrent episodes with HSV or HSV antibodies were present in complement-fixation test, but no seroconversion and no IgM antibodies were detected. Sera of 66 patients with culture positive HSV-1 infections, sera of 12 patients with recurrent herpes labialis and sera of 19 patients with Varicella zostervirus infections were tested. The control group consisted of 50 schoolchildren between the age of 6-10 years, because HSV-2 infections would be unlikely at this age.

Antigen preparations. HEp2- cells were grown in Optimem medium (Gibco, UK)

supplemented with 4% fetal bovine serum and 1% glutamine. Hep-2 cells were maintained in Optimem supplemented with 2% fetal bovine serum and 1% glutamine. Semi-confluent monolayers in 75 cm^2 plastic flasks were infected with HSV-1 of HSV-2 (5-10 pfu/cell) isolated from two patients. When cytopathogenic effect was observed in 75% of the cells (for HSV-1 after approximately 24h, for HSV-2 after approximately 72h), the cells were scraped into the medium, centrifuged for 10 min at 500 x g and washed three times with cold phos-phate-buffered saline (PBS, pH 7.2). The pellet was then resuspended in 0.6 ml PBS per flask and the protein concentration was measured (Biorad protein assay microprocedure). The suspension was pelleted again. For WBA [2,3] the pellet was resuspended in electrophoresis sample buffer (ESB) containing 1% Sodium dodecyl sulphate (SDS), 1% mercaptoethanol, 0.0625 M Tris, 10% glycerol, 0.001% bromphenol blue at a final protein concentration of 700-1000 µg/ml, boiled for 10 min and stored at -80 ^{0}C until use. For immunodotting [14] the pellet was resuspended in 4 ml 1% Triton X100 in Tris-buffered saline (TBS) pH 7.5. After extraction in the cold for 10 min, the suspension was centrifuged for 60 min at 100,00 x g at 4 ^{0}C. The supernatant was stored at -80 ^{0}C until use. Mock infected cells were treated in the same way.

Western Blot Analysis. The frozen HSV- and Mock infected cell-lysates in ESB were thawed and loaded onto a 9% discontinous polyacrylamide gel containing 0.1% SDS and cross-linked with 0.23% methylenbisacrylamide [2,3]. After electrophoresis, the proteins were transferred onto nitrocellulose (NC) sheets. Dried NC sheets were stored at -20 ^{0}C

Lectin affinity chromatography. Helix pomatia Lectin-Sepharose 6MB columns (Pharmacia, Sweden) in 2 ml plastic syringes (bed volume 1ml) with a filter paper (nr 3, Whatman, England) on the bottom were washed with 10 bed volumes starting buffer (1% Triton X100 in TBS containing 0.15M NaCl, 0.02M Tris, pH 7.5). One ml of the thawed HSV-1, HSV-2 or control antigen was added and incubated for 15 min at room temperature. After incubation, the non-bound glycoproteins were washed away with 10 bed volumes of starting buffer. The bound glycoproteins were eluated with 5mM N-acetylgalactosamine (Sigma, USA) in the same buffer [11-15].

Fractions of 0.05 ml were collected and fractions 2 and 3 were pooled and stored in glass ampules at -80 C. One µl of the eluate of HSV-1, HSV-2 and Mock infected cells was placed on NC strips of about 7 x 50 mm. The strips were dried overnight at room temperature and used immediately or stored at -20 ^{0}C.

Enzyme assay. Nitrocellulose strips for WBA and NC strips carrying the HPA lectin-purified antigens for DIA were incubated together with 10 ml blocking buffer (PBS pH 7.2 with 5% goat serum and 5% non-fat dried milk) during 10 min at room temperature on a rotating platform. The 100 µl of test serum was added and incubation was continued overnight at room temperature. Thereafter, the strips were washed three times for 5 min in washing buffer (PBS pH 7.2, 0.05% Tween-20). Alkaline phosphatase-conjugated rabbit anti-human IgG (Dakopatts, Denmark), in blocking buffer was added and the strips were incubated for 2 h at room temperature on a rotating platform. Strips were washed four times with washing buffer, after which the strips were rinsed with substrate buffer (0.1M Tris, 0.1M NaCl, 50mM MgCl2) and stained with Nitroblue Tetrazolium Chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, p- toluidine salt) (Bethesda Research Laboratories Life Technologies, USA) for approximately 20 min at room temperature. The strips were dried overnight before they were read [6,16]. For DIA, a reaction was regarded to be positive for HSV-2 antibodies when a purple dot was visible where the HSV-2 antigen had been dotted. For WBA, a blot was regarded to be positive for HSV-2 antibodies when the 92 KD band in the HSV-2 antigen lane was visible.

Results.

To demonstrate the reaction patterns in WBA and DIA, some results obtained with sera from patients with either early or recurrent HSV-1 or HSV-2 infections are shown in Figures 1 and 2. Sera from patients with either recurrent HSV-1 or HSV-2 infections reacted strongly with many HSV-1 and HSV-2 proteins in a WBA (Figure 1, strips 1, 2 and 6). Only weak or no reactions were detected with sera from patients with early HSV-2 infections (Figure 1, strips 3, 4, 5).

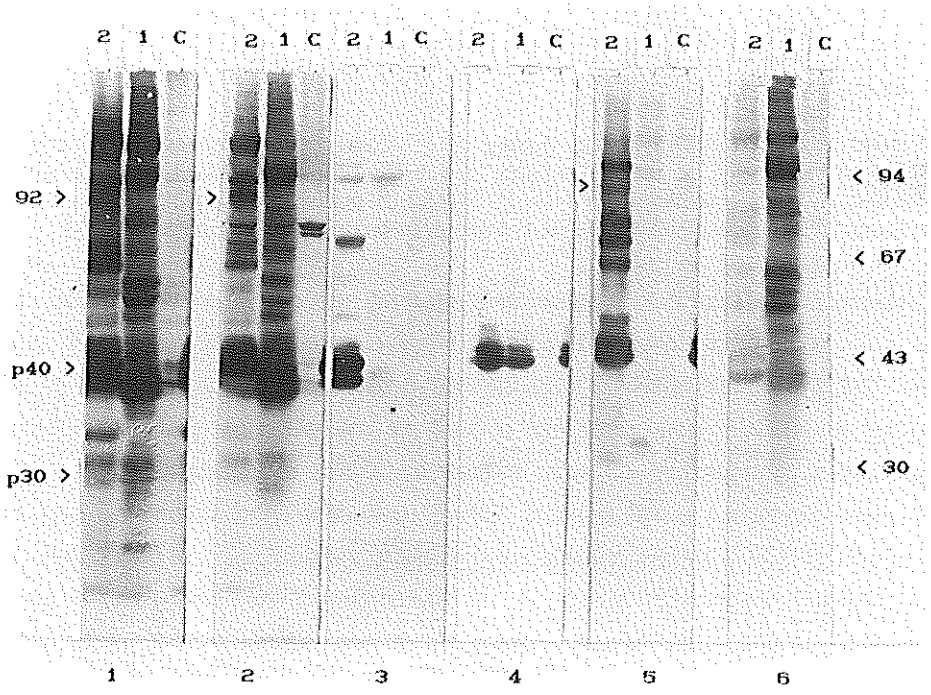

Figure 1. Western Blot Analysis. Strip 1 and 2: patients with recurrent HSV-2 infections. Strips 3-5: patients with early HSV-2 infections (21, 18, 26 days after the onset respectively). Strip 6: patient with recurrent HSV-1 infection.
1 = HSV-1 antigen, 2 = HSV-2 antigen, C = Control antigen
arrows left: glycoprotein G2 (92K), proteins P40 and P30 are indicated.
arrows right: molecular weight markers are indicated

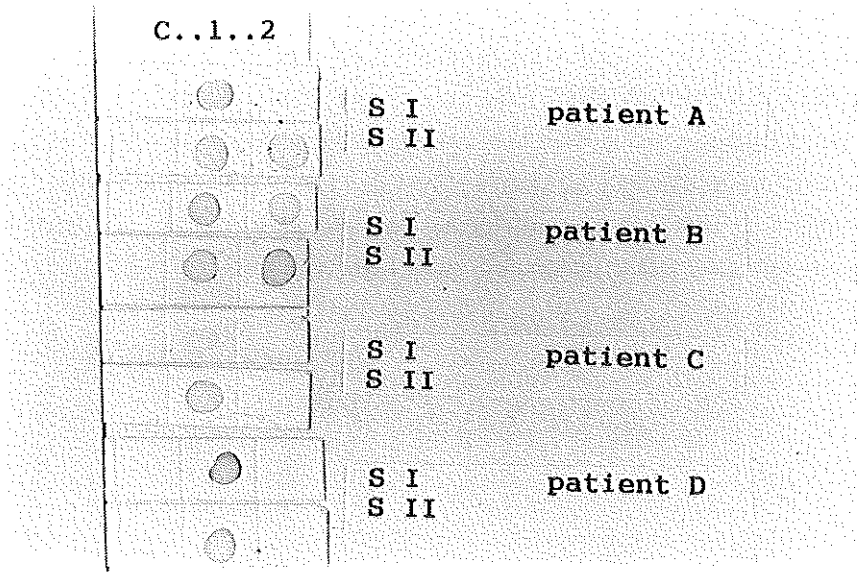

Figure 2. Dot Immunobinding Assay. SI = acute phase serum, SII = reconvalescence serum, C = control antigen, 1 = HSV-1 antigen, 2 = HSV-2 antigen. Patient A: early infection with HSV-2. Patient B: recurrent infection with HSV-2. Patient C: early infection with HSV-1. Patient D: recurrent infection with HSV-1.

However, in a serum from a patient with recurrent episodes of HSV-1 infection, no reaction could be demonstrated with a 92 KD glycoprotein of HSV-2 (Figure 1, strip 6). This glycoprotein is known to be the HSV-2 type-specific glycoprotein G2 [5]. In order to facilitate easy screening of sera for the presence of HSV-2-specific antibodies, glycoprotein G2 was isolated by affinity chromatography on Helix pomatia Sepharose 6 MB columns. Others have shown that chromatography of extracts of HSV-1 in Helix pomatia columns led to the purification of glycoprotein C of molecular weight 125-130 KD [11, 13]. Glycoprotein C from HSV-1 carries type-common antigenic determinants. Purified glycoprotein G2 from HSV-2, glycoprotein C from HSV-1, and control cell extracts were used for a DIA. Sera from patients with either HSV-1 or HSV-2 infections reacted with the type-common HSV-1 glycoprotein C (Fig 2, A-D), but only sera from patients with HSV-2 infections reacted with the type-specific HSV-2 glycoprotein G2 (Figure 2, A and B). No reactions were detected with control antigens.

Sera taken one to five weeks after the onset of clinical symptoms from patients with early HSV-2 infections and from patients with recurrent infections were tested in WBA and DIA. The results are presented in Table 1. In Table 1, the differences in reactivity between sera from patients with late and early infections are shown. In 93% of the cases, sera from patients with late HSV-2 infections reacted in the WBA with the 92 KD protein and in 89% of the cases in the DIA with purified glycoprotein G2. Patients with early infections showed a lower reactivity: 40% reacted in WBA, 20% reacted in DIA, but there was a tendency for additional reactions if a longer period has elapsed between the onset of clinical symptoms and the date the serum was taken. Some patients developed anti-glycoprotein G2 antibodies only after 3-5 weeks, whereas others did not develop anti-glycoprotein G2 antibodies at that moment. The same sera were used for the results presented in Table 2. From 40 sera with a reciprocal titer in CF ≤4, 8 (20%) reacted in WBA and 4 (10%) in DIA. From the 42 sera with a reciprocal titer in CF ≥8, 25 (60%) reacted with glycoprotein G2 in WBA and 12 (29%) in DIA.

In order to evaluate the specifity of the WBA and the DIA, sera from patients with HSV-1 or VZV infections and sera from schoolchildren were tested.

Table 1: Results of 82 sera from 41 patients with early HSV-2 infections and in 44 sera of 44 patients with late HSV-2 infections using WBA and DIA.

patient group	N	WBA HSV-2 positive (%)	DIA gG2 positive (%)
Early infection			
week 1	35	6 (17%)	3 (9%)
week 2	17	8 (47%)	5 (29%)
3-5 weeks	30	19 (63%)	8 (27%)
Total	82	33 (40%)	16 (20%)
Late infection	44	41 (93%)	39 (89%)

Table 2: Results of 41 acute phase and 41 reconvalescence sera obtained from 41 patients with early HSV-2 infection using WBA and DIA. The same sera were divided according to the reciprocal titer in CF ≤ 4 or ≥ 8.

Serum	N	WBA HSV-2 positive (%)	DIA HSV-2 positive (%)
Acute Phase	41	7 (17%)	5 (12%)
Reconvalescence	41	26 (63%)	11 (27%)
CF ≤ 4	40	8 (20%)	4 (10%)
CF ≥ 8	42	25 (60%)	12 (29%)
Total	82	33 (40%)	16 (20%)

Table 3: Results of the sera from patients with genital or extragenital HSV-1 infections, in patients with recurrent herpes labialis, in schoolchildren, and in patients with Varicella Zoster virus infection using WBA and DIA.

Patient group	N	WBA HSV-2 positive (%)	DIA HSV-2 positive (%)
HSV-1 infection	66	7 (11%)	4 (6%)
Recurrent herpes labialis	13	2 (15%)	2 (15%)
Schoolchildren	50	0 (0%)	0 (0%)
Varicella Zoster	19	0 (0%)	0 (0%)

The results are shown in Table 3. There were no cross-reactions with sera from schoolchildren and sera from patients with VZV infection. However, seven patients with culture positive HSV-1 infection and two patients with recurrent herpes labialis reacted with glycoprotein G2. The results of the patients with discordant serology are presented in Table 4. In a number of cases, a history of HSV-2 infection could not be totally excluded.

Table 4: Discordant results using WBA and dot immunobinding assay in patients with genital of extragenital HSV-1 infection and patients with recurrent herpes labialis.

Patient nr	Age years	WBA HSV-2	Dot 92K	Clinical information	Culture
Genital or extragenital HSV-1 infection					
54	22	+	-	m[1], urethral vesicles	HSV-1
241	6	+	-	f[2], stomatitis	HSV-1
248	18	+	+	f, vulvar ulcerations	HSV-1
252	21	+	-	f, vulvar vesicles	HSV-1
254	61	+	+	f, herpes labialis, TPHA +	HSV-1
257	22	+	+	f, vulvar ulcerations	HSV-1
53	19	+	+	f, vulvar vesicles	HSV-1
Recurrent herpes labialis					
149	26	+	+	f, rec.h.lab[3]/h.gen[4]	n.d.
152	24	+	+	f. rec.h.lab	n.d.

[1]m = male
[2]f = female
[3]rec.h.lab = recurrent herpes labialis
[4]rec.h.gen = recurrent herpes genitalis
n.d. = not done

Discussion

Western Blot Analysis has been described by several authors [2-6] and is more successful for identifying antibodies against HSV-2 then virus neutralization and plaque reduction tests. However, a lot of sera reacted with HSV-1 and HSV-2 on NC strips in WBA. In the cases of atypical reaction patterns, sera had to be absorbed with HSV-1 and HSV-2 antigens. Ashley et al [5] used a quantative method by weighing the cut-out tracings from scanning densitometry. However, using this method it was difficult to distinguish between sera containing HSV-1 antibodies and sera containing dual antibodies. In qualitative sense, densitometry was found to be useful in identifying the band corresponding to the HSV-2-specific glycoprotein G2 in the presence of HSV-1 antibody. Purified glycoprotein G2 can be used in glycoprotein G2-specific assays.

Comparing WBA and glycoprotein G2-specific Immunodot Enzyme Assay, Ashley

et al [6] detected antibodies to HSV-2 in 25% of the sera using WBA and in 8% of the sera using Immunodot Enzyme Assay with sera obtained earlier than 21 days after the onset of primary HSV infection. Ninety-nine percent of samples were positive for HSV-2 in WBA en 96% were positive in Immunodot Enzyme Assay with sera obtained at least 21 days after onset of primary HSV infection.

Lee [7] observed that 17% of the sera obtained within 10 days after onset and 95% of sera obtained more than 10 days after onset were positive in Immunodot Enzymatic Assay. The test was found to be very specific. None of the 631 sera from HSV-seronegative adults, children, nuns and patients with primary genital HSV-1 infections were reactive with glycoprotein G2 antigen.

Lee [7] and Ashley [6] used immuno-affinity purified antigen for Immunodot Enzymatic Assay.

We used Helix pomatia (HPA) lectin-purified HSV-2 glycoprotein G2 in dot immunobinding assay. Using dot immunobinding assay, we observed that 39 out of the 44 (89%) sera were positive from patients with late HSV-2 infections and 41 out of the 44 (93%) sera were positive using WBA.

Swennerholm [11] used HPA lectin-purified antigens in an ELISA system. From the 26 sera of patients with verified HSV-1 infections, eight contained antibodies against HSV-2 type-specific antigen. From the 26 sera of patients with verified HSV-2 infections, all demonstrated positive titers against HSV-2 specific antigen.

Suchánková [12] used HPA lectin-purified antigens in Solid Phase RIA for detection of HSV type-specific antibodies. Antibodies to HSV-2 were detected in 14 out of the 16 subjects treated for genital herpes lesions (not cultured). Antibodies to HSV-2 were not detected in any of the 57 children.

In our hands the WBA was slightly more sensitive than DIA.

In another study [5], the WBA was also observed to be slightly more sensitive. The difference between WBA and dot immunobinding assay is more prominent when early sera were used. This could be explained by the fact that reaction to antigens other than HSV-2 glycoprotein G2 occurred earlier. We used very stringent criteria for a positive WBA. Only a WBA with a reaction to HSV-2 glycoprotein G2 was regarded as positive.

In patients with early HSV-2 infections, 6 out of the 35 (17%) sera taken in the first

week after the onset of clinical symptoms were positive using WBA, 3 (9%) were positive using DIA (Table 1). From the 17 sera taken in the second week after the onset of clinical symptoms 8 (47%) and 5 (29%) were positive using WBA and DIA respectively. From the 30 sera taken 3-5 weeks after the onset 19 (63%) were positive using WBA and 8 (27%) were positive using DIA. Twenty percent of the sera with a reciprocal CF titer ≤ 4 were positive using WBA and 10% were positive using DIA, whereas 60% of the sera with CF titer ≥ 8 were positive using WBA and 29% were positive using DIA (Table 2). The best method of recognizing type-specific HSV-2 antibodies is to test sera taken three weeks or later after the onset of clinical symptoms or sera with CF titer ≥ 8.

In our study, sera taken more then five weeks after onset of clinical symptoms of first episode were not available. From this study we are unable to answer the question whether HSV-2-specific antibodies in patients who once had a clinical episode can be detected. Neither the percentage of patients who once had a symptomless infection could be detected nor could the length of time the antibodies existed after one clinical or symptomless episode be established.

From other studies [5, 7, 16], the WBA and glycoprotein G2 immunodot assay turns out to be very specific. We observed that 7 sera were positive from a total of 66 patients with proven HSV-1 infection. In the study by Samarai [18] in patients with recurrent genital herpes, in one episode HSV-1 was isolated, in another episode HSV-2 was isolated. Therefore it is possible to detect HSV-2-specific antibodies in patients from which HSV-1 was isolated from the genitals. From sexually active adults with herpes labialis, a part will be once infected by HSV-2. Therefore it is not surprising to find HSV-2 specific antibodies in adults with herpes labialis. We have no explanation for HSV-2-specific antibodies in a 6 years old child hospitalized with stomatitis, from whom HSV-1 was isolated.

The use of immuno-affinity columns to purify glycoprotein G2 is more effective, and the product is more concentrated, than by using HPA-lectin columns. However, monoclonal antibodies needed for immuno-affinity are not commercially available at present. It is relatively simple to purify glycoprotein G2 with HPA-lectin columns and the HPA bound lectin is commercially available. Recently, glycoprotein G1 and glycoprotein G2 were expressed in recombinant-baculovirus-infected insect cells

[19], and in E. coli [20].

In conclusion, the dot immunobinding assay using glycoprotein G2 is very useful for testing large numbers of sera rapidly. Both using WBA and DIA, the best results were obtained in patients with recurrent infections. For patients with an early HSV-infection the best results were obtained using sera with a reciprocal titer in CF ≥ 8 or sera taken more than two weeks after the onset of clinical symptoms.

References

1. Rawls WE, Iwamoto K, Melnick JL. Measurements of antibodies to herpes virus types 1 and 2 in human sera. J Immunol 1970; 104: 599-606.
2. Bernstein DI, Garratty E, Lovett MA, Bryson YL. Comparison of Western Blot Analyses to microneutralization for the detection of type specific herpes simplex virus antibodies. J Med Virol 1985; 15: 223-230.
3. Bernstein DI, Lovett MA, Bryson YL. The effects of acyclovir on antibody response to herpes simplex virus in primary genital herpetic infections. J Infect Dis 1984; 150: 7-13.
4. Sherlock CH, Ashley RL, Shurtleff ML, Mack KD, Corey L. Type specific of complement-fixing antibody against herpes simplex virus type 2 and 4 early antigen in patients with asymptomatic infections. J Clin Microbiol 1986; 24: 1093-1097.
5. Ashley RL, Militoni J. Use of densitometric analysis for interpreting HSV serologies based on Western Blot. J Virol Meth 1987; 18: 159-168.
6. Ashley RL, Militoni J, Lee F, Nahmias A, Corey L. Comparison of Western Blot (Immunoblot) and Glycoprotein G Specific Immunodot Enzyme Assay for Detecting Antibodies to Herpes Simplex Virus Types 1 and 2 in Human Sera. J Clin Microbiol 1988; 26: 662-667.
7. Lee FK, Coleman M, Pereira L, Bailey PD, Tatsuno M, Nahmias AJ. Detection of Herpes Simplex Virus Type 2-Specific Antibody with Glycoprotein G. J Clin Microbiol 1985; 50: 547-554.
8. Marsden HS, Stow ND, Preston VG, Timbury MC, Wilkie NM. Physical mapping of Herpes Simplex Virus induced polypeptides. J Virol 1978: 624-642.
9. Marsden HS, Buckmaster A, Palfreyman JW, Hope RG, Minson AC. Characterization of the 92.000-Dalton Glycoprotein Induced by Herpes Simplex Virus Type 2. J Virol 1984; 50: 547-554.
10. Roizman B, Norrild B, Chan C, Pereira L. Identification and Preliminary Mapping with Monoclonal Antibodies of a Herpes Simplex Virus 2 Glycoprotein Lacking a Known Type 1 Counterpart. Virology 1984; 133: 242-247.
11. Svennerholm B, Olofsson S, Jeansson S, Vahlne A, Lycke E. Herpes Simplex Virus Type-Selective Enzyme Linked Immunosorbent Assay with Helix pomatia lectin purified Antigens. J Clin Microbiol 1984; 19: 235-239.
12. Suchánková A, Hirsch L, Kremár M, Vonka V. Determination of Herpes Simplex Viruc Type-Specific Antibodies by Solid-Phase RIA on Helix pomatia Lectin-Purified Antigens. The J Infect Dis 1984; 149: 964-972.
13. Olofsson S, Lundström M, Marsden H, Jeansson S, Vahlne A. Characterization of a Herpes Simplex Virus Type 2-specified Glycoprotein with Affinity for N-Acetylgalactosamine-specific lectins and its Identification as g92K or gG. J Gen Virol 1986; 67: 737-744.
14. Olofsson S, Jeansson S, Lycke E. Unusual Lectin-Binding Properties of a Herpes Simplex Virus Type 1-Specific Glycoprotein. J Virol 1981; 38: 564-570.
15. Olofsson S, Norrild B, Andersen AB, Pereira L, Jeansson S, Lycke E. Populations of Herpes Simplex Virus Glycoprotein gC with and without Affinity for the N-Acetyl-Galactosamine Specific lectin of Helix Pomatia. Arch Virol 1983; 76: 25-38.
16. Lee FK, Pereira L, Griffin C, Reid E, Nahmias A. A novel glycoprotein for detection of herpes simplex virus type 1-specific antibodies. J Virol Meth 1986; 14: 111-118.
17. Sullender WM, Yasukawa LL, Schwartz M, et al: Type-Specific Antibodies to Herpes Simplex Virus Type 2 (HSV-2) Glycoprotein G in Pregnant Women, Infants Exposed to Maternal HSV-2 infections at Delivery and Infants with Neonatal Herpes. J Infect Dis 1988; 157: 164-171.
18. Samarai AMA, Shareef AA, Kinghorn GR, Potter CW. Sequential genital infections with herpes simplex virus type 1 and 2. Genitourin Med 1989; 65: 39-41.
19. Sanchex-Martinez D, Pellet PE. Expression of HSV-1 and HSV-2 glycoprotein G in Insect cells by using a novel Baculovirus expression vector. Virology 1991, 182: 229-238.
20. Parkes SL, Smith CM, Rose JM, Brandis J, Coates SR. Seroactive recombinant Herpes simplex virus type 2-specific glycoprotein G. J Clin Microbiol 1991; 29: 778-781.

CHAPTER 9

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

A review of the various sexually transmitted diseases (STDs) is presented in **chapter 1**. In addition, the importance of quick and accurate laboratory diagnostics is also discussed. At the time of writing, the laboratory diagnostics of the STDs such as *Chlamydia trachomatis* infections, gonorrhoea and herpes genitalis still mainly depend on the relatively slow and complicated culturing techniques for the causative microorganisms.

An overview of the clinical manifestations and complications of the three above mentioned STDs is presented in **chapter 2**.

Several problems which are still confronted in the serological diagnostics of the above mentioned 3 STDs are summarized in **chapter 3**. Uncomplicated infections of *C. trachomatis*, *N. gonorrhoeae* and herpes simplex virus (HSV) are superficial skin and/or mucous membrane infections. These diseases evoke an inadequate systemic antibody response to enable diagnosis at the moment they become clinically manifest. Nevertheless, antibodies are detectable during the later stages and can remain in circulation for a number of years. This leads to the problem in distinguishing between an acute infection and a previously suffered infection. An acute infection can be recognized by the differences in the titres of the acute phase serum and the convalescent serum obtained at approximately two weeks interval. An additional problem is the high antibody titres which may also occur in healthy individuals as a result of exposure to related microorganisms which show a high antigenic similarity, e.g. *N. catharrhalis* & *N. meningitides* with *N. gonorrhoeae*, *C. pneumonia* & *C. psittaci* with *C. trachomatis* and HSV-1 with HSV-2.

In the combat against STDs, a sensitive and rapid diagnostic method is important for the quick therapeutic intervention in order to break the infection chain. A rapid and sensitive method would be ideal because it can be performed while the patient waits so that the diagnosis and the treatment can be established at the same consultation. However, the culture and the serological methods for these STDs are not quick enough for this purpose. Besides, such methods are mostly too complicated to be available at the location where the diagnosis is undertaken. This imposes special demands on the transportation because the microorganisms responsible for causing the STDs survive poorly under non-optimal conditions and

can adversely affect the results of the culture. For this reason, a search has been and is being made for alternative methods for detecting the causative agents. Such methods are based on:

1. detection of antigens: antibodies against the microorganism raised in experimental animals are used to detect the pathogen in samples taken from the patients.
2. The detection of the microbial DNA or RNA.
3. The replication of the DNA or RNA which is subsequently used as proof for the presence of the microorganism.

Besides, in developing the diagnostic methods, it was attempted to make redundant the for the patient unpleasant examination of the cervix, the urethra, the rectum and the oropharynx. In addition, the specific and sensitve tests would enable the screening of certain groups at risk.

During the period in which the investigations described in chapters 4-7 were being conducted, attention was mainly focussed on the methods to detect antigens which were mostly performed in an ELISA set-up. Advantages of this type of tests are the speed and the simplicity of execution, the possibility for automation and also the possibility for no longer having to detect living microorganisms as causative agents for STDs. The ELISA method for detecting *C. trachomatis* and to a lesser extent, the ELISA for detecting *N. gonorrhoeae* have penetrated as far as the routine - laboratory. However, such methods have reached no further than the research laboratory for detecting HSV. The commercially available ELISAs are not cheap. The advantages may only show up well when the number of samples from the patients is large enough. When the number of samples is relatively small, these tests are conducted only in a "batch-wise" manner, whereby the advantage of obtaining quick results is lost. The balance between advantages and disadvantages of the test for each microorganism may be different for each laboratory.

The molecular biology-based detection methods such as hybridization and PCR are of prominent interest. The sensitivity of the commercially available hybridization tests, the probe assays for *C. trachomatis* and *N. gonorrhoeae* appeared not to be higher than that of the respective ELISAs. This was in contrast to the sensitivity of the PCR. This technique is extremely sensitive and in principle a single

microorganism can be detected in the samples from the patients. Initially, the poor specificity of the PCR was an important disadvantage which appears to have been overcome by the use of closed systems. The possibility to detect additional microorganisms in a single sample using different primers is a further advantage of this test. Using this test, it would be possible to establish an infection with *C. trachomatis* and *N. gonorrhoea* in urine samples from men in the future. Thus, it would be unnecessary to take samples from the urethra thereby reducing the burden imposed on the patient. The presence of living microorganisms is unnecessary in these tests so that there is no decrease in the sensitivity because of non-optimum transportation conditions.

These methods also have disadvantages. As such, the microorganism is not available for typing and testing for susceptibility which is particularly important for *N. gonorrhoeae*. Besides, the quantification of the detected microorganism is not possible. The result is either positive or negative without any gradation. The possibility to detect a very low number of microorganisms in case of *C. trachomatis* is considered by some to be a disadvantage because it only involves a commensal. However, such a view is questionable because it involves a sexually transmissible microorganism whereby it is also important to trace and treat the asymptomatic carriers.

Alternative methods for detecting chlamydia have been mainly searched for because there are many disadvantages associated with culturing this microorganism. Initially, these alternatives were the ELISA and the IF for detecting antigen. Later the probe assay and the PCR were added. A review of the different techniques is presented in **chapter 3**. In **chapters 4 and 5**, an ELISA for detecting *Chlamydia trachomatis* antigen is evaluated.

In **chapter 4**, an investigation into 403 urethra samples from men is described. The patients were divided into groups according to the seriousness of the symptoms and the abnormalities present in the urine sediment. The sensitivity of the ELISA as compared with the culture varied from 50% in the group without symptoms or abnormalities in the urine sediment to 82% in the group with post-gonococcal urethritis. Other authors using urethra samples from men reported the sensitivity of the ELISA to vary from 66% to 100% [1-8].

In **chapter 5**, an investigation into the ELISA using samples from the cervix of 246 women including the 135 women mentioned in chapter 4 is described. The number of leucocytes was semi-quantitatively determined in the smears of the cervix and scored from 0 (no leucocytes present) to 4 (more than 20 leucocytes per microscopic field). The sensitivity of the ELISA as compared with the culture varied from 50% in the group with no leucocyte to 100% in the group with more than 20 leucocytes. In addition, the patients were divided on the basis of the results of the culture. There was a significant difference in the results of the ELISA between both groups: The sensitivity was 54% in samples with less than 20 inclusions in the culture and 100% in samples with more than 20 inclusions in the culture. These results indicated that the sensitivity of the ELISA depended on the amount of antigen. The ELISA had an overall sensitivity of 67% as compared with the culture. In other studies, using samples from the cervix, the sensitivity of the ELISA varied from 71% to 98% [1,3,4,7-10].

The results of the sensitivity varied considerably and could have been due to the following:

1. The reference test. Most researchers used the culture as the reference. However, this is not standardized and may be undertaken using different cell lines such as the HeLa 229 and McCoy cell lines which may also have been pre-treated in different manners, e.g. with DEAE-dextran or Cytochalazine B. In addition, cultures may also have undergone more than one passage. Finally, the culture may also have been confirmed in various ways. Staining with iodine is relatively unsensitive, whereas staining with Giemsa followed by evaluation using dark-field illumination is more sensitive. Confirmation of the culture using immunofluorescence is the most sensitive method. It is clear that the observed sensitivity of the ELISA would depend on the sensitivity of the used culture technique.
2. The composition of the tested group of patients. Classification according to the clinical presentation, leucocyte score or abnormality in the urine sediment are very important for determining the sensitivity of the ELISA as illustrated in our study and in those by others [5,6,11-13].
3. A correct sampling technique is essential for the diagnostics of Chlamydia.

The sample must contain enough cells because an intracellular microorganism is involved. An inadequate sampling would result in a low amount of antigen. The sequence in which the samples for the culture and the ELISA were taken also appeared, at least in men, to influence the results [4,11]. In a mouse model, Taylor-Robinson observed that the sensitivity of the ELISA for detecting *C. trachomatis* as compared with the culture was dependent on the sequence in which the samples were taken [14].

The manner of transportation may also have an effect because non-optimum conditions would have an adverse effect on the viability of the micro-organism which in turn would adversely effect the results of the culture. Unfamiliarity with these various factors hampers the direct comparison of the results of the various studies.

The specificity of the ELISA was determined using samples from patients consulting the outpatient department and in whom the results of the Chlamydia culture were negative. The specificity was observed to be 98.5% in men and 92% in women. These figures corroborate those reported in the literature which varied from 94.7% to 98% [6,13,15].

In summary, the value of the ELISA in detecting antigen for the diagnostic of *C.trachomatis* infections is limited mainly because of the low sensitivity of the test. The test may be useful in situations where the culturing of the microorganism is not possible or when the transportation of the sample is not possible under optimal conditions. However, as shown in the studies described in **chapters 5 and 6**, one should realize that in half of the patients with a low amount of chlamydia antigen and/or few clinical symptoms, the results of the test may turn out to be negative.

In **chapter 6,** a modified version of an earlier commercially available ELISA test, the Gonozyme for detecting gonococcal antigen was evaluated. In this study, the test was evaluated using samples of the cervix and the urethra obtained from 266 prostitutes because the results of an earlier study in women had been disappointing [16]. The prevalence of gonorrhoea on the basis of the culture was 9.3% which was rather high taking into account that these prostitutes underwent regular medical check-ups. It was decided to randomize the sequence of taking samples

because it was suspected that the sequence in which the samples were taken may influence the results. The sensitivity of the ELISA was observed to be 88% (urethra) and 91% (cervix) provided that the cotton-swabs for the ELISA were taken first and 56% and 85% respectively if the cotton-swabs for the culture were taken first. The overall sensitivity and specificity of the ELISA using samples from the urethra were 71% and 98.9% respectively. For the samples from the cervix, the overall sensitivity and the specificity of the ELISA as compared with the culture were observed to be 88% and 99.2% respectively. The predictive value for a negative cervix test was high (98.9%), so that this test was considered to be suitable for screening this group of women for gonorrhoea. These results corroborated the results (sensitivity 87.5% and specificity 98%) of another study [17]. However, Papasian et al observed that using samples from the cervix, the Gonozyme had a much lower sensitivity of 74.4%, but the specificity was 99.2% [18]. In earlier studies, it appeared that using samples of the urethra from men, the Gonozyme performed well with a sensitivity of 90% to 100% [17] and 97.3% [18].

A comparison of the results of various investigations is difficult due to the factors similar to those mentioned in the previous paragraph: differences in the reference test, differences in the composition of the patient groups and differences in the manner in which the samples were taken. Similarly, strains that are susceptible to vancomycin may be missed if a selective medium is used for culturing. The sensitivity of Gonozyme was also observed to decrease in similar manner to that of Chlamydiazyme when the samples from the patients containing little antigen were used [17,18].

In summary, excellent results with the ELISA were obtained in men with symptoms of urethritis. However, in this group of patients, the ELISA has hardly any advantages as compared with the much cheaper and quicker Gram-stained preparation. The situation is different for women, because the Gram-stained preparation of the samples from the urethra and the cervix are insensitive. In this case, the ELISA is a suitable alternative for the Gram-stained preparation and the culture. The last is especially true in situations where the transportation of the samples under optimum conditions is not possible and because the results of the ELISA are not influenced by non-optimum transportation conditions or cold storage

of the samples for a period of 14-26 days. In addition, the ELISA has advantages if quick diagnosis is required. This advantage is used in Rotterdam, where the ELISA is used for the weekly screening of prostitutes and women working in sex-clubs.
The culture is the most sensitive and specific method to detect HSV and in combination with IF, can also be used to type the virus. However, it may take 1 to 10 days for the CPE to appear. Various ELISA systems have been developed so that the results are quickly available. In the investigations described in **chapter 7,** an ELISA for detecting HSV (Abbot, USA) was compared with the culture in patients with genital lesions suspected of an HSV infection. The sensitivity of the ELISA as compared with that of the culture was 76.9% and the specificity was 100 %. When different groups of patients were compared, it appeared that the sensitivity (87%) of the ELISA was the highest using samples obtained from patients with blisters or ulcers. The sensitivity of the ELISA was also higher using samples in which CPE was observed within 48 hours (100% for HSV-1 and 92% for HSV-2). This indicated that the results of the ELISA improved with an increasing number of virus particles in the sample.
Various ELISA systems (Dako, Ortho, Herpcheck, Welcome, Novo) were evaluated by different investigators [19-27]. The specificity of all the tests was high (93% to 100%). However, the sensitivity of the tests varied (34.3% to 98.6%) depending on the type of the test, the investigator and the group of patients that was investigated. In general, a low sensitivity was observed using samples of the cervix obtained from asymptomatic women [21,22,26]. The sensitivity was higher using samples taken from lesions of patients with symptoms [25,26]. In this last group of patients, it also appeared that the sensitivity of the ELISA was higher when the samples used were taken from lesions at an early stage [25].
It appeared that the specificity of ELISA tests for detecting HSV antigen was good, but the sensitivity left a lot to be desired for. Highest sensitivity was observed using samples from patients with symptoms and lesions at an early stage which contained a high amount of antigen. However, these are the same samples in which CPE is observed within 48 hours in the culture, so that in such cases, the ultimate time gained is limited. This test adds little to the already available diagnostics for patients without symptoms or with lesions at a late stage. However

for lesions at a late stage, serological investigations do provide additional information as described in studies in **chapter 8.**

In these studies, two serological tests for detecting type-specific antibodies against HSV-2 were evaluated. The results of the dot immunobinding assay (DIA) with affinity-purified gG2 as the antigen were compared with the results of the Western Blot Analyses (WBA) and the culture. For this purpose, sera of 219 patients were divided into groups according to the type of the virus that was cultured and the symptoms. From the 44 patients with recurrent HSV-2 infections, 39 (88%) were positive using the DIA and 41 (93%) were positive in the WBA. Using 82 sera from patients, taken 1-5 weeks after the appearance of the first symptoms, the sensitivity of both the tests was much lower: 20% for the DIA and 40% for the WBA. Both the tests seemed to be very specific: antibodies against HSV-2 were not detected in any of the 50 sera from schoolchildren using either the DIA or the WBA. The WBA was more sensitive than the DIA, but is difficult to perform at a routine laboratory. The DIA is much simpler. However, at present, the purified glycoprotein G2 is also not commercially available. A type-specific serological test is a useful supplement to the already available diagnostics. The type-specific serology may provide valuable information in situations in which culturing is not or no longer possible such as in patients with lesions at a late stage or in patients without symptoms, but in whom there may be a specific query such as in risky pregnancy.

References

1. Cherneski MA, Mahony JB, Castriciano S, et al. Detection of *Chlamydia trachomatis* antigens by enzyme immunoassay and immunofluorescence in genital specimens from symptomatic and asymptomatic men and women. J Infect Dis 1986; 154: 141-8.
2. Howard LV, Coleman PF, England BJ, Herrman JE. Evaluation of Chlamydiazyme for the detection of genital infections caused by *Chlamydia trachomatis*. J Clin Microbiol 1986; 23: 329-32.
3. Ryan RW, Steingrimsson O, Guamundsson J, Thorarinsson H, Tilton RC. Rapid detection of *Chlamydia trachomatis* by an enzyme immunoassay method. Diagn Microbiol Infect Dis 1986; 5: 225-34.
4. Backman M, Ruden A-KM, Ringerts O, Sandstrom EG. Evaluation of a commercial enzyme immunoassay versus culture for the detection of *Chlamydia trachomatis*. Eur J Clin Microbiol Infect Dis 1989; 8: 778-82.
5. Hipp SS, Han Y, Murphy D. Assessment of enzyme immunoassay and immunofluorescence test for detection of *Chlamydia trachomatis*. J Clin Microbiol 1987; 25: 1938-43.
6. Moi H, Danielsson D. Diagnosis of genital *Chlamydia trachomatis* infection in males by cell culture and antigen detection test. Eur J Clin Microbiol Infect Dis1986; 5: 563-8.
7. Mumtaz G, Mellars BJ, Ridgeway GL, Oriel JD. Enzyme immunoassay for the detection of *Chlamydia trachomatis* antigen in urethral and endocervical swabs. J Clin Pathol 1985; 38: 740-2.
8. Levy RA, Warford AL. Evaluation of the modified Chlamydiazyme immunoassay for the detection of chlamydial antigen. Am J Clin Pathol 1986; 86: 330-5.
9. Lefebre J, La Perriere H, Rousseau H, Masse R. Comparison of three techniques for detection of *Chlamydia trachomatis* in endocervical specimens from asymptomatic women. J Clin Microbiol 1988; 26: 726-31.
10. Baselski VS, McNeeley SG, Ryan G, Robinson MA. A comparison of nonculture-dependent methods for detection of Chlamydia in pregnant women. Obstet Gynecol 1987; 70: 47-52.
11. Hall CJ, Nelder C. Comparison of three non-culture techniques for detection of *Chlamydia trachomatis* in genital tract specimens. Eur J Clin Microbiol Infect Dis 1989; 8: 866-70.
12. Taylor Robinson D, Thomas BJ, Osborn MF. Evaluation of enzyme immunoassay (Chlamydiazyme) for detecting *Chlamydia trachomatis* in genital tract specimens. J Clin Pathol 1987; 40: 184-9.
13. Pothier, P. Kazmierczak A. Comparison of cell culture with two direct chlamydia tests using immunofluorescence or enzyme linked immunosorbent assay. Eur J Clin Microbiol Infect Dis 1986; 5: 569-72.
14. Taylor-Robinson D, Tuffrey M. Comparison for detection procedures for *Chlamydia trachomatis*, including enzyme immunoassays in a mouse model of genital infection. J Med Microbiol 1987; 24: 169-73.
15. Jones MF, Smith TF, Houglum AJ, Herrmann JE. Detection of *Chlamydia trachomatis* in genital specimens by the Chlamydiazyme test. J Clin Microbiol 1984; 20: 465-7.
16. Aardoom HA, de Hoop D, Iserief COA, Michel MF, Stolz E. Detection of *Neisseria gonorrhoeae* antigen by a solid phase enzyme immunoassay. Brit J Vener Dis 1982; 58: 359-63.
17. Demetrion E, Sackett R, Welch DF, Kaplan DW. Evaluation of an enzyme immunoassay for detection of *Neisseria gonorrhoeae* in an adolescent population. JAMA 1984; 252: 247-50.
18. Papasian CJ, Bartholemew WR, Amsterdam D. Modified enzyme immunoassay for detecting *Neisseria gonorrhoeae* antigens. J Clin Microbiol 1984; 20: 641-3.
19. Land SA, Skurrie IJ, Gilbert GL. Rapid diagnosis of herpes simplex virus infections by enzyme-linked immunosorbent assay. J Clin Microbiol 1984; 19: 865-9.

20. Alexander I, Ashley CR, Smith KJ, Harbour J, Roome APCH, Darville JM. Comparison of ELISA with virus isolation for the diagnosis of genital herpes. J Clin Pathol 1985; 38: 554-7.
21. Warford AL, Levy RA, Rekrut KA. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of herpes simplex virus antigen. J Clin Microbiol 1984; 20: 490-93.
22. Warford AL, Levy RA, Rekrut KA, Steinberg E. Herpes simplex virus testing of an obstetric population with an antigen enzyme-linked immunosorbent assay. Am J Obstet Gynecol 1986; 154: 21-8.
23. Sewell DL, Horn SA. Evaluation of a commercial enzyme-linked immunosorbent assay for the detection of herpes simplex virus. J Clin Microbiol 1985; 21: 457-8.
24. Lee SF, Pepose JS. Sandwich enzyme immunoassay and latex agglutination test for herpes simplex virus keratitis. J Clin Microbiol 1990; 28: 785-6.
25. Emsbroek JA, Overdiek P, Coutinho RA. Rapid amplified enzyme linked immunosorbent assay evalued for detection of herpes simplex virus. Genitourin Med 1988; 64: 107-9.
26. Kudesia G, van Hegan A, Wake S, van Hegan RJ, Kinghorn GR. Comparison of cell culture with an amplified enzyme immunoassay for diagnosing genital herpes simplex infection. J Clin Pathol 1991; 44: 778-80.
27. Silles M. Clinical Evaluation of enzyme immunoassay in rapid diagnosis of herpes simplex virus infections. J Clin Pathol 1992; 45: 165-7.

SAMENVATTING EN DISCUSSIE

SAMENVATTING EN DISCUSSIE

In **Hoofdstuk 1** is een overzicht gegeven van de verschillende SOA's. Tevens is het belang van een goede en vooral snelle laboratorium-diagnostiek besproken. Op moment van het schrijven van dit proefschrift berust voor de SOA's veroorzaakt door *C. trachomatis*, *N. gonorrhoeae* en herpes simplex virus de laboratorium-diagnose nog steeds voor een belangrijk deel op de relatief trage en gecompliceerde kweekmethode van deze ziekteverwekkers.

In **Hoofdstuk 2** is een overzicht gegeven van de klinische manifestaties en complicaties van de drie bovengenoemde SOA's.

De serologische diagnostiek van de genoemde drie SOAs, die is samengevat in **Hoofdstuk 3**, levert enkele specifieke problemen op. Ongecompliceerde infecties met *C. trachomatis*, *N. gonorrhoeae* en herpes simplex virus zijn oppervlakkige huid en/of slijmvlies infecties. Op het moment dat zij klinisch manifest worden, hebben zij tot een nog onvoldoende sterke systemische antilichaam respons geleid om deze een rol te laten spelen bij de diagnose. Niettemin zijn in latere stadia wel antilichamen detecteerbaar, die meerdere jaren in de circulatie aanwezig kunnen blijven. Dit geeft problemen bij het onderscheid tussen een acute infectie en een vroeger doorgemaakte infectie. Door gebruik te maken van twee serummonsters welke ca. 2 weken na elkaar worden afgenomen, het acute fase serum en het reconvalescentie serum, kan een acute infectie vaak als zodanig worden herkend aan de titerverschilen van deze twee monsters. Een bijkomend probleem vormen de hoge antilichaamtiters, die bij gezonde personen kunnen voorkomen en die veroorzaakt worden door contact met verwante microorganismen die een grote antigene overeenkomst vertonen zoals *N. catharrhalis* en *N. meningitidis* met *N. gonorrhoeae*, *C. pneumonia* en *C. psittaci* met *C. trachomatis*, en HSV1 met HSV2. Bij de bestrijding van de sexueel overdraagbare aandoeningen is een gevoelige en snelle diagnostische methode belangrijk om door een snelle behandeling de infectie-keten te kunnen doorbreken. Het ideaal is een snelle en gevoelige methode die verricht kan worden terwijl de patiënt er op wacht, zodat diagnostiek en behandeling in een bezoek kan plaatsvinden. Voor dit doel zijn zowel de kweekmethode als de serologie voor deze SOAs onvoldoende snel. Daarnaast zijn zij meestal te gecompliceerd om beschikbaar te zijn op de plaats waar de diagnose

gesteld moet worden. Dit stelt eisen aan het transport omdat sexueel overdraagbare ziekteverwekkers slecht overleven onder niet optimale transport omstandigheden en het resultaat van de kweek ongunstig beinvloed kan worden. Dit zijn de redenen dat er gezocht is en wordt naar alternatieve methoden ter opsporing van de ziekteverwekkers. Deze berusten op:

1. antigeendetectie: antilichamen tegen het microorganisme, opgewekt in proefdieren worden gebruikt om in patiëntenmateriaal de ziekteverwekker aan te tonen.
2. het aantonen van het DNA of RNA van het microorganisme.
3. de vermeerdering van het DNA of RNA, dat vervolgens wordt aangetoond als bewijs voor de aanwezigheid van het microorganisme.

Daarnaast is getracht om bij het ontwikkelen van diagnostische methoden de voor de patiënten belastende onderzoeken van de cervix, de urethra, het rectum en de oropharynx overbodig te maken. Bovendien kunnen specifieke en sensitieve testen screening van bepaalde bevolkingsgroepen mogelijk maken.

Ten tijde dat het in de hoofdstukken 4-7 beschreven onderzoek verricht werd, was de aandacht sterk gericht op antigeen detecterende methoden, die veelal werden uitgevoerd in een ELISA opstelling. Voordelen van dit type testen is de snelheid en eenvoud van uitvoering, de mogelijkheid tot automatiseren en de mogelijkheid ook niet meer levende sexueel overdraagbare ziekteverwekkers aan te tonen. De ELISA methode voor het aantonen van *C. trachomatis* en in mindere mate de ELISA voor het aantonen van *N. gonorrhoeae* zijn doorgedrongen tot het routine-laboratorium. Voor herpes simplex virus zijn deze methoden niet verder gekomen dan het research laboratorium. De commercieel beschikbare ELISAs zijn niet goedkoop. De voordelen komen slechts volledig tot hun recht bij een voldoende groot aanbod van patiëntenmonsters. Bij een minder groot aanbod worden deze testen uit economische overwegingen "badge-gewijze" uitgevoerd, waardoor het voordeel van het snel verkrijgen van de uitslag vervalt. Per laboratorium en per microorganisme kan de balans tussen voor- en nadelen anders uitvallen.

De moleculair biologische detectiemethoden zoals hybridisatie en PCR staan thans sterk in de belangstelling. De sensitiviteit van de commercieel verkrijgbare hybridisatie testen, de probe assays voor *C. trachomatis* en *N. gonorrhoeae* bleek

niet groter dan die van de respectievelijke ELISAs, dit in tegenstelling tot die van de PCR. Deze techniek is zeer gevoelig, in principe kan één organisme in het patiëntenmateriaal gedetecteerd worden. Aanvankelijk was de slechte specificiteit van de PCR een belangrijk nadeel, dat overwonnen lijkt te zijn door het toepassen van gesloten systemen. Een verder voordeel is de mogelijkheid in één monster met behulp van verschillende primers meerdere microorganismen aan te tonen. Met deze test zal het in de toekomst mogelijk zijn een infectie met *C. trachomatis* en *N. gonorrhoea* bij mannen vast te stellen mbv urinemonsters. Hierdoor kan het afnemen van het urethramonster worden vermeden, waardoor de belasting voor de patiënt verder afneemt. Voor deze testen is de aanwezigheid van levende microorgansmen niet noodzakelijk, zodat geen verlies van sensitiviteit optreedt door niet optimale transport omstandigheden.
Er kleven ook nadelen aan deze methoden. Zo is het microorganisme niet beschikbaar voor gevoeligheidsbepalingen en typeringen, met name van belang bij *N. gonorrhoeae.* Daarnaast is een kwantificering van het gevonden microorganisme niet mogelijk, het resultaat is positief of negatief, zonder gradaties. De mogelijkheid zeer kleine aantallen microorganismen op te sporen wordt in het geval van *C. trachomatis* door sommigen wel als een nadeel beschouwd, aangezien het slechts een commensaal zou betreffen. Echter, daar het hier gaat om een sexueel overdraagbaar microorganisme, waarbij het van belang is ook asymptomatische dragers op te sporen en te behandelen, dient bij deze opvatting een vraagteken geplaatst te worden.

Wegens de vele nadelen die aan de kweek van chlamydia kleven, is vooral voor dit organisme naar alternatieven gezocht. Dit waren aanvankelijk de ELISA en de IF voor antigeen detectie, later zijn daar de probe assay en PCR bijgekomen. Een overzicht van de verschillende technieken is gegeven in **Hoofdstuk 3.** In de **Hoofdstukken 4 en 5** werd een ELISA voor detectie van *Chlamydia trachomatis* antigeen geëvalueerd.
In **Hoofdstuk 4** is het onderzoek van 403 urethramonsters van mannen beschreven. De patiënten werden ingedeeld in groepen, afhankelijk van de ernst van de symptomen en afwijkingen in het urine-sediment. De sensitiviteit van de ELISA

t.o.v. de kweek varieerde van 50% in de groep zonder symptomen of sedimentsafwijkingen tot 82% in de groep met postgonococcal urethritis. Andere auteurs vonden voor de ELISA, verricht met urethra monsters van mannen een sensitiviteit, die varieerde van 66-100% [1-8].

In **Hoofdstuk 5** is het onderzoek met de ELISA van cervixmonsters, afkomstig van 246 vrouwen, waaronder begrepen de 135 vrouwen, die in Hoofdstuk 4 werden vermeld, beschreven. In de cervix uitstrijkjes werd een semi-quantitatieve bepaling van het aantal leucocyten verricht, waarbij werd gescoord van 0 (geen leucocyten aanwezig) tot 4 (>20 leucocyten/microscopisch veld). De sensitiviteit van de ELISA t.o.v. de kweek varieerde van 50 % in de groep met 0 leucocyten tot 100 % in de groep met meer dan 20 leucocyten. Daarnaast werd een indeling gemaakt op basis van de resultaten van de kweek. Er bleek een significant verschil tussen beide groepen in de resultaten van de ELISA: de sensitiviteit bedroeg 54 % bij patiëntenmonsters met < 20 inclusies en 100 % bij >20 inclusies in de kweek. Deze resultaten suggeren dat de sensitiviteit van de ELISA afhangt van de hoeveelheid antigeen. Er werd een overall sensitivity van 67% voor de ELISA t.o.v. de kweek gevonden. In studies van anderen varieerde de sensitiviteit van de ELISA verricht met cervixmonsters van 71 tot 98% [1,3,4,7-10].

De gevonden resultaten voor de sensitiviteit lopen nogal uiteen. Hiervoor zijn verschillende oorzaken aan te wijzen:

1. De referentietest. Door de meeste onderzoekers wordt de kweek als referentie gebruikt. Deze is echter niet gestandaardiseerd en kan plaats vinden op diverse cellijnen zoals de HeLa 229 en Mc Coy cellijnen, die ook nog op diverse manieren voorbehandeld kunnen zijn met bijv. DEAE-dextran of Cytochalazine B. Tevens kunnen er slechts één, maar ook meerdere passages worden uitgevoerd. Tenslotte kan de identificatie op diverse manieren plaatsvinden. Kleuring met jodium is relatief ongevoelig, kleuring met Giemsa, gevolgd door beoordeling onder donkerveld-belichting is gevoeliger. Identificatie met behulp van immunofluorescentie is de gevoeligste methode. Het zal duidelijk zijn dat de gevonden sensitiviteit van de ELISA af zal hangen van de sensitiviteit van de gebruikte kweek-methode.

2. De samenstelling van de geteste patiëntengroep. Het maken van onderscheid

naar klinische beeld, leucocytenscore of sedimentsafwijkingen is van groot belang bij de bepaling van de sensitiviteit van de ELISA, zoals blijkt uit onze studie en die van anderen [5,6,11-13].

3. Een juiste monsterafname is bij de Chlamydia diagnostiek essentieel. Omdat het een intracellulair microorganisme betreft moet het monster voldoende cellen bevatten. Een slechte monster-afname zal resulteren in een lager antigeen aanbod. Ook de volgorde waarin de monsters voor de kweek en de ELISA werden afgenomen bleek, althans bij mannen, de resultaten te beinvloeden [4,11]. Taylor Robinson toonde in een muizen-model aan dat de sensitiviteit van de ELISA voor het aantonen van *C. trachomatis* vergeleken met de kweek afhankelijk was van de volgorde van het afnemen van de monsters [14].

Ook de wijze van transport kan van invloed zijn, doordat aantasting van de viability van de microorganismen slechts invloed heeft op de resultaten van de kweek. De onbekendheid met deze verschillende factoren belemmert de mogelijkheid tot een directe vergelijking van de resultaten van de diverse studies.

De specificiteit van de ELISA werd bepaald met behulp van monsters verkregen van bezoekers van de polikliniek, die negatief scoorden in de Chlamydia kweek. Voor mannen bedroeg de specificiteit 98.5 % en voor vrouwen 92 %. Deze getallen zijn in goede overeenstemming met die in de literatuur, welke varieren van 94.7% tot 98% [6,13,15].

Samenvattend, de waarde van de ELISA voor antigeen detectie bij de diagnostiek van *C.trachomatis* infecties is beperkt, met name door de lage sensitiviteit van de test. Daar waar de kweek niet beschikbaar is of het transport van het monster onder niet-optimale omstandigheden verloopt, zou de test een plaats kunnen hebben. Echter, zoals in **Hoofdstukken 5 en 6** is aangetoond, dient men zich te realiseren dat de test bij de helft van de patiënten met een lage hoeveelheid chlamydia-antigeen en/of weinig klinische verschijnselen negatief zal uitvallen.

In **Hoofdstuk 6** werd een gemodificeerde versie van een reeds eerder op de markt verschenen ELISA test voor het aantonen van gonokokken antigeen, de Gonozyme, geevalueerd. Omdat in eerdere studies het resultaat bij vrouwen teleurstellend was [16], werd in deze studie de test geevalueerd met behulp van cervix-

monsters en urethramonsters van 266 prostituées. De prevalentie van gonorrhoea op basis van de kweek was ondanks de regelmatige controles van deze vrouwen vrij hoog, namelijk 9.3%. Wegens het vermoeden dat de volgorde waarin de monsters werden afgenomen de resultaten zou kunnen beinvloeden, werd besloten de afname-volgorde te randomiseren. De sensitiviteit van de ELISA bedroeg 88% (urethra) en 91% (cervix) indien de wattenstok voor de ELISA het eerste werd afgenomen en respectivelijk 56% en 85% als de wattenstok voor de kweek het eerste werd afgenomen. De overall sensitiviteit van de ELISA verricht met urethra monsters was 71%, de specificiteit was 98,9%. Voor de cervixmonsters werd voor de ELISA ten opzichte van de kweek een overall sensitiviteit van 88% en een specificiteit van 99.2% gevonden. De voorspellende waarde van een negatieve cervix test was hoog (98.9%), zodat deze test geschikt werd geacht voor het screenen op gonorrhoea bij deze groep vrouwen. Deze resultaten komen overeen met de resultaten (sensitiviteit 87.5%, specificiteit 98%) van een andere studie [17]. Echter, Papasian et al. vonden een veel lagere sensitiviteit voor de Gonozyme in cervix monsters, namelijk 74.4%. De specificiteit was eveneens 99.2% [18]. In eerdere studies was reeds gebleken, dat de Gonozyme bij urethra-monsters van mannen uitstekend voldeed met een sensitiviteit van 90-100% [17] en 97.3% [18].
Het vergelijken van de resultaten van de diverse onderzoekers wordt bemoeilijkt door soortgelijke factoren als in de vorige paragraaf: verschil in referentietest, verschillen in de samenstelling van de patiëntengroepen en verschil in monster-afname. Zo zullen vancomycine gevoelige stammen in de kweek gemist kunnen worden als een selectieve voedingsbodem wordt gebruikt. Net als bij de Chlamydiazyme blijkt ook de sensitiviteit van de Gonozyme af te nemen, wanneer patiëntenmonsters, die weinig antigeen bevatten, worden gebruikt [17, 18].
Samenvattend, met de ELISA worden uitstekende resultaten verkregen bij mannen met symptomen van urethritis. Echter, de ELISA heeft in deze groep nauwelijks voordelen boven het veel goedkopere en snellere Grampreparaat. Voor vrouwen is de situatie anders, omdat het Grampreparaat van urethra en cervix ongevoelig is. Hier is de ELISA een goed alternatief voor het Grampreparaat en de kweek. Dit laatste geldt met name wanneer het transport van de monsters niet optimaal is, daar de resultaten van de ELISA niet worden beinvloed door niet-optimale transpor-

tomstandigheden of gekoeld bewaren van de monsters gedurende een periode van 14-26 dagen. Verder heeft de ELISA voordelen als de diagnose snel gewenst wordt. Van dit voordeel wordt gebruik gemaakt in Rotterdam, waar de ELISA wordt gebruikt voor de wekelijkse screening van sex-clubvrouwen en prostituées.

Voor het detecteren van HSV is de kweek de meest gevoelige en specifieke methode, door de combinatie met IF wordt het virus tevens getypeerd. Echter, het duurt 1 tot 10 dagen voor het CPE zichtbaar wordt. Er zijn diverse ELISA systemen ontwikkeld, waarbij het resultaat sneller bekend is. In de studie beschreven in **Hoofdstuk 7** werd een ELISA voor het aantonen van HSV (Abbot, USA) vergeleken met de kweek, bij patiënten met genitale laesies verdacht voor HSV infectie. De sensitiviteit van de ELISA ten opzichte van de kweek bedroeg 76.9%, de specificiteit 100 %. Bij vergelijken van de diverse patiëntengroepen bleek de sensitiviteit van de ELISA het hoogste in de monsters afkomstig van patienten met blaasjes of ulcera (87%). De sensitiviteit van de ELISA was ook hoger in monsters die in de kweek binnen 48 uur een CPE veroorzaakten (100% voor HSV1, 92% voor HSV2). Dit suggereert dat de resultaten met de ELISA beter zijn naarmate de monsters meer virusdeeltjes bevatten.
Diverse ELISA systemen (Dako, Ortho, Herpcheck, Welcome, Novo) zijn geevalueerd door verschillende onderzoekers [19-27]. De specificiteit was bij alle testen hoog (93-100%), echter de gevonden sensitiviteiten lopen nogal uiteen, (34.3-98.6%) afhankelijk van de soort test, de onderzoeker en de onderzochte patiëntengroep. In het algemeen was de gevonden sensitiviteit laag bij cervix-monsters van asymptomatische vrouwen [21,22,26]. De sensitiviteit was hoger bij monsters van laesies, afgenomen bij patiënten met symptomen [25,26]. Bij deze laatste groep bleek bovendien dat de sensitiviteit van de ELISA hoger was naarmate de monsters afkomstig waren van laesies in een vroeger stadium [25].
Het blijkt dat de ELISA testen voor het aantonen van HSV-antigeen een goede specificiteit hebben, maar dat de gevoeligheid te wensen over laat. De sensitiviteit is het hoogste in monsters van patiënten met symptomen en laesies in een vroeg stadium, welke veel antigeen bevatten. Dit zijn echter dezelfde monsters die in de kweek binnen 48 uur CPE vertonen, zodat in deze gevallen de uiteindelijke tijdwinst

gering is. Bij patiënten zonder symptomen of met laesies in een laat stadium voegt deze test weinig toe aan de bestaande diagnostiek. Echter, hier biedt serologisch onderzoek extra informatie, zoals blijkt in **Hoofdstuk 8**.

Hierin wordt de evaluatie van twee serologische testen voor het aantonen van type-specifieke antilichamen tegen HSV-2 beschreven. De resultaten van de dot immunobinding assay (DIA) met gG2 als antigeen, dat is gezuiverd door affiniteits-chromatografie met behulp van gel-gebonden Helix pomatia lectine, zijn vergeleken met de resultaten van de Western Blot Analyse (WBA) en de kweek. Hiertoe werden sera van 219 patiënten, ingedeeld in diverse groepen, afhankelijk van het gekweekte virustype en de symptomen, getest. Van 44 patiënten met recidiverende HSV-2 infecties waren er 39 (88%) positief in de DIA en 41 (93%) in de WBA. In 82 sera van patiënten, afgenomen 1-5 weken na het begin van de eerste symptomen, was de gevoeligheid van beide testen veel lager: 20% voor de DIA en 40% voor de WBA. De testen bleken beide zeer specifiek: in 50 sera van schoolkinderen werden noch met de DIA noch met de WBA antilichamen tegen HSV-2 gevonden. De WBA is gevoeliger dan de DIA, maar is in een routine laboratorium nauwelijks uitvoerbaar, omdat hiervoor specialistische apparatuur vereist is. De DIA is veel eenvoudiger. Echter, gezuiverd glycoproteine G2 is tot op heden niet commercieel verkrijgbaar. Type specifieke serologische diagnostiek is een nuttige aanvulling op de bestaande diagnostiek. Daar waar de kweek niet of niet meer mogelijk is, zoals bij patiënten met laesies in een laat stadium of bij patiënten zonder symptomen maar met een specifieke vraagstelling, zoals bij risico-zwangeren, kan de type-specifieke serologie waardevolle informatie geven.

Referenties

1. Chemeski MA, Mahony JB, Castriciano S, et al. Detection of Chlamydia trachomatis antigens by enzyme immunoassay and immunofluorescence in genital specimens from symptomatic and asymptomatic men and women. J Infect Dis 1986; 154: 141-8.
2. Howard LV, Coleman PF, England BJ, Herrman JE. Evaluation of Chlamydiazyem for the detection of genital infections caused by Chlamydia trachomatis. J Clin Microbiol 1986; 23: 329-32.
3. Ryan RW, Steingrimsson O, Guamundsson J, Thorarinsson H, Tilton RC. Rapid detection of Chlamydia trachomatis by an enzyme immunoassay method. Diagn Microbiol Infect Dis 1986; 5: 225-34.
4. Backman M, Ruden A-KM, Ringerts O, Sandstrom EG. Evaluation of a commercial enzyme immunoassay versus culture for the detection of Chlamydia trachomatis. Eur J Clin Microbiol Infect Dis 1989; 8: 778-82.
5. Hipp SS, Han Y, Murphy D. Assessment of enzyme immunoassay and immunofluorescence test for detection of Chlamydia trachomatis. J Clin Microbiol 1987; 25: 1938-43.
6. Moi H, Danielsson D. Diagnosis of genital Chlamydia trachomatis infection in males by cell culture and antigen detection test. Eur J Clin Microbiol Infect Dis 1986; 5: 563-8.
7. Mumtaz G, Mellars BJ, Ridgeway GL, Oriel JD. Enzyme immunoassay for the detection of Chlamydia trachomatis antigen in urethral and endocervical swabs. J Clin Pathol 1985; 38: 740-2.
8. Levy RA, Warford AL. Evaluation of the modified Chlamydiazyme immunoassay for the detection of chlamydial antigen. Am J Clin Pathol 1986; 86: 330-5.
9. Lefebre J, La Perriere H, Rousseau H, Masse R. Comparison of three techninques for detection of Chlamydia trachomatis in endocervical specimens from asymptomatic women. J Clin Microbiol 1988; 26: 726-31.
10. Baselski VS, McNeeley SG, Ryan G, Robinson MA. A comparison of nonculture-dependent methods for detection of Chlamydia in pregnant women. Obstet Gynecol 1987; 70: 47-52.
11. Hall CJ, Nelder C. Comparison of three non-culture techniques for detection of Chlamydia trachomatis in genital tract specimens. Eur J Clin Microbiol Infect Dis 1989; 8: 866-70.
12. Taylor Robinson D, Thomas BJ, Osborn MF. Evaluation of enzyme immunoassay (Chlamydiazyme) for detecting Chlamydia trachomatis in genital tract specimens. J Clin Pathol 1987; 40: 184-9.
13. Pothier, P. Kazmierczak A. Comparison of cell culture with two direct chlamydia tests using immunofluorescence or enzyme linked immunosorbent assay. Eur J Clin Microbiol Infect Dis 1986; 5: 569-72.
14. Taylor-Robinson D, Tuffrey M. Comparison for detection procedures for Chlamydia trachomatis, including enzyme immunoassays in a mouse model of genital infection. J Med Microbiol 1987; 24: 169-73.
15. Jones MF, Smith TF, Houglum AJ, Herrmann JE. Detection of Chlamydia trachomatis in genital specimens by the Chlamydiazyme test. J Clin Microbiol 1984; 20: 465-7.
16. Aardoom HA, de Hoop D, Iserief COA, Michel MF, Stolz E. Detection of Neisseria gonorrhoeae antigen by a solid phase enzyme immunoassay. Brit J Vener Dis 1982; 58: 359-63.
17. Demetrion E, Sackett R, Welch DF, Kaplan DW. Evaluation of an enzymeimmunoassay for detection of Neisseria gonorrhoeae in an adolescent population. JAMA 1984; 252: 247-50.
18. Papasian CJ, Bartholemew WR, Amsterdam D. Modified enzyme immunoassay for detecting Neisseria gonorrhoeae antigens. J Clin Microbiol 1984; 20: 641-3.
19. Land SA, Skurrie IJ, Gilbert GL. Rapid diagnosis of herpes simplex virus infections by enzyme-linked immunosorbent assay. J Clin Microbiol 1984; 19: 865-9.
20. Alexander I, Ashley CR, Smith KJ, Harbour J, Roome APCH, Darville JM. Comparison of ELISA with virus isolation for the diagnosis of genital herpes. J Clin Pathol 1985; 38: 554-7.
21. Warford AL, Levy RA, Rekrut KA. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of herpes simplex virus antigen. J Clin Microbiol 1984; 20: 490-93.

22. Warford AL, Levy RA, Rekrut KA, Steinberg E. Herpes simplex virus testing of an obstetric population with an antigen enzyme-linked immunosorbent assay. Am J Obstet Gynecol 1986; 154: 21-8.
23. Sewell DL, Horn SA. Evaluation of a commercial enzyme-linked immunosorbent assay for the detection of herpes simplex virus. J Clin Microbiol 1985; 21: 457-8.
24. Lee SF, Pepose JS. Sandwich enzyme immunoassay and latex agglutination test for herpes simplex virus keratitis. J Clin Microbiol 1990; 28: 785-6.
25. Emsbroek JA, Overdiek P, Coutinho RA. Rapid amplified enzyme linked immunosorbent assay evalued for detection of herpes simplex virus. Genitourin Med 1988; 64: 107-9.
26. Kudesia G, van Hegan A, Wake S, van Hegan RJ, Kinghorn GR. Comparison of cell culture with an amplified enzyme immunoassay for diagnosing genital herpes simplex infection. J Clin Pathol 1991; 44: 778-80.
27. Silles M. Clinical Evaluation of enzyme immunoassay in rapid diagnosis of herpes simplex virus infections. J Clin Pathol 1992; 45: 165-7.

List of publications

1. Schuller JL, Piket-van Ulsen J, van der Veeken I, Michel MF, Stolz E. Antibodies against Chlamydia of lymphogranuloma-venereum type in Crohn's disease. Lancet 1979; 6: 19-20.
2. van Ulsen J. Nieuwe snelle diagnostische methoden voor het aantonen van *N. gonorrhoeae* en *Chlamydia trachomatis*. Soa-bulletin 1984; 5: 15.
3. van Ulsen J, van Zuuren-van der Horst A, Tjiam KH, van der Heijst Y, Michel MF, van Eijk RVW, van Joost T, Stolz E. Solid-phase enzyme immunoassay for detection of *Chlamydia trachomatis*. Eur J Clin Microbiol Infect Dis 1985; 4: 397-399. This article is used in Chapter 4.
4. Stolz E, Tjiam KH, van Ulsen J, van Eijk RVW, van Joost T. De waarde van sneldiagnostiek van sexueel overdraagbare aandoeningen, in het bijzonder van gonorrhoe, *Chlamydia trachomatis*-infecties en herpes genitalis. Ned T Geneesk 1985; 129: 2101-2104.
5. van Ulsen J, Michel MF, van Strik R, van Joost T, Stolz E, van Eijk RVW. Ervaringen met een solid phase enzyme immunoassay voor het aantonen van gonorroe bij promiscue vrouwen. RIVM 1985, rapportnummer 842089001.
6. van Ulsen J, Michel MF, van Strik R, van Eijk RVW, van Joost T, Stolz E. Experience with a modified solid-phase enzyme immunoassay for detection of gonorrhea in prostitutes. Sex Transm Dis 1986; 13: 1-4. This article is used in Chapter 6.
7. Schift R, van Ulsen J, Ansink-Schipper MC, van Joost T, Michel MF, Woudstra RK, Stolz E. Comparison of oral treatment of uncomplicated urigenital and rectal gonorrhoea with cefuroxime axetil ester or clavulanic acid potentiated amoxycillin (Augmentin). Genitourin Med 1986; 62: 313-7.
8. van Ulsen J, Dumas AM, Wagenvoort JHT, van Zuuren A, van Joost T, Stolz E. Evaluation of an enzyme immunoassay for detection of herpes simplex virus antigen in genital lesions. Eur J Clin Microbiol Infect Dis 1987; 6: 410-413. This article is used in Chapter 7.
9. van Ulsen J, Stolz E, van Joost T, Geursen-Reitsma AM. Allergy to spermacidal lubricant in a contraceptive. Contact Dermatitis 1987; 17: 115-

116.

10. van Ulsen J, van Loon LAJ, van Joost T. Overgevoeligheid voor materialen in kunsthars gebitsprothesen als een oorzaak voor het Burning Mouth Syndroom. Ned Tijdschrift voor Tandheelkunde 1987; 94: 529-33.
11. van Joost T, Stolz E, Piket-van Ulsen J. Sensibilisatie voor Tromantadine (Vuru-Merz) bij herpes simplex infecties. Ned T Geneesk 1987; 131: 21-22.
12. van Ulsen J, van Eijk RVW, Wagenvoort JHT, Stolz E. Experience with a solid phase enzyme immunoassay for the detection of non-complicated cervical infection in women caused by Chlamydia trachomatis. Eur J Clin Microbiol Infect Dis 1988; 7: 672-675. This article is used in Chapter 5.
13. van Ulsen J, Stolz E, van Joost T. Chromate dermatitis due to a chromium containing oral homeopathic drug. Contact Dermatitis 1988; 18: 56-57.
14. van Ulsen J, Stolz E, van Joost T. Lokale vasculitis met purpura door benzoylperoxide bij acne vulgaris. Bulletin Contactdermatosen 1988; 2: 65-66.
15. van Joost T, van Ulsen J, van Loon LAJ. Hypersensitivity to denture materials as a possible cause of a burning mouth (Burning Mouth Syndrom). Contact Dermatitis 1989; 18: 97-99.
16. van Joost T, van Ulsen J, Vuzevski VD, Naafs B, Tank B. Purpuric contact dermatitis to benzoyl peroxide. JAAD 1990; 22: 359-61.
17. de Groot AC, van Ulsen J, Weyland JW. Allergisch contacteczeem rond de anus met dyshidrotisch eczeem van de handen door Kathon CG in vochtige toiletdoekjes. Ned T Geneesk 1991; 135: 1048-9.
18. de Groot AC, Beretty PJM, van Ginkel CJW, den Hengst CW, van Ulsen J, Weyland JW. Allergic contact dermatitis from tocopheryl acetate in cosmetic creams. Contact dermatitis 1991; 25: 302-304.
19. Stricker BHC, de Jong PAC, Schreuder F, Bijlmer-lest JC, Herrmann WA, van Ulsen J. Smaakverlies tijdens gebruik van terbinafine. Ned T Geneesk 1992; 136: 2438-40.
20. van Ulsen J, Buitenwerf J, Dumas AM, Stolz E. Evaluation of a dot immunobinding assay for detection of herpes simplex virus antibodies. Submitted for publication. This article is used in Chapter 8.

Abstracts

1. van Ulsen J, Notowicz A, van der Maas. Crohn's disease of the vulva. Brit J Dermat 1986; 114: 721-2.
2. van Ulsen J, van Zuuren A, Tjiam KH, Michel MF, van Joost T, Stolz E. The use of solid phase enzyme immunoassay (EIA) for the detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and herpes simplex virus. Brit J Dermat 1986; 114: 728.
3. van Ulsen J, Buitenwerf J, Dumas AM, Stolz E. Analyses of the type-specific serological response in herpes simplex virus infections. International Congress on Sexually transmitted disease: viral infections as a cause of STD. November 17-18, 1989, Amsterdam, The Netherlands. Abstract Book.

Dankwoord

Op deze plaats wil ik een ieder die betrokken is geweest bij de tot standkoming van dit proefschrift hartelijk danken. Enkele personen wil ik met name noemen.

Prof. Dr. E. Stolz wil ik danken voor de wijze waarop hij mij enthousiast heeft weten te maken voor onderzoek op venereologisch gebied. De vele ideeen die hij had hebben een grote bijdrage geleverd aan de kwaliteit en de orginaliteit van het in dit proefschrift beschreven onderzoek.

Dr. J. Buitenwerf ben ik veel dank verschuldigd voor de intensieve begeleiding van het onderzoek op het gebied van de HSV serologie en het geven van waardevolle opmerkingen betreffende het manuscript.

Prof. Dr. M.F. Michel wist in 1974 mijn belangstelling voor *Chlamydia trachomatis* te wekken, een belangstelling die nooit meer over is gegaan. Hij had het op zich genomen mij te leren hoe je een artikel moet schrijven, na dat eerste artikel zijn er gelukkig nog meer gevolgd. Ik dank hem tevens voor het kritisch lezen van het manuscript.

Prof. Dr. A. Meheus en **Prof. Dr. N. Masurel** wil ik danken voor het kritisch lezen van het manuscript en voor hun nuttige opmerkingen.

De samenstelling van het proefschrift in de huidige vorm is in zeer grote mate beinvloed door **Dr. J.J. van der Sluis** die met veel geduld mij in de eindfase van het schrijven heeft begeleid. De persoonlijke samenwerking met hem heeft een enorme stimulans teweeg gebracht en zal niet snel door mij worden vergeten.

Prof. Dr. Th. van Joost en **Prof. Dr. E. Stolz** dank ik voor de ruimte die ik tijdens de opleiding kreeg om onderzoek te doen.

Dr. A.M. Dumas wil ik danken voor haar vele leerzame opmerkingen over HSV diagnostiek en de begeleiding van het onderzoek over HSV serologie. Zij heeft samen met **Dr. J. Buitenwerf** een zeer waardevolle rol gespeeld bij het initieren van het virologisch deel van het onderzoek.

Dr. B. Tank wil ik danken voor het uitstekende vertaalwerk en het leveren van waardevol inhoudelijk commentaar.

Prof. Dr. R. van Strik en **Dr. P. Mulder** wil ik danken voor hun dynamische statistische begeleiding en de vergroting van mijn begrip over de juiste toepassing van statistische technieken.

Johan van der Stek dank ik voor de uitstekende wijze waarop de dia's voor lezingen en foto's voor het proefschrift werden verzorgd.
Voor het vele typewerk in diverse fasen van het manuscript wil ik met veel plezier bedanken **Magda de Ridder-Goetjaer**, **Ada Beukelmans**, **Joke Peperkamp** en vooral **Marijke Leenders-Sterk**, die in de eindfase het grootste deel van het proefschrift in de tekstverwerker heeft gezet.
Een groot deel van het beschreven onderzoek berustte op laboratoriumwerk, daarom wil ik de medewerkers van het laboratorium voor Klinische Microbiologie AZR Dijkzigt, met name **Anneke van Zuuren-van der Horst** en het Virologisch Laboratorium van de GGD te Rotterdam op deze plaats bedanken voor de prettige samenwerking en het enthousiasme waarmee de vele extra testen waarmee ik ze opzadelde werden uitgevoerd.
Last but not least zou er geen proefschrift gekomen zijn als mijn steun en toeverlaat, mijn echtgenoot **Bram Piket** mij niet vele malen letterlijk "op weg" geholpen had.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 16 januari 1951 te Diemen. Zij behaalde het diploma HBS-B in 1969. Zij was werkzaam als medisch microbiologisch analiste tot zij in 1977 begon aan de studie geneeskunde aan de Medische Faculteit van de Erasmus Universiteit Rotterdam. Het docteraal examen in de geneeskunde werd behaald in 1981 en het artsexamen in 1983. Alvorens de specialisatie tot Dermato-Venereologe op de afdeling Dermatologie en Venereologie (Prof. Dr. E. Stolz en Prof. Dr. Th. van Joost) van het Academisch Ziekenhuis Rotterdam-Dijkzigt in 1984 werd aangevangen, was zij één jaar als wetenschappelijk medewerker aan deze afdeling verbonden. In 1988 vond registratie plaats als Dermato-venereologe. Van 1988 tot 1989 deed zij onderzoek naar serologie bij HSV-infecties op het Virologisch Laboratorium van de GGD te Rotterdam (Hoofd: Dr. A.M. Dumas), onder begeleiding van Dr. J. Buitenwerf. Van 1988 tot 1990 was zij staflid op de afdeling Dermatologie en Venereologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt en was zij tevens verbonden als consulent aan de Dr. Daniel Den Hoed Kliniek te Rotterdam en het Delta Ziekenhuis te Rhoon. Sinds 1990 is zij werkzaam als Dermatolo-venereologe in het Bosch Medicentrum (lokatie Willem Alexander Ziekenhuis) te 's-Hertogenbosch.