Sexually Transmitted Infections

Serological, microbiological and microscopical aspects

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This thesis is dedicated to all those
who believe in the ideology of romantic love

- and to the ones who don´t

Make Love, Not War

ABSTRACT

The prevalence of sexually transmitted infections (STI) is high in the adult populations world-wide but varies between populations and time periods. Since a high proportion of infected individuals are asymptomatic, diagnostic approaches to reduce further transmission and complications are essential.

The three main topics of this thesis are (1) the prevalence of the herpes viruses: herpes simplex type 1 (HSV-1) and type 2 (HSV-2), Epstein-Barr virus (EBV) and cytomegalovirus (CMV) in different populations; (2) the clinical spectrum of genital infection with HSV-2; and (3) the connection between different criteria of cervicitis and female urethritis and a positive chlamydia test.

Herpes simplex virus type 2 infections, diagnosed by type-specific serology, were common in both STI patients and pregnant women. Of the pregnant women 10% were seropositive for HSV-2, and of female and male STI patients 23% and 12% had HSV-2 antibodies, respectively. Infection with HSV-2 was often asymptomatic and only 41% of seropositive patients had a history of genital herpes. Atypical manifestations, so-called unrecognised infections, were common and are of clinical importance.

Among 112 male patients with urethritis no cases of herpes simplex virus were found. Instead, Epstein-Barr virus was detected by PCR in urethral samples in a significantly higher proportion of the subjects than in the controls (21% vs. 6%). EBV was also detected in 10.5% of cervical samples from young women attending for Cervical Cancer Screening. In a similar proportion of these women, 11.5%, cytomegalovirus was found in the cervical specimens.

In female STI patients a significant correlation with a positive *C. trachomatis* test was shown for mucopurulent discharge in the cervical portio, for easily induced bleeding from the same locus, and for more polymorph nuclear leucocytes (PMNL) than epithelial cells in the vaginal wet smear. However, no correlation was demonstrated between microscopical cervicitis or urethritis and *C. trachomatis*.

In conclusion, diagnostic tests for HSV-2 should be performed generously in patients with recurring genital symptoms of unknown cause. The detection of EBV in urethral samples from men with urethritis and the demonstration of EBV and CMV in the cervix of young women support genital transmission of these viruses. Epstein-Barr virus was significantly correlated to male urethritis, which has not been demonstrated previously. However, further studies are needed to elucidate a possible causality between EBV and urethritis. Since an elevated number of PMNL in stained smears from the cervix or the urethra was not correlated with a positive test for *C. trachomatis*, routine sampling for microscopy from these loci in unselected female STI patients is questionable.

Key words: Herpes simplex virus, seroprevalence, Epstein-Barr virus, cytomegalovirus, urethritis, cervicitis, *Chlamydia trachomatis*, STI

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Gun-Britt Löwhagen, **Matilda Berntsson**, Ellen Bonde, Petra Tunbäck, Ingela Krantz
Acceptance and Outcome of Herpes Simplex Virus Type 2 Antibodies Testing in Patients Attending an STD Clinic – Recognized and Unrecognized Infections.
Acta Derm Venereol 2005; 85: 248–252

II. **Matilda Berntsson**, Petra Tunbäck, Agneta Ellström, Ingela Krantz and Gun-Britt Löwhagen
Decreasing Prevalence of Herpes Simplex Virus-2 Antibodies in Selected Groups of Women in Sweden
Acta Derm Venereol 2009; 89: 623–626

III. **Matilda Berntsson**, Gun-Britt Löwhagen, Tomas Bergström, Lejla Dubicanac, C Welinder-Olsson, G Alvengren, Petra Tunbäck
Viral and bacterial aetiologies of male urethritis: findings of a high prevalence of Epstein-Barr virus
Int J STD AIDS. 2010 Mar; 21(3):191-4

IV. **Matilda Berntsson**, Lejla Dubicanac, Petra Tunbäck, Agneta Ellström, Gun-Britt Löwhagen, Tomas Bergström
Frequent detection of CMV and EBV in cervical secretions in healthy young women
(In manuscript)

V. **Matilda Berntsson**, Petra Tunbäck
Clinical and microscopical signs of cervicitis and urethritis and the correlation with *Chlamydia trachomatis* infection in female STI patients
(In manuscript)

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ABBREVIATIONS

AV	Adenovirus
CI	Confidence interval
CMV	Cytomegalovirus
Ct	Cycle threshold
EBV	Epstein-Barr virus
FDA	the US Food and Drug Administration
gG-1	Glycoprotein G1 (in HSV-1)
gG-2	Glycoprotein G2 (in HSV-2)
GH	Genital herpes
HPF	High power field
HSV	Herpes simplex virus
KOH	Potassium hydroxide
Mg	Mycoplasma genitalium
MSM	Men who have sex with men
NAAT	Nucleic acid amplification test
NaCl	Natrium chloride
Ng	Neisseria gonorrhoeae
NGU	Non-gonococcal urethritis
NGNCU	Non-gonococcal-non-chlamydial urethritis
PCR	Polymerase chain reaction
PhHV	Phocid herpes virus
PID	Pelvic inflammatory disease
PMNL	Polymorph nuclear cells
SD	Standard deviation
SDA	Strand displacement assay
STD	Sexually transmitted diseases
STI	Sexually transmitted infections
Uu	Ureaplasma urealyticum
VZV	Varicella zoster virus

INTRODUCTION

The term "sexually transmitted infections" (STI) encompasses infections caused by a broad range of pathogens, including viruses, bacteria and protozoa. The transmission route of these infections is sexual contact between human beings. The number of known STI is about thirty and the prevalence of chronic viral STI such as herpes simplex virus infections and human papilloma virus infections is very high in the adult populations world-wide. The highest incidence rates of STI are generally found in urban populations between the ages of 15 and 35. It is not unlikely that the majority of adults in the world have one or more STI (1). Since many of these infections are asymptomatic, the term STI is preferable to the formerly used STD (sexually transmitted diseases).

This research work began with epidemiological studies of herpes simplex virus type 2 in STI patients and pregnant women (papers I-II). The starting point of the study of male urethritis (paper III) was the question to what extent herpes simplex virus is an etiological agent in urethritis. The results showed no cases of herpes simplex virus but instead a relatively high proportion of men with Epstein-Barr virus. The next step was to investigate the occurrence of the different herpes viruses, including Epstein-Barr virus, in the uterine cervix of young women (IV). The last study (V) comprises STI patients with cervicitis, the female counterpart to urethritis in men. The driving forces for this study were the lack of established criteria for cervicitis in combination with a growing interest in health economy.

Viral agents

Herpes viruses

Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) all belong to the herpesviridae. This family consists of large double-stranded DNA viruses with an icosahedral capsid. Outside the capsid is an amorphous layer of viral

proteins called the tegument surrounded by a lipid bilayer envelope containing viral glycoproteins. The ability to reactivate after period/periods of latency is a common feature, but otherwise the biology and the clinical pictures of the produced infections vary between the different herpes viruses. The most severe infections are seen in immunocompromised patients. Three subfamilies, alpha-, beta- and gamma-herpesviridae, have been classified according to details of replication, the cells in which they establish latency, gene content and gene organization. HSV-1, HSV-2 and VZV constitute the alpha-herpesviruses and establish latent infection in dorsal root ganglia.

Herpes simplex type 1 and type 2

Herpes simplex viruses are large double-stranded DNA viruses. Outside the core of viral DNA is an icosahedral capsid surrounded by an envelope, in which glycoproteins necessary for viral entry are located. All glycoproteins in the envelope induce antibody production. There is a high degree of homology between the glycoproteins in HSV-1 and HSV-2 and the only known type-specific antibody responses are the ones directed against glycoprotein G1 (gG-1) and glycoprotein G2 (gG-2).

HSV is transmitted through damaged skin and intact mucosa. Following entry both HSV-1 and HSV-2 infect nerve endings and through the neuronal axons the viruses reach the neuronal nuclei, where latency is established. In mice the rate of reactivation has been correlated to the number of latently infected neurons in the ganglia (2). Latent HSV infection in humans causes a chronic inflammation without signs of neuronal destruction (3). During the latency phase the virus persists either in a true latent state or in a condition of low-level replication. HSV-1 predominates in oro-facial lesions and is commonly found in the trigeminal ganglia, whereas HSV-2 is often located in the sacral root ganglia. Reactivation leads to transport of the virus through the axon back to the epithelial cells of the skin or mucosa producing prodromal symptoms, clinical lesions or subclinical shedding. Described triggers are sun-light exposure (HSV-1), psychosocial stress (4), hormonal factors (menstruation), fever, infections, immunosuppression and mechanical friction.

Fig.1. Age-related seroprevalence of HSV-2 in the Swedish general population in 1990-91

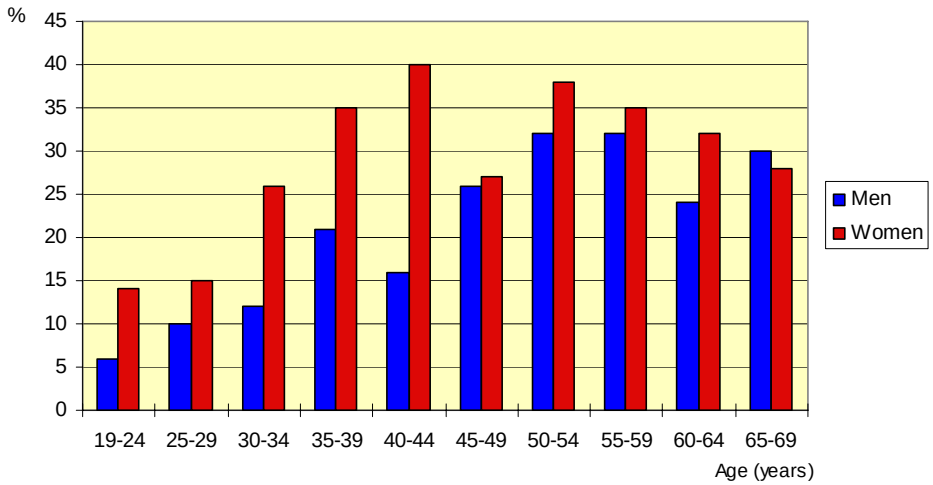
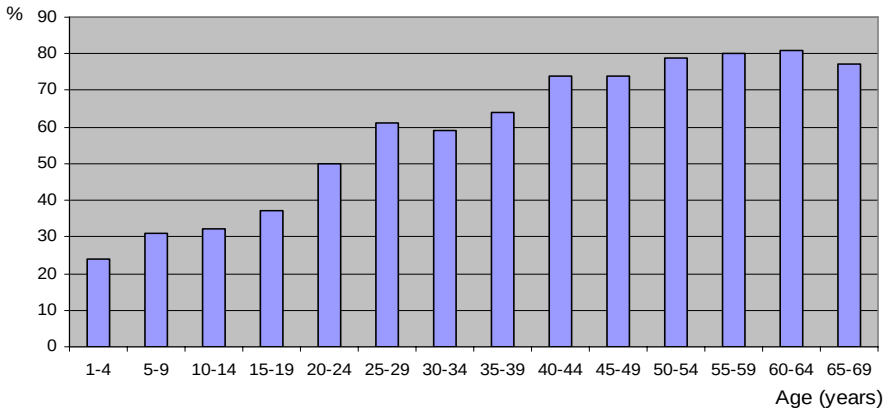


Fig.2. *Age-related seroprevalence of HSV-1 in the Swedish general population in 1990-91*



Source to Fig. 1-2 : Tunbäck 2004 (5)

Epidemiology

Genital herpes (GH) is one of the most widely spread sexually transmitted viral diseases and is caused by HSV-2 and HSV-1. The seroprevalence of HSV-2 is used as a marker for genital herpes, whereas HSV-1 antibodies reflect oro-labial and/or genital herpes.

In childhood and adolescence the HSV-2 seroprevalence is very low. It rises with increasing age after coitarche until about 40 years of age (6). Older age and female sex are factors associated with a higher seroprevalence of HSV-2. The age-related seroprevalence of HSV-2 in the Swedish general population in 1990-91 is shown in Figure 1 (5). The frequency of HSV-2 antibodies is higher in patients attending STI clinics than in the general population, with figures from 17% in Italy (7) to 40% in a study from Australia (8). Indeed, there is a correlation to sexual behaviour, especially to the number of sexual partners (9). In addition, ethnicity and low socio-economic status are known risk factors for HSV-2. The highest reported seroprevalence of HSV-2 in Sweden, 39%, has been found in middle-aged women who were teenagers during the 1960s, a decade when the incidence of gonorrhoeae was high as well (10). The occurrence of HSV-2 antibodies is higher in sub-Saharan Africa than in other developing countries with a prevalence of 30-80% in women and 10-50% in men (11). In Asia the prevalence in the general population is lower, between 10-30% (12).

Regarding HSV-1 the major transmission route is by transfer of saliva during childhood and the seroprevalence increases with age. The age-related seroprevalence of HSV-1 in the general population in Sweden 1990-90 is shown in Figure 2 (5). In recent years HSV-1 infection during childhood has decreased in many countries (13) and the proportion of first episodes of genital herpes caused by HSV-1 is increasing, especially shown in young women (14, 15). In the US the proportion of GH caused by HSV-1 in the age group 16-21 years of college students was 78% in 2001 compared to 31% ten years earlier (15). This increase has been attributed to change in sexual behaviour. In a report from the National Institute of Public Health in 1997 oral sex was more frequently reported in younger age groups (16). Another explanation might be a higher

susceptibility in young adults due to a decrease in the seroprevalence of HSV-1 (17). Also HSV-1 antibodies are to some extent correlated to low socio-economic status and sexual behaviour (18). In the adult population seroprevalence figures vary from <=70% in northern Europe and non-black Americans to >85% in southern Europe and Africa (18).

Clinical picture

(1) First episode primary HSV infection (no HSV antibodies)

Oro-labial herpes simplex: Clinical symptoms of primary oro-labial HSV infection vary from a mild local irritation or a few vesicles turning into crusts to gingivo-stomatitis with wide-spread ulcerations, lymphadenopathy, fever and malaise. Symptoms appear after an incubation period of 2-12 days (mean 4 days) and excretion of virus can be detected for an average time of 7-10 days (19).

Genital herpes simplex: After an incubation time of 2-20 days (mean 6 days) for both HSV-1 and HSV-2 the clinical picture ranges from mild erythema to vesicles, more often seen as ulcerations in the genital area, especially in the mucosa. Bilateral location of lesions is not uncommon. Extra-genital lesions on the buttocks, groins or thighs are more common in primary HSV-2 genital herpes, whereas concomitant lesions in the face are more common in genital herpes caused by HSV-1 (20). Primary anal and peri-anal localisation is particularly seen in men who have sex with men (MSM). Symptoms of systemic illness, like fever, malaise, urinary retention and lymphadenopathy, are more common in women than men (21).

(2) First episode non-primary HSV infection (antibodies against the other subtype of HSV)

This designation is mainly used for HSV-1 seropositive patients, who acquire HSV-2 genital herpes. In this case the HSV-1 antibodies seem to modify the clinical picture, making the symptoms milder and of shorter duration (22).

(3) Recurrent HSV infection

A clinical recurrence of HSV infection is the appearance of symptoms due to reactivation of latent virus in the neural ganglia in a person with HSV antibodies. The clinical signs and symptoms are milder compared to the primary infection and subside faster. Prodromal symptoms such as itch, pain or a burning sensation occur 4-48 hours before clinical manifestations in about 50% of patients with GH. In oral HSV-1 infection vesicles commonly appear on the lip margin rapidly turning into dry crusts, which are usually completely healed in a week's time in immunocompetent individuals. Recurrent episodes of genital herpes are mostly caused by HSV-2. In a Swedish study from 2001, 94% of cases of recurrent genital herpes were due to HSV-2 (23). Common manifestations of genital recurrences are unilateral vesicles or shallow ulcers in the genital area. Extra-genital lesions can occur, especially in patients with extra-genital location of lesions during the primary infection (20). Recurring fissures, erythema and oedema can be atypical manifestations of HSV-2 (24). The occurrence of clinical recurrences is dependent on both virus type and site of infection. The highest frequency of recurrences is seen in genital HSV-2 infections, followed by oro-labial HSV-1, genital HSV-1 and oro-labial HSV-2 (25).

The chronic nature of genital herpes in combination with genital location, asymptomatic shedding of virus and unpredictable recurrences leads to psychosocial/psychosexual distress in many patients, especially those with HSV-2 (26, 27).

(4) Asymptomatic HSV infection ─ unrecognised infections

The majority of HSV-1 and HSV-2 infections are asymptomatic or give non-specific symptoms that are not recognized as herpes (28). In a prospective study of 2393 sexually active HSV-2 seronegative persons followed for 18 months, 63% (98/155) of the newly acquired HSV-2 infections were asymptomatic. Asymptomatic seroconversion to HSV-2 was more common in men than women. Of 19 acquired HSV-1 infections, diagnosed by type-specific seroconversion or viral culture for HSV-1, 7 (37%) were asymptomatic (22). Another study by Langenberg et al. showed that approximately 50% of women with initially reported asymptomatic HSV-2 infection did in fact have clinical symptoms of GH (29).

(5) Neonatal HSV infection

Neonatal HSV infections can result from either HSV type 1 or 2. According to the literature 50-70% of the cases are caused by HSV-2 (30). Asymptomatic cervical shedding of virus during a primary infection in the mother is regarded as a main risk factor for HSV transmission to the neonate. The risk of vertical transmission has been estimated to 25-50% during a primary HSV-1 or HSV-2 infection compared with a risk of <1% in women with longstanding HSV-2 infection (31). Since recurrences are much more common than primary HSV infections, 30-50% of infants with neonatal herpes are born to mothers with established HSV infection, though in many cases unrecognised (1). Clinical manifestations of neonatal herpes infection ranges from disease localised to the skin, eye or mouth, to encephalitis and disseminated disease with high mortality (32).

Viral shedding and transmission

The most important factors for the transmission of genital herpes are asymptomatic shedding of HSV and unrecognised infections. A prospective study of 144 HSV-2 discordant heterosexual couples reported an annual seroconversion rate of 10% (33). In 11 of the 14 couples in which transmission occurred, the male was the source partner. The higher susceptibility to HSV-2 in

women than men has been shown in other studies and is partly explained by the larger exposed mucosal surface in women (34). In 69% of cases the transmission occurred without reported symptoms in the HSV-2 positive partner (33). In a prospective study of women with genital herpes, genital shedding was detected by virus isolation in 55% of HSV-2 infected women and 29% of women with HSV-1 genital herpes. Subclinical shedding of HSV-2 was shown on a mean of 2% of the days (35). The detection rate of subclinical shedding is affected by the utilised test method, the number of anatomical sites sampled and the duration and frequency of sampling.
With more sensitive PCR methods HSV DNA has been detected on up to 20% of days in women with HSV-2 genital herpes (36) and similar shedding data have been published for oral HSV-1 infection. Such high frequencies of HSV shedding support the view of HSV as a chronic rather than intermittent infection.

A study from 2008 demonstrated that the frequency of symptomatic recurrences of GH was not related to the risk of transmission of HSV-2 to sexual partners (37). Analogously, in quantitative PCR analyses, the number of viral copies detected in subclinical reactivation is often as high as during symptomatic recurrences (1).

Interaction between HSV-1 and HSV-2
Previous infection with HSV-1 does not give protection against acquisition of HSV-2 GH, but implies a higher likelihood of asymptomatic seroconversion to HSV-2 (22), in most cases a milder first episode of GH, fewer symptomatic recurrences and less genital shedding of HSV-2 (38). However, a study of pregnant women reported a HSV-2 seroconversion rate during pregnancy in HSV seronegative women with HSV-2 positive partners of 33% compared to a 5% risk in women with HSV-1 antibodies (39). A protective effect of genital HSV-2 against genital HSV-1 infection has also been proposed (40).

Concurrent genital infections with HSV-1 and HSV-2 are occasionally seen and case reports have been published (41).

Prevention of HSV infections

A pooled analysis of the effect of condom use in preventing acquisition of HSV-2 demonstrated a moderate protective effect of condom use in both men and women with no gender differences. Individuals who reported a 100% use of condoms had a 30% lower risk of catching an HSV-2 infection compared with those who never used condoms (42). Vaccination remains the ideal method to prevent viral infections, but HSV vaccination trials have not yet succeeded. A delicate problem is the occurrence of recurrent episodes in the presence of specific antibodies.

Educational efforts directed to populations at high risk of acquiring genital herpes are thought to be important in preventing HSV transmission. Type-specific serological tests and polymerase chain reaction (PCR) tests, information and counselling to help patients identify atypical genital symptoms as recurrences of HSV infection might reduce the transmission rate.

Diagnostic methods

For the detection of herpes viruses in clinical specimens, molecular assays based on PCR amplification have been shown to be more sensitive and more specific than virus culture. However, culture methods are used to detect resistance to antiviral drugs, mostly seen in immunocompromised patients.

Type-specific serological tests, based on glycoprotein G as antigen, are of value in patients with recurring genital symptoms, when the results of PCR tests for HSV have been negative. Some authors also recommend serological methods to find out whether a first-episode of GH is a primary infection or a first clinical recurrence (14, 22). The use of serological analyses of HSV in clinical practice is limited. However, the value of serology in epidemiological studies cannot be overestimated.

Treatment of HSV

Acyclovir, valacyclovir and famciclovir are all effective drugs to reduce symptoms and the duration of symptoms in primary and recurrent episodes of genital infections with HSV. Antiviral therapy should be administered as soon as the first symptoms appear.

Treatment of primary genital herpes does not affect the frequency or severity of recurrences. In patients with frequent recurrences long-term daily oral administration of the above-mentioned drugs reduces the number and the intensity of recurrent episodes. Although not an indication for treatment, valacyclovir has been shown to decrease transmission, when seropositive partners in HSV-2 discordant couples were treated (43).

Cytomegalovirus (CMV)

Cytomegalovirus is a large double-stranded DNA virus. The name is derived from the characteristic of this virus to enlarge the infected cells. CMV infects primarily leukocytes. The virus replicates for a long period in humans followed by latency and intermittent reactivation. The cellular site (s) of the latency phase is not fully elucidated, but endothelial cells and white blood cells are strong candidates (44, 45).

Epidemiology/clinical picture

Primary CMV infection is usually asymptomatic (77-100%) or associated with a mild mononucleosis-like illness. The incubation time is 4-8 weeks. Severe pneumonia and generalized, fatal infections are a threat to immunocompromised individuals (46). CMV pneumonitis and retinitis were feared complications in HIV patients before the introduction of highly-active antiretroviral therapy (44). A serious complication of CMV is congenital infection. Among newborns in Sweden about 0.5% have detectable CMV in the urine (47). Vertical transmission occurs both in women with primary CMV infection during pregnancy and in seropositive women with reactivation of the disease. About

80% of the children born with CMV are asymptomatic. Ten to twenty percent of neonatal infections with CMV result in neurological complications.

In 4-year-old children in Sweden the prevalence of CMV antibodies was about 40 % in 1998 (47) and the seroprevalence increases with age. The prevalence is higher in women than in men (48). Studies on pregnant women have shown CMV antibodies in 35-77% with higher prevalences in low-income groups (49). Reactivation of CMV during pregnancy due to hormonal factors has been reported (50).

Transmission

CMV has been detected in saliva, urine, breast milk (51), blood, cervical secretion and semen (52). Reported transmission routes of CMV are through saliva, droplet infection, blood transfusion, sexual contact and congenital or perinatal infection (44). Several reports have shown frequent transmission between children in day-care centers and from children to their caretakers.

CMV as an STI

Many studies have shown an association between CMV antibodies and sexual behaviour, such as early sexual debut, many sexual partners and occurrence of STI like chlamydia and gonorrhoeae (53, 54). No attempts have been made, however, to separate genital sexual practices from deep kissing, an activity undoubtedly associated with sexual activity and also with CMV transmission. The sexual transmission of HIV and HSV-2 has been shown to be more efficient from men to women than from women to men, partly explained by the larger exposed mucosal surface in women. Analogously, the higher seroprevalence of CMV in women than men at ages after coitarche compared with no reported gender difference during childhood is indicative of sexual transmission beyond oral-oral contact. Cervical CMV excretion was less frequent in women using barrier contraceptive methods (55). Coonrod et al. followed 608 CMV seronegative women in an STI clinic and of the 245 patients who seroconverted two women had CMV isolated from the genito-urinary tract before seroconversion suggesting genital transmission (53). Moreover, the

seroconverters were more likely to have symptoms and signs of upper genital tract infection, including cervical friability and cervical motion tenderness in absence of chlamydia and gonorrhoeae. CMV has been detected from endometrial samples in women with uterine bleeding and intrauterine device (56) and in chronic endometritis (57). CMV inclusion bodies have also been demonstrated in a recurrent vaginal ulcer (58) and in four cases of CMV cervicitis (cervical biopsies) (59).

Diagnostic methods

In clinical practice CMV is primarily detected by PCR methods. Quantification of DNA can be utilised to estimate the effect of antiviral treatment. Virus isolation is mainly used for estimation of antiviral drug resistance. Serological methods are useful, but a negative result does not rule out infection with CMV (44).

Treatment

Valacyclovir and ganciclovir are usually effective treatment against infection with CMV. Medical treatment is given to patients with severe infections. The use of ganciclovir/valganciclovir is sometimes limited due to haematological adverse effects.

Epstein-Barr virus (EBV)

EBV enters the body through mucosal surfaces and infects B cells, T cells, monocytes and epithelial cells (60). The virus then establishes a life-long latent infection in B lymphocytes, which become immortalised by the virus.

Epidemiology/clinical picture

Primary infection with EBV usually occurs subclinically during childhood and is correlated to low socio-economic status in the parents (60). Symptomatic infectious mononucleosis, also called “kissing disease”, appears mostly at ages 15-25 years after an incubation period of about two weeks. EBV infection is associated with two malignant diseases: Burkitt’s lymphoma seen in Africans

with concurrent malaria infection, and nasopharyngeal carcinoma primarily seen in Chinese people in south-east China. Our knowledge of congenital EBV infections and possible effects on the foetus is insufficient. A prospective controlled study on 126 women with primary and recurrent EBV during pregnancy showed no significant teratogenic effects, but stated the need of further studies with a larger sample size (61).

The reported prevalence of EBV antibodies in pre-adolescent children in developed countries is 40-80% (62). Between 7 and 14 years of age the prevalence is stable followed by a gradual increase during adolescence and early adulthood. The reported seroprevalence in teenage girls was 96% in the UK and 82% in Sweden (63, 64). In adult populations world-wide the reported seroprevalence is more than 90-95% (60).

Transmission

The transmission between children is related to transfer of saliva during pre-school years. A study of 24 healthy seropositive donors followed for 15 months demonstrated EBV shedding in the throat in 22/24 (92%) (65).

Epstein-Barr virus as an STI

Sexual activity has been identified as a significant risk factor for EBV seropositivity and infectious mononucleosis. Higgins et al. showed correlation of EBV to numerous sex partners and intercourse without a condom (66). Shared EBV strains between sexual partners have also been demonstrated (67). Besides in saliva EBV has been detected in genital secretions from the uterine cervix (68), the male urethra (67, 69) and in semen (52). In addition EBV has been demonstrated in vulvar mucosa (70), sulcus coronarius (68) and anal mucosa in homosexual HIV-positive men (71). Thomas et al. drew the conclusion that genital transmission is a minor route, based on the observation that the levels of EBV in genital secretions were lower than in saliva (67). Furthermore, fractionation of genital samples into cellular and supernatant fluid components revealed EBV to be mainly cell-associated, which might indicate passive transport in B-lymphocytes related to latent infection rather than active

viral replication. However, transmission of EBV via transfer of latently infected cells has been demonstrated in connection with blood transfusions (1). The detection of EBV in the genital area of both sexes supports genital transmission of this virus. As described for CMV, the higher frequency of EBV in women than men at ages after coitarche, compared to no difference between the sexes during childhood, is indicative of sexual transmission beyond oral-oral contact.

Real-time PCR techniques have made it possible to estimate the EBV viral load in specific samples. The demonstration of high numbers of EBV in cervical secretions of some individuals has been interpreted as shedding of cell-free virus rather than a passive carrier state of lymphocytes (63).

Correlation between the detection of EBV from the male urethra and microscopical urethritis was shown in paper III, but the causal connection with urethritis is still to be shown. Non-herpetic acute general ulcers in young women have been related to primary EBV infection and occasionally EBV has been isolated directly from the vulvar ulcer (72, 73). However, this type of acute genital ulcers associated with signs of systemic infection is not thought, by most authors, to be sexually transmitted. Whether such cases of acute vulvar ulcers reflect a primary genital EBV infection remains to be shown.

Diagnostic methods

EBV is detected in blood or cerebrospinal fluid by PCR methods in patients with severe infections. Acute EBV infection is diagnosed through demonstration of IgM antibodies. Heterogenetic antibodies that agglutinate erythrocytes (Monospot test), antibodies to viral capsid antigen (VCA), early antigen (EA) and Epstein-Barr nuclear antigen (EBNA) are serological analyses indicating different phases of EBV infection.

Treatment

There is no effective treatment in clinical practice.

Adenovirus

Adenovirus (AV) belongs to the adenoviridae family and the name is derived from the "adenoidal tissues" (tonsils) in which it was first identified. It is a double-stranded DNA virus of medium size (60-90 nm) without an envelope. In humans more than 50 different serotypes have been identified (60).

Epidemiology/clinical infections

AV accounts for about 10 % of acute airway infections and is the second most frequent cause of gastroenteritis in children. The incubation time is about one week and lymphadenopathy is common (74). Adenoviral conjunctivitis with seasonal outbreaks is often seen. AV infections complicated with meningitis and encephalitis have been described (75, 76). Severe infections with AV are seen in immunocompromised individuals, especially children (44).

Transmission

The primary transmission route of respiratory AV infections is by circulating infected droplets, but virus particles are also excreted in stool. Eye infections are often contracted through bathing water or direct contact. Gastroenteritis is transmitted via the faecal-oral route.

Adenovirus as an STI

Adenovirus as a cause of non-gonococcal urethritis in men has been reported, often with simultaneous conjunctivitis or upper respiratory tract infection. Adenoviruses were detected in 12 men (4%) with NGU compared to one case (0.3%) in the control group without urethritis in a study by Bradshaw et al. in 2006. The two symptoms, severe dysuria and meatitis, were both associated with detection of adenovirus in this study (77).

Our knowledge of adenovirus as an STI in women is limited. Of 175 female patients in an STI clinic in Australia, none of the cervical samples were positive for adenoviruses (A to E) with a multiplex PCR method (78). Adenoviruses have only rarely been detected in the female genital tract (79).

Diagnostic methods

Adenovirus can be detected by PCR assays, antigen detection or serologic methods. Antibody assays are utilised in respiratory infections but are not adequate for intestinal and eye infections. Quantitative PCR methods can be used to estimate the amount of virus in blood in immunocompromised children with suspected chronic AV infection. Although rarely used, virus isolation methods are still performed in some laboratories (44).

Treatment

There is no treatment available in clinical practice.

Bacterial agents

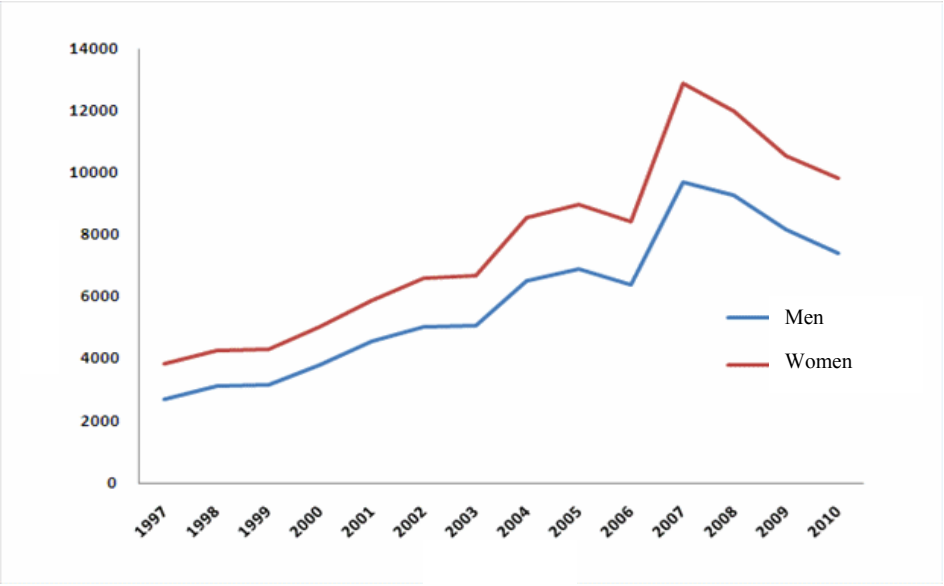
Chlamydia trachomatis

Chlamydia trachomatis is a small obligate intracellular organism, which is classified as a bacterium, although it cannot multiply outside the host cell. The serovars (genotypes) B-K produce genital infections, whereas A-C are the cause of trachoma. The main locus is columnar and transitional epithelium in the urogenital region, but *C. trachomatis* also infects squamous epithelial cells of the conjunctiva.

Epidemiology

C. trachomatis is the most common bacterial sexually transmitted infection world-wide. Incidence rates of genital chlamydia infection in the general population in Sweden are not known. The reported number of cases reflects the proportion of infected people in the tested populations and the extent of surveillance programs. In 1988 *C. trachomatis* was included in the Swedish Communicable Diseases Act. This implies that all diagnosed cases are reported, both from clinicians and laboratories. Patients with suspected or explicit *C. trachomatis* infection are given medical care and treatment free of charge, and partner tracing and notification are mandatory. Screening for genital chlamydia infection in Sweden started in the 1980s and the number of reported cases during the last decades is shown in Figure 3 (80).

Fig.3. The number of C. trachomatis cases reported to the Swedish Institute for Communicable Disease Control 1997-2010 (80)



The number of *C. trachomatis* cases increased during the late 90s, reaching a maximum of 47000 in 2007. A false decline, due to a mutant strain of *C. trachomatis* not detected by some of the diagnostic tests, was noted in 2006 (81). Of patients tested for chlamydia in STI clinics in Gothenburg approximately 10% were positive in 2007 (82). Among tested individuals in Sweden the male: female ratio is 1:3, i.e. the number of unrecorded cases may be higher among men. Population-based studies of the *C. trachomatis* prevalence in the US and sub-Saharan Africa indicate higher prevalences in women than men (83, 84). As for HIV and HSV-2, the transmission of *C trachomatis* may be more efficient from men to women than from women to men, attributed to the larger exposed mucosal area in women (1). Furthermore, the female genital mucosa is often exposed to semen for prolonged periods. The prevalence rate also depends on age, geographic location, sexual behaviour and diagnostic test methodology.

Clinical picture

Clinical manifestations in men are urethritis and epididymitis and in women urethritis, cervicitis and pelvic inflammatory disease (PID). Notably, the majority of infected women and men are asymptomatic. In symptomatic males dysuria and urethral discharge are common. Some infected women suffer from abnormal vaginal discharge, intermenstrual vaginal bleeding or vaginal bleeding after intercourse but these symptoms are unspecific (85).

Cases of chlamydia conjunctivitis in adults are seen occasionally. Rectal chlamydia infection is usually asymptomatic and mostly seen in MSM (86). *C. trachomatis* has been detected in the pharynx, but the clinical significance of oro-pharyngeal *C. trachomatis* is uncertain (87, 88). Occasional cases of sexually acquired reactive arthritis and Reiter's syndrome following genital chlamydia infection are seen.

The more invasive variant of *C. trachomatis*, subtype L, causing lymphogranuloma venereum, seems to be increasing among MSM in many European cities (89).

The extent to which chlamydia is the cause of long-term complications like tubal factor infertility and ectopic pregnancy is being debated (90). Pelvic inflammatory disease (PID) is caused by chlamydia (and other bacteria) ascending from the vagina and cervix into the upper genital tract (the uterus, fallopian tubes and the peritoneal cavity). Symptoms of PID include vaginal discharge and bleeding, dyspareunia and abdominal pain. One of the problems in assessing the risk of long-term complications caused by *C. trachomatis* is the lack of established diagnostic criteria of PID. Infertility as a result of *C. trachomatis* in men has also been proposed (91).

Transmission

Infection with *C. trachomatis* is mainly spread through sexual intercourse, but transmission in other intimate situations including transfer of genital secretions cannot be excluded. Transmission from mother to infant during labour can result in conjunctivitis and pneumonia in the newborn child.

Diagnostic methods

The development of diagnostic methods has yielded highly sensitive nucleic acid amplification tests (NAAT) for *C. trachomatis* as polymerase chain reaction (PCR) and strand displacement assays (SDA). These methods synthesize high amounts of DNA copies from bacterial chromosomal or plasmid DNA. In Sweden two PCR methods, Roche Diagnostics and Abbot Laboratories, and one SDA method, Becton Dickinson, are equally recommended.

In male patients samples for the diagnosis of *C. trachomatis* are collected from the urethral orifice with a cotton swab or as first voided urine. In women diagnostic samples from the uterine cervix, the vagina, the urethra and first voided urine are used separately or in different combinations to increase the sensitivity of the test. The specificity is high irrespective of the source of the sample in both men and women. A report from the SBU (Swedish Council on Health Technology Assessment) in 2010 demonstrated a higher sensitivity of vaginal samples than samples from other origins in women. The lowest sensitivity was shown for urine samples in women (92). On the contrary, first-voided urine samples have a slightly higher sensitivity than urethral samples in males (93). Earlier used culture methods have mostly been replaced by NAAT, but are still performed in some laboratories.

Treatment

The recommended treatment in Sweden is tetracyclines for 9-10 days, i.e. doxycycline 100 mg twice a day the first day and then once daily for another eight days. An alternative regimen is a single dose of azithromycin (1g).

Mycoplasma genitalium

Both mycoplasma and ureaplasma species belong to the class of bacteria called Mollicutes, which means "soft skin". The mycoplasmas lack a cell wall and are surrounded by a cell membrane. *Mycoplasma genitalium* (Mg) has the smallest genome of all self-replicating microorganisms (94). Like *C. trachomatis* Mg is incapable of replication outside the host cell and has affinity for columnar and transitional epithelium in the urogenital region.

Epidemiology

The incidence of genital infections with *M. genitalium* in the general population is not known. Among STI patients in Sweden the reported frequency of Mg is 3.5-7% in both sexes (95-97), in most studies about half as common as *C. trachomatis*. Bjartling et al. found a prevalence of Mg in 7598 female attendees in a gynaecology outpatient clinic of 2.1% (98).

Clinical picture

Mg is an etiological agent of male urethritis, both non-gonococcal urethritis (NGU) and non-chlamydial-non-gonococcal urethritis (NGNCU) (99). The role of Mg in prostatitis is a matter of debate, although a few small studies have found the organism in urine and semen of prostatitis patients (100, 101). Even less is known regarding Mg epididymitis. A case report of a male patient with concomitant unilateral conjunctivitis and urethritis with positive PCR for Mg from both locations has been published (102). The reported prevalence of Mg in rectal samples from 500 MSM in San Francisco was 5% (103).

There are studies suggesting Mg as an etiological agent in mucopurulent cervicitis (104), in endometritis (105), in post-abortion PID (106) and in salpingitis (107), but additional work in this field is needed.

Transmission

M. genitalium has become established as an STI pathogen. Falk et al. detected Mg in a significantly higher proportion of male and female sexual partners to patients with Mg (108).

Diagnostic methods

M. genitalium is detected by in-house PCR methods in first-void urine and samples from the urethra and/or the cervix.

Treatment

Recommended therapy is a 5-day regimen of azithromycin, with 500 mg the first day followed by 250 mg for another 4 days. Increasing resistance to this drug has been noted in some countries (109, 110). In cases of resistance to azithromycin, moxifloxacin, in oral doses of 400 mg once daily for 7-10 days, has proved to be highly effective (111).

Ureaplasma urealyticum

Ureaplasma urealyticum (Uu) was detected more than half a century ago (112) and has been demonstrated to be common in the genital tract of both men and women. It was shown to be associated with an increased number of sexual partners in a study from 1973 (113) and its significance in urethritis has been a matter of debate ever since (114-116). Some studies have shown association between Uu biovar 2 and urethritis in men (117, 118), but after multiple logistic regression Povlsen et al. only found independent correlation between Uu and age<=24 years but not between Uu and NGU. Uu as a sexually transmitted pathogen in women has not been fully investigated. It was detected in 3% of cervical samples from female STI patients in Australia (78) and its correlation to cervicitis is not known. In a study of male STI patients, Uu was detected in 30% of both men with and without NGU (117).

THE CONCEPTS OF URETHRITIS AND CERVICITIS

Urethritis

The term *urethritis* means inflammation of the urethra. Common symptoms are urethral discharge, pain during micturition and urethral pruritus or discomfort. A high proportion of men and women with urethritis are asymptomatic. Persistent or recurrent urethritis following treatment of acute non-gonococcal urethritis (NGU) occurs in 10-20% of male patients (119).

Epidemiology

Urethritis is the most common clinical syndrome in male patients in STI clinics. Our knowledge of the prevalence of urethritis in the general population is limited. In 2009 the prevalence of urethritis among 5447 young men aged 18-27 years in the US was estimated; In this study 1.2% of the participants reported symptoms of urethritis (120).

Microscopical urethritis

Microscopically, urethritis is defined as an increased number of polymorph nuclear leukocytes (PMNL) in a Gram or methylene-blue stained smear from the distal part of the urethra. The most established definition of microscopic urethritis is ≥5 PMNL per high power field (HPF) in ≥5 fields in 1000x magnification (121).

In men a PMNL count of ≥5 in a stained urethral smear has been correlated with detection of *C. trachomatis*. Among men with *C. trachomatis* infection 63-82% display this microscopic criterion of urethritis. Studies have shown that 94% of male patients with Ng and 77% of men with Mg have PMNL counts ≥5 in stained urethral smears (122, 123).

The signs and symptoms of urethritis in women are less generally defined. However, in most instances, clinically and scientifically, the same definition as for men has been used. In a study by Falk et al. women with 5-9 PMNL per HPF in urethral Gram stain were categorized as “grey-zone-urethritis” (108).

Etiology

Also the etiology of urethritis has mainly been investigated in male patients. *Neisseria gonorrhoeae* was the etiological pathogen first identified in male urethritis. Cases negative for Ng were defined as non-gonococcal urethritis (NGU). *C. trachomatis* was discovered in the 1970s and is detected in 11-43% of patients with NGU. In recent years *M. genitalium* has been identified as a causal agent in 9-25% of NGU cases. Other reported agents in NGU are adenoviruses, *T vaginalis* and herpes simplex virus detected in 2-45, 1-20% and 2-3%, respectively (124). EBV has been detected in urethral smears from men with *N. gonorrhoeae* (125). However, in 20-50% of patients with NGU no pathogen is isolated (1) and the diagnosis in such cases is "non-specific urethritis". This diagnosis most probably includes both men with non-infectious urethritis and men infected with not yet identified pathogens.

Partner tracing

An evaluation of partner notification of men with asymptomatic NGNCU stated that female contacts with a high prevalence of *C. trachomatis* were identified, but at the cost of quite high resources (126). In another study only 1/41 (2.4%) female partners of men with asymptomatic NGNCU were diagnosed with *C. trachomatis* (127). There is also some concern that invasive urethral screening in STI clinics might reject asymptomatic men from attending.

Cervicitis

Cervicitis is the term for inflammation of the uterine cervix, but there is no widely accepted definition of this condition. The clinical signs mostly used for cervicitis are visible mucopurulent secretion and friability (easily induced bleeding) of the cervical portio. Various microscopical criteria have been used, such as >10 or >30 PMNL per HPF in a stained cervical smear (1). Inflammatory cells in a vaginal wet smear in 40x magnification have been interpreted as reflecting inflammation of the cervix with the limits 10 PMNL per HPF (128) or a ratio of PMNL:epithelial cells >1 judged as cervicitis (129).

AIMS OF THE STUDIES:

I. To estimate the age-related seroprevalence of HSV-2 in male and female STI patients. To assess the proportion of symptomatic, unrecognised and true asymptomatic patients with HSV-2 infection, initially and after receiving the serological results and counselling

II. To estimate the age-related seroprevalence of HSV-2 in pregnant women and compare with contemporary female STI patients

III. To investigate the role of viral organisms in male urethritis

IV. To investigate asymptomatic shedding from the uterine cervix of five human herpes viruses; cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV) type 1 and type 2 and varicella zoster virus (VZV) in young women

V. To estimate the association of the microscopical and clinical signs of inflammation routinely used in Scandinavian STI clinics with a positive test for *C. trachomatis* in female STI patients

MATERIALS AND METHODS

The studies were conducted at the STI clinic of Sahlgrenska University Hospital (I, III, V), Gothenburg, at the STI clinics in Skövde and Borås (III) and at two Maternity Centers in Western Sweden: Västra Frölunda (II, IV) and Vänersborg (IV). The microbiological analyses were performed in the virological and bacteriological laboratories at the Department of Clinical Microbiology, both in Sahlgrenska University Hospital.

Subjects and samples

Paper I: Patients (first visitors) who attended the STI clinic of Sahlgrenska University Hospital during the recruitment period were asked to participate in the study. The catchment period ran from January 2000 to May 2001, except during three summer months in 2000 and some additional days, when the clinic was too busy. All included patients were given written information about the study and the natural course of genital herpes infections. Of 1769 patients invited to participate, 1014 (57%) agreed to be included. Demographics and data on sexual behaviour and previous STI are shown in paper I, Table I. The age and sex distribution was similar between the included patients and the ones declining, with a mean age of 30 years for the men and 28 years for the women. The motives for abstaining were not consecutively investigated, but among 53 patients asked by a nurse common reasons were unwillingness to have a blood test or to know about a silent HSV-2 infection. One third did not want to tell the reason.

Paper II: In paper II the HSV-2 seroprevalence in pregnant women was investigated. Sera were collected from women attending an antenatal clinic in Western Sweden during 2002. As is routine in Sweden blood was drawn for serological analyses of HIV and rubella from all the 661 women who attended. A random sample of 299 sera from these 661 frozen blood samples was selected and tested anonymously for HSV-2 antibodies. The mean age of the pregnant women was 30.5 years (range 17-45 years).

For comparison 290 female STI patients, included in the STI patient population in paper I, were separately analysed.

Paper III: Male attendees at the STI clinics of Sahlgrenska University Hospital, Södra Älvsborgs Hospital and Skövde Hospital were included from 2004 to 2007. Patients were not included when the clinics were too busy. The inclusion criterion was more than four PMNL per HPF in the urethral smear in at least five HPF. Initially 124 men met this criterion. Since 12 samples were lost in the laboratory, 112 patients with urethritis were analysed. 100 of these were included in the STI clinic at Sahlgrenska University Hospital. As controls, 103 male patients, who attended the STI clinics for other reasons than urethritis and had 0-2 PMNL per high power field in their urethral smears, were included. None of the control patients had symptoms of urethritis. None of the included men had visual lesions of genital herpes or balanitis involving meatus. Demographic data for patients with microscopic urethritis and for the controls are shown in paper III, Table 1. The median age was 28 years in the group with urethritis and 30 years in the control group. The number of partners during the last six months was similar between the groups, but a history of STI was more common among the patients with urethritis.

Collection of smears for microscopy

The samples for microscopy were collected with a plastic loop of 1 μl from the distal part of the urethra. The methylene-blue smear was then examined by light microscopy at magnification x1000. The used definition of microscopical urethritis was ≥5 PMNL per high power field in ≥5 fields. Specimens for virological analyses were collected with a thin cotton-tipped swab from the distal urethra and put in a tube with 1 ml of sterile NaCl. First-voided urine samples were collected for detection of *Chlamydia trachomatis, Mycoplasma genitalium* and *Ureaplasma urealyticum*. For all patients the time since last micturition was >1 hour.

Paper IV: 305 young Swedish women, who attended two Maternity Centers in Western Sweden, were included. The enrolment periods ran from October 2004 to May 2005 and November 2005 to April 2006. All women, who were invited to the population based Cervical Cancer Screening Programme at the age of 23 and 26 years and attended during these periods were included. Cervical specimens were obtained by putting the cytobrush used for Pap smear in a tube with 1 ml ethanol (95%). The tubes were then stored at 8°C until analyzed. The samples were marked with the name of the study but with no patient identification.

Paper V: Ninety-nine female patients, who attended the STI clinic of Sahlgrenska University Hospital due to chlamydia partner notification from February 2005 to October 2007, were included. In Sweden, according to the Communicable Diseases Act, all cases of chlamydia infection are reported and partner tracing is mandatory.

A genital examination was performed on all patients and the cervix was initially cleaned of excess mucus with a large cotton swab. Samples for microscopy were collected before the specimens for *C. trachomatis* SDA in all cases. Time from the latest micturition was > one hour for all patients.

Collection of samples for detection of Chlamydia trachomatis

The specimens for detection of Chlamydia trachomatis were sampled by separate standard cotton swabs from the cervix and the distal urethra after the smear from each site. Both swabs were put in the same tube with BD ProbeTec ET transport medium, which was sent to the Department of Bacteriology, Sahlgrenska University Hospital the same day.

Collection of smears for microscopy

From endocervix a sample was taken by a cotton swab and from urethra by a plastic loop of 1 µl; these specimens were stained with methylene blue and visually examined through a light microscope (x1000).

For wet smear examination samples were taken from the vaginal wall by a plastic loop and diluted in 10% KOH and 0.9% NaCl, respectively. The wet

smear was examined with a phase contrast microscope (x400). In addition to recording the number of PMNL in relation to epithelial cells the presence of clue cells, amine odour and signs of candidiasis were noted.

Laboratory methods / Diagnostic methods

Paper I: Antibodies for HSV-2 were determined by an ELISA test using a Helix pomatia lectin-purified glycoprotein G2 antigen (gG-2) described by Svennerholm et al. (130). A high titrating HSV-1 positive serum was used as control and the cut-off was set to the mean value of this control plus 0.2 absorbance units. Positive sera were further analysed by the ELISA test Gull/Meridian. Sera with discordant results between the two ELISA tests were confirmed by a Western blot assay previously characterised in the Virological Laboratory at Sahlgrenska University Hospital and considered as gold standard at that time (131). Virus isolation and typing of HSV were performed as previously described (132). The PCR assay for detection of HSV was based on primers from the type-unique promoter region of the glycoprotein D gene (133).

Paper II: Sera were initially tested by the in-house ELISA test based on the helix pomatia lectin-purified gG-2 antigen described for paper I (above). For verification, all positive sera, both from the pregnant women and from female STI patients, were confirmed by a commercial test approved by the US Food and Drug Administration: HerpeSelect HSV-2 ELISA (Focus Diagnostics), which is based on recombinantly produced gG-2. When making a comparison with older studies the results of the in-house gG-2 ELISA without further confirmation were used.

Paper III: Real-time PCR (TaqMan, Applied Biosystems, Foster City, CA, USA) was used for the detection and quantification of DNA of EBV, CMV, HSV-1, HSV-2 and adenovirus as well as ß-globin and PhHV-1 DNA. The specific methods for detection of each of these viruses have been described previously (134-137). As internal controls, to assess an efficient extraction of

DNA, PhHV-1 primers and probe were used as earlier reported by Niesters (138). The method for analysis of human ß-globin, used to guarantee a sufficient amount of DNA in the samples, was published in 2001 (139).
Chlamydia trachomatis was detected by the DNA amplification technique strand displacement amplification (SDA) using BD ProbeTec ET System *C. trachomatis* amplified DNA assay (Becton Dickinson Diagnostic Systems) (140). This assay amplifies a single target region in the *C. trachomatis* chromosomal CDA cryptic plasmid and detects both the *C. trachomatis* wild-type and the mutant strain described in 2006 (81). This method is used as the standard method for detection of *C. trachomatis* in the laboratory of Microbiology, Sahlgrenska University Hospital.
PCR identifications of *Mycoplasma genitalium* and *Ureaplasma urealyticum* were made as previously described (141, 142).

Paper IV: PCR detection and quantification of EBV, CMV, HSV-1 and HSV-2 and VZV were made by a quantitative real-time (TaqMan) PCR assay as described in paper III. Analyses of ß-globin and PhHV-1 were performed as in paper III.

Paper V: NAAT detection of *C. trachomatis* (Becton Dickinson) was performed as described in paper III (samples from the uterine cervix and the urethra in paper V). This assay also detects the mutant strain of *C. trachomatis* described in 2006.

Statistical analysis

Paper I: Statistical calculations were performed in the Epi Info program. Differences of proportions were compared using the Chi-squared test with the level of significance set to 5%.

Paper II: Point estimates of proportions were given with 95% CI. When comparing the HSV-2 seroprevalence of the pregnant women in paper II with a previous study population in Malmö 1990-93 our population was age-adjusted to the age distribution of the Malmö cohort.

Paper III: Fisher's exact test was used to compare clinical and laboratory data between patients and controls. Possible confounding factors, including co-variations of pathogens, were investigated by multivariate logistic regression. In the multivariate analysis the included variables were "chlamydia infection", since it was found to be significant in the univariate analysis and "previous chlamydia infection", a factor shown to be associated with urethritis in the literature and also significant in the univariate analysis of paper III.

Paper IV: Point estimates of proportions were given with 95% CI.

Paper V: Fisher's exact test was used for univariate associations. Statistical significance was set to $p<0.05$.

Ethical approval

The studies I-III and V were approved by the Ethics Committee of the Medical Faculty at the University of Gothenburg. For paper IV ethical approval was not needed, since the study did not include any patient-identifiable data or samples and all samples were destroyed at the end of the study.

SUMMARY OF THE RESULTS AND DISCUSSION

Seroprevalence of herpes simplex type 2 (Papers I-II)

Serological tests discriminating herpes simplex type 2 from type 1 have made it possible to study the specific prevalence of HSV-2 in different populations and to follow changes in the epidemiology of HSV-2 infections. Serological evidence of HSV-2 is a marker of genitally acquired infection and HSV-2 prevalence is negligible in individuals who have never been sexually active.
In papers I and II the seroprevalence of HSV-2 in two different populations has been studied: STI patients (first visitors) of both sexes in our STI clinic 2000-2001 and pregnant women at their first visit in an antenatal clinic in Gothenburg in 2002.

Among 1014 STI patients giving a blood test for detection of HSV-2 antibodies, 152 (15%) were seropositive: 12% (86/724) of the male patients and 23% (66/290) of the females. Comparison between studies is difficult due to differences in both diagnostic methods and the studied populations; e.g. patients with symptoms of and/or history of genital herpes are included by some authors and excluded by others. Moreover, since HSV-2 seroprevalence is related to sex, age and ethnical background, accurate information about the studied population is essential (143). Reported figures on HSV-2 seroprevalence in STI patients from France 1994, Italy 1997-98, Spain 1996-97 and the UK 1992 were for females/males 67/45%, 25/25%, 30/12% and 25/17%., respectively * (144).

Of the pregnant women 10.4% (31/299) were seropositive in the type-specific in-house HSV-2 ELISA. Of these, 27/31 were verified as positive in the HSV-2 Focus test. Hence, the resulting seroprevalence of HSV-2 was 9.0% (27/299), which was at a level similar to contemporary studies of pregnant women from Norway and Finland (145, 146).

** References of seroprevalence in different populations have been selected among those which report separate figures for gender and age groups and with a sample size of >100. Studies of HIV positive populations and MSM populations have been excluded, since they are not relevant for comparison in this context.*

During the 1980s high figures on the seroprevalence of HSV-2 in pregnant women were reported from Stockholm and Malmö (Table II, paper II). These high prevalences of HSV-2 antibodies, >30% in most age groups, were considerably higher than reported from other northern European countries around the same period of time. The reported HSV-2 seroprevalences in Germany, Finland and Norway were 8.9%, 16% and 27%, respectively (147-149).
Epidemiological data on time trends of HSV-2 seroprevalence are limited. A population-based cross-sectional study from the US demonstrated a significant decrease from 21% in 1988-1994 to 17% in 1999-2004 (150). However, a cross-sectional study from Sweden found no significant change in HSV-2 seroprevalence during the first six years of the 1990s (151). Comparison of the pregnant women described in paper II with the latest cohort studied by Persson et al. from Malmö (152) showed a significant decrease in HSV-2 seroprevalence from 20.7% in 1990-93 to 9.4% in 2002 (after age-adjusting the population from Gothenburg to the age distribution of the Malmö cohort).

The comparison of HSV-2 seroprevalence with female STI patients in paper II showed, as expected, a much higher frequency of HSV-2 antibodies in this population. This illustrates the importance of distinct report of patient selection. Although these two populations were of female gender and from the same part of Sweden, a high-risk sexual behaviour is more common in STI patient populations and is mirrored by a higher seroprevalence of HSV-2.

In papers I and II sera were initially tested by the in-house ELISA test based on the helix pomatia lectin-purified gG-2 antigen. In paper I positive sera were further analysed by the ELISA test Gull/Meridian. Sera with discordant results between the two ELISA tests were confirmed by a Western blot assay. In paper II positive results of the in-house gG-2 test were confirmed by HerpeSelect HSV-2 ELISA/Focus Diagnostics, since the Gull/Meridian ELISA test, used for confirmation in paper I, was no longer available. Both the commercial Focus ELISA and the in-house gG-2 assay have a high reported sensitivity and specificity in high-prevalence populations (5, 153). Also the Gull/Meridian

ELISA test was approved by the FDA and had a high reported sensitivity and specificity (154).
The use of gG-2-based assays in populations with a low prevalence of HSV-2 is more controversial. In a study from 2002 the authors stated that only initial testing by a gG-2 ELISA confirmed by Western blot testing of the positive results would give a PPV over 80% in a population with a HSV-2 seroprevalence of 10% (155). Western blot is the diagnostic gold standard according to the 2010 European guidelines for the management of genital herpes, but the method is labour-intensive and not available in most clinical settings. Results of Western blot can be difficult to interpret as illustrated by a recent study from Canada: Of 364 tested sera, 14 (3.8%) had to be excluded due to inconclusive results of the Western blot (153). Golden et al. demonstrated a higher risk of false positive results of HSV-2 ELISA tests in HSV-1 seropositive individuals (156) and it is recommended to confirm positive HSV-2 ELISA tests with an additional unrelated test, especially when the positive results are in the low-titer range.

When commercial type-specific serological tests became available, there was a debate concerning screening for HSV-2 infection in high-risk populations such as STI patients, MSM and HIV positive individuals. The motives for screening were that diagnosing asymptomatic and unrecognised HSV-2 positive patients in high-risk settings should allow these individuals to practise safer sex and thereby prevent further transmission of HSV-2 (157). It has also been stated that STI patients seeking medical care should have the right to be tested for a sexually transmissible infection such as HSV-2. Against screening for HSV-2 in STI clinics stands the knowledge that a positive serological result of HSV-2 might have negative psychosocial and psychosexual consequences, especially for vulnerable individuals (158). On the other hand, other studies have reported no apparent psychosocial impact of diagnosing subclinical HSV-2 infection serologically in STI patients (158, 159). The ethical considerations regarding serotesting of HSV-2 were discussed by Krantz and Löwhagen in 2004 (160). Their conclusion was that a correct diagnosis provides ethical benefits for

patients with symptoms. For asymptomatic individuals, however, the disadvantages may outweigh the advantages.

Health economy consequences are to be considered as well. A study estimating the costs and benefits of screening heterosexual couples for asymptomatic infection with HSV-2 estimated that screening could prevent a new case of HSV-2 infection at a cost of 8000-9000 USD. The authors stated that screening would result in a decrease in the incidence of HSV-2 infections and an increase in health care costs. Whether this cost-benefit analysis would justify HSV-2 screening compared to other preventative health interventions was uncertain, since the impact of HSV-2 infection on quality of life has not been sufficiently studied (161). Another problem is that the results of serological tests are difficult to interpret due to varying time to seroconversion and demonstrated fluctuation in estimated antibody levels over time in patients with established HSV-2 infections (162).

Serological HSV-2 screening of STI patients or other high-risk groups is no longer recommended. Remaining clinical indications for HSV-2 antibody testing are recurring genital symptoms, including those typical of clinical herpes, and atypical lesions like fissures, when PCR tests have been negative. As described by Löwhagen et al., serological testing to distinguish between recently acquired HSV-2 infections and clinical recurrences of former asymptomatic infections might be useful in selected patients (14). Whether partners of patients with genital herpes should be tested for HSV antibodies will have to be decided from case to case.

Routine serological testing of pregnant women for HSV-2 has been discussed and advocated especially in the US (163). Considering the low prevalence of HSV-2 in pregnant women, the PPV of a serological test will be low. Furthermore, to identify the women at highest risk, the male partners have to be tested as well, in order to find HSV serodiscordant couples with women susceptible for and exposed to HSV. A health economy study of screening pregnant women and their partners for HSV estimated a resulting decrease in HSV-1 and HSV-2 infections in women and infants and a save of costs (164).

Acceptance of herpes simplex type 2 antibody testing (Paper I)

Patients (first visitors) of both sexes attending the STI clinic at Sahlgrenska University Hospital were offered a blood test for detection of HSV-2 antibodies. Of 1769 patients asked to participate, 1014 (57%) agreed to be included. Age and sex did not differ between those who chose to be tested and those who abstained. The participation rate in similar studies from STI clinics in Seattle and the UK were 52% and 90%, respectively (165, 166).

Acceptance of HSV-2 antibody testing has been shown to be independently correlated to female sex, older age, a history of STI, perceived vulnerability to HSV-2 infection and the test being free of charge (165, 167). The acceptance of HSV-2 antibody testing is higher among patients in STI clinics than in general medical clinics. In a study from Leeds to assess patients' attitudes toward HSV-2 antibody testing and the effect of written information regarding genital herpes, 200 clinic attenders were randomly divided into two groups; Half of the patients were given a detailed three-page sheet of information on genital herpes, and the others were simply asked to fill in a questionnaire and give a blood sample for HSV-2 antibodies without additional information. The authors could not demonstrate any differences between the groups in the willingness to perform a blood test for HSV-2 antibodies, and the overall refusal rate for testing was only 2% (166).

There is reason to believe that patients' willingness to be tested is affected by what aspects of HSV-2 infection are emphasised in written and oral information. In the work by Mullan et al. only 41% of the males and 37% of the females chose to be tested, and in that study the information stressed the disadvantages of performing the test (168). The relatively low participation rate in our study, compared to the one from Leeds, is probably due to the fact, that all clinicians involved in our study were observant of patients' reluctance to be tested and scrupulous about not trying to persuade anyone. The tendency to abstain from HSV-2 antibody testing might be correlated to certain personality traits. These might co-vary with sexual risk-taking behaviour. However, this possible

personality type selection bias could not be avoided without the risk of causing psychological and psychosexual morbidity in sensitive patients.

In the present study, drop-outs were not consistently asked about their reasons to abstain from serological testing. However, of 53 patients asked by the nurse why they did not want to participate: 34% gave the answer "don't like blood tests", 17% of the drop-outs did not want to know about a possible silent HSV-2 infection and one third of the patients did not want to tell their reason for not participating. Fear of needles is a logical reason to avoid a blood test and has been associated with abstaining in other studies (167). In paper I this is illustrated by a significant difference in the proportion of patients having an HIV test; 85% of the patients tested for HSV-2 compared to 36% of the ones abstaining. In some cases, recent high-risk sexual behaviour in combination with the knowledge that an HIV test should be postponed to be reliable, might have been the cause for abstaining.

Demographics and data of sexual behaviour and STI of included patients and drop-outs are shown in Table I, paper I. A higher proportion of included patients had a history of genital herpes: 9% compared to 4% of the drop-outs. It is not surprising that individuals with experience of symptoms consistent with GH are more inclined to have a serological test for HSV than other patients. However, the proportion of patients tested for *C. trachomatis* and *N.gonorrhoeae* was significantly higher in the group not tested for HSV-2 antibodies and so was the occurrence of positive tests for these bacterial STI. Since there is co-variation between the prevalence of HSV-2 and other STI, this might, to some extent, compensate for the bias of the higher number of patients with symptomatic genital herpes in the study population. Data on the number of sexual partners and condom use did not differ between the patients tested for HSV-2 antibodies and the drop-outs.

Recognised and unrecognised infections with herpes simplex type 2 (Paper I)

Among 1014 patients giving a blood test for detection of HSV-2 antibodies 152 (15%) were seropositive: 12% of the male patients and 23% of the females. According to the questionnaire, 62 (41%) of the seropositive patients answered that they had a history of genital herpes. The proportion of symptomatic infections among HSV-2 seropositive individuals varies between studies (28, 169-172). In general, higher figures have been shown in STI clinics than in antenatal clinics or among blood donors (28). This could be explained by the fact that individuals with symptoms of genital herpes seek medical care in STI clinics. Moreover, as shown in the present study, patients with symptomatic genital herpes are more inclined to have a serological test for HSV-2 than more unselected populations.

Twelve seropositive patients without a known history of genital herpes presented with symptoms and signs typical of genital herpes at the initial visit. In seven of these patients HSV-2 was detected, by PCR or culture, and no further follow-up was performed within the study.

Of 83 seropositive patients without a documented history of genital herpes invited to a counselling visit, 65 (78%) showed up. Interviews with these patients revealed that 20/65 (31%) of the patients had had symptoms compatible with genital herpes defined as "recurring vesicles and/or ulcers in the genito-anal region". This group of patients illustrates the definition of "unrecognised infections". Six patients with most of their recurrences in the peri-anal or gluteal region had not realised that it could be genital herpes, due to the localisation. After counselling, some of the patients admitted that they had had a slight suspicion of genital herpes, which they had tried to suppress.

Uncharacteristic genital symptoms with low suspicion of herpes, such as periods of itching, eczema, balanitis and urethritis were reported by 11/65 patients.

Fifty-two percent (34/65) of the patients attending for a counselling visit had not had any genital symptoms.

A similar study of recognised and unrecognised HSV-2 infections showed that, after receiving a positive result of HSV-2 antibodies and counselling, approximately 50% of women with reported asymptomatic HSV-2 infection declared symptoms of GH (29). This illustrates the importance of HSV-2 serological testing and information to help patients recognising clinical recurrences of HSV in order to practise preventive measures.

The proportion of "unrecognised symptomatic infections" is related to the extent and contents of the given oral and written information preceding the question "do you have genital herpes". If the patients in the present study had received the information and counselling given during the counselling visit at the initial visit before answering the questionnaire, some of those might have been put in the group with previous history of genital herpes. However, the most important factor for recognition of previously unspecific genital symptoms as manifestations of genital herpes is most probably the positive result of HSV-2 antibodies.

Asymptomatic HSV-2 seropositive patients had a history of genital candida more often than HSV-2 seronegative patients (42% vs. 26%). A similar result was seen in a study of pregnant women in 1993 (173). To a question about sensitive skin defined as tendency to redness, itching and pain after friction and/or washing, 45 % of the asymptomatic patients with HSV-2 antibodies answered yes compared to 28% of HSV-2 seronegative patients. These answers could represent atypical manifestations of genital herpes (i.e. unrecognised recurrences), but could also be due to a more vulnerable skin barrier predisposing both for non-specific irritative symptoms, HSV infection and candida. However, there was no difference in the occurrence of genital eczema between asymptomatic seropositive and seronegative patients.

Urethritis in men (Paper III)

Signs or symptoms of urethral inflammation are common in male STI patients. However, the etiology is unknown in the majority of cases. In paper III 112 male patients with microscopical urethritis, defined as ≥5 PMNL per HPF, were tested for *C. trachomatis, M. genitalium, U. urealyticum*, HSV-1, HSV-2, CMV; EBV and adenovirus. For comparison, 103 male patients, who attended for other reasons than urethritis, with a maximum of 2 PMNL per HPF were included. None of the control patients had symptoms consistent with urethritis, whereas 98/112 (88%) of the patients with microscopical urethritis reported discharge and/or dysuria.

EBV was detected in 24/112 (21%) patients with urethritis compared with 6/103 (6%) in the control group. This correlation between EBV and microscopical urethritis was statistically significant ($p<0.01$). None of the viruses, HSV-1, HSV-2, CMV and adenovirus, were found in the urethritis patients and therefore these analyses were not performed in the control group. As expected, a significant correlation was demonstrated for *C. trachomatis*, an established pathogen in urethritis; 17/112 (15%) compared to 3/103 (3%) in the controls. *M. genitalium* was detected in 7/112 (6%) of the urethritis patients compared to 1/103 (1%) of the controls. This difference was not significant ($p=0.067$), probably due to the low prevalence in the urethritis group and to a relatively small sample size. *U urealyticum* was found in 10% of the patients in both groups. Uu subtype determination showed a similar distribution in patients with urethritis and controls.

Our inclusion criterion of ≥5 PMNL per HPF in urethral smear was chosen to study male patients with "microscopical urethritis". We regarded this method of diagnosis of urethritis as more objective and unbiased than including patients with symptoms of urethritis. The control group consisted of patients with confirmed absence of urethral inflammation estimated by a maximum of 2 PMNL per HPF in the urethral smear. A control group as well defined as in paper III is hard to obtain, since sampling from the urethral orifice is feared by many patients.

At the time of inclusion of patients in the study of urethritis, all male STI patients were offered an evaluation of a urethral smear as part of the clinical examination and very few patients abstained.

The limit of ≤2 PMNL per HPF in the control group was set to exclude patients with borderline urethritis, since it has been shown that intra- and inter-observer variation in the interpretation of smears is quite high. The reason for excluding men with balanitis involving meatus was to make sure that the observed PMNL originated from "true" urethral inflammation rather than from inflammation of the terminal urethra concomitant with balanitis.

In previous studies of male urethritis, the inclusion criteria of patients and controls and the used diagnostic methods vary, making direct comparison difficult. In a comprehensive case-control study of urethritis by Bradshaw et al., 329 symptomatic men were compared with 307 men without symptoms of urethritis regardless of microscopic evidence of urethral inflammation. As in our study (paper III) men with visual lesions of genital herpes were excluded, but meatitis, defined as "meatal inflammation and/or oedema" was noted in 38% of the patients with urethritis (sic!). First-stream urine was tested by SDA or PCR for *C. trachomatis*, *M. genitalium*, HSV-1 and -2, *T. vaginalis*, and adenoviruses. A significant correlation to urethritis was demonstrated for *C. trachomatis, M. genitalium*, adenoviruses and HSV-1, which were found in 20%, 9%, 4% and 2% of the cases, respectively (77).

The negative results of HSV and adenovirus in paper III could partly be explained by the exclusion of meatitis, which has been correlated with detection of these viruses in other studies (77). Regrettably, no analysis of drop-outs was carried out in our study of urethritis, but to our knowledge very few patients were excluded due to meatitis. In the above-mentioned study by Bradshaw et al., adenoviruses and HSV-1 were associated with sex with men and only one of the included urethritis patients in paper III defined himself as MSM. It is also possible that a microscopic inclusion criterion of presence of monocytes rather than PMNL would be more sensitive for viral infections.

The correlation between EBV and male urethritis has not been demonstrated earlier. Quantitative real-time PCR analysis of the 24 samples positive for EBV showed viral loads from 133 to 11500 copies/mL with a mean of 1343 copies/mL. The number of viral copies was not higher in the 15 EBV positive patients with >10 PMNL per HPF than in the 9 patients with 5-9 PMNL per HPF, a finding that somewhat supports EBV as a pathogen in urethritis rather than a passive passenger in lymphocytes. Moreover, although EBV was found in 10 patients with concomitant *C. trachomatis*, logistic regression analysis demonstrated an independent correlation between EBV and urethritis, unrelated to both actual and previous chlamydia infection.

A study of male STI patients from 1975 detected HSV by virus isolation in 1/114 men with urethritis, 1/61 without urethritis and 3/53 men with gonorrhoeae* (174). An Israeli study from 2003 identified HSV by antigen detection of urethral samples in 7/238 (2.9%) men with urethritis (175). A Swedish study of male patients also demonstrated HSV by virus isolation in 7/688 (1.0%) male first visitors in an STI clinic in Stockholm* (176). However, it is difficult to interpret whether such low figures represent asymptomatic shedding of HSV unrelated to urethritis or, in fact, rare cases of HSV urethritis.

To our knowledge, PCR detection of CMV from urethral samples in men with urethritis has not been published. In 1978 a study of 107 men with urethritis could not demonstrate CMV by virus isolation in any of the cases (121).

When it comes to bacterial etiology of urethritis, *C. trachomatis* has been reported to account for 30-50% of the cases and *M. genitalium* for 10-30% (77). The relatively low prevalence of *C. trachomatis* and *M. genitalium* in the men with urethritis in paper III, 15 and 6%, respectively, was somewhat unexpected. One explanation could be that 26% of the patients with urethritis had received antibiotic treatment in the last three months. In a previous study from our clinic comprising 115 men with urethritis, the detection rates of *C. trachomatis* and *M. genitalium* were 36 and 14%, respectively (95). In this former study from 2000, antibiotic treatment during the last three months was an exclusion criterion. However, the low detection rate of *M. genitalium* in paper III could not be fully

explained by antibacterial medication, since the vast majority of the recently treated patients in that study had received tetracyclines, drugs which do not eradicate *M. genitalium* in most cases. A more likely reason is that the high proportion of patients with previous episodes of urethritis (63%) reflects the fact, that most patients were included during booked consulting hours rather than in the walk-in STI clinic, which has been increasingly busy in recent years. There is reason to believe that the prevalence of pathogen-positive urethritis is higher in patients seeking instant medical care than in patients accepting a booked appointment later on.

U urealyticum biovar 2 has been correlated to urethritis in some studies, whereas other authors did not find any association, and the role of *U urealyticum* biovar 2 in urethritis is still uncertain (77, 114, 117, 118). Although the detection of *U. urealyticum* in the male urethra has been correlated to the number of sexual partners (177), it is not known whether this represents colonisation or infection by *U. urealyticum*.

The PCR assays utilised to analyse the prevalence of the different herpes viruses and adenovirus are highly sensitive and specific. Although not accredited for analyses of urethral specimens, all samples tested for ß-globin and phocid herpes virus type 1 were positive, showing that human DNA was amplified and that the DNA extraction procedure was efficient.

The utilised diagnostic NAAT methods for *C. trachomatis* and *M. genitalium* are the ones used in clinical practice and shown to be both sensitive and specific. Analysis of first-void urine samples has proved to be more sensitive than urethral swab specimens in detecting both chlamydia and mycoplasma in male patients (93). The incubation time was > one hour in all patients in paper III, but a study from 2009 demonstrated that shortening the incubation time to 20 minutes did not reduce the reliability of highly sensitive NAATs for *C. trachomatis* (178).

* *After exclusion of patients with external genital herpes lesions*

Asymptomatic genital shedding of herpes viruses in young women (Paper IV)

Viral DNA was detected in 66 (21.6%) of the cervical samples. The most common findings were CMV DNA, detected in 35 (11.5%) and EBV DNA found in 32 (10.5%) of the women. These two herpes viruses are widely spread in the general population with reported seroprevalence figures of 60-90% for CMV (179) and above 90% for EBV (180). HSV-1 DNA was detected in 5 (1.7%) and HSV-2 DNA in 4 (1.4%) of the cervical specimens. Corresponding seroprevalence rates in young women were 60% for HSV-1 and 15% for HSV-2 in 1990 (5). VZV DNA was not detectable in any of the specimens from the uterine cervix despite a seroprevalence of >95% in most European countries (181). The absence of VZV shedding was not surprising, since VZV has the least ability to reactivate of all herpes viruses.

The estimated DNA level for the detected viruses was similar with a mean DNA quantity of 2.6 log genome equivalents (GE)/mL for CMV (range 1.7-4.3), 2.5 log GE/mL for EBV (range 1.7-4.7), 2.4 log GE/mL for HSV-1 (range 1.7-3.5) and 2.6 log GE/mL for HSV-2 (range 1.7-4.1). The simultaneous presence of DNA from two or more herpes viruses was detected in 8 specimens. The simultaneous presence of two DNA viruses; CMV/EBV, HSV-1 /HSV-2 and CMV/HSV-2, was detected in 5 (1.6 %), 1 (0.3%) and 1 (0.3%) specimens, respectively. One woman had four detectable viruses (CMV, EBV, HSV-1 and 2). The DNA viral loads within the CMV group were evenly distributed, whereas in a few samples of EBV and HSV a higher number of viral DNA copies were detected (Fig.1, paper IV). High viral loads may indicate a higher risk of transmission, both to a child during pregnancy and delivery and to a sexual partner. However, the amount of HSV or other herpes viruses required for sexual or perinatal transmission is not known (2).

CMV DNA was detected in 11.2% of the cervical specimens, which is in accordance with previous studies. In the US cervical secretion of CMV was found in 13.6% of female STI patients (55); i.e. a population with an expected higher prevalence of pathogens associated with sexual behaviour. A study from

Italy demonstrated CMV DNA in 21% of cervical specimens from healthy women (17-25 years) with secretion rates decreasing with age (182).

EBV DNA was detected in the uterine cervix in 10.5% of the women. In a previous Swedish study EBV was found in 8.9% (10/112) of the cervical samples in female STI patients (183), while an earlier investigation from our STI clinic demonstrated EBV cervical shedding in 38% of 91 female patients (184). From Italy the reported occurrence of EBV DNA in the uterine cervix of healthy women between 17 and 70 years of age was 18-19% and similar in all age groups (182).

HSV-1 DNA was found in 5 (1.7%) and HSV-2 DNA in 4 (1.4%) of the cervical samples, respectively. Genital shedding of HSV is known to be intermittent and more frequent in HSV-2 than in HSV-1 GH. In a study by Koelle et al. cervical or external genital shedding during the first year after primary genital infection was detected in 1.25% of all days for HSV-1 and 4.3% for HSV-2 (185). The relatively high proportion of genital HSV-1 compared to HSV-2 in the cervical samples in the present study might be explained by the fact that HSV-1 has been shown to cause about 80% of first episodes of GH in young women (14).

PCR analysis of cervical shedding of VZV, a virus with a seroprevalence at a level similar to EBV, >90% in the adult population, has, to our knowledge, not been published earlier. The demonstrated absence of VZV in the present study argues against general shedding of herpes viruses in the cervix.

The seroprevalence of CMV, EBV, HSV-1 and HSV-2 is related to socio-economic status. These viruses spread by close contact while VZV, which is not correlated to socio-economic factors, spreads mainly by the much quicker airborne route. A limitation in the design of the study is that the specific socio-economic status of the included women is not known. The Cervical Cancer Screening Programme is population-based, i.e. all women of certain ages are invited. The catchment area of the Maternity Centers involved in this investigation comprises districts of both high and low socio-economic status. An analysis of drop-outs might have shown a relationship between socio-economic

status and the willingness to attend for screening, but this has not been carried out.

In 2006 the participant rate in cytological cervical screening was 54-81% in different parts of Gothenburg, with a higher tendency to attend in districts with higher socio-economic status (Agneta Ellström: personal communication). Nevertheless, since no collected samples or data were patient-identifiable, all attending women were consecutively included.

The utilised PCR methods have high sensitivity and specificity. Although most scientists are confident of these methods, some concern has been raised regarding the risk of contamination giving falsely positive results (186). However, this risk must now be considered to be very small since better routines and methods have been developed. The high sensitivity of PCR methods implies that even small amounts of DNA are detectable, and the identification of a possible pathogen might represent a remainder of a past infection, which is no longer active. This issue is important, for example in patients with *C. trachomatis*, who might receive falsely positive results if a NAAT test is performed too soon after antibiotic treatment.

Detection of certain viruses in the genital area of both sexes is supportive of genital transmission. EBV has been detected in the genital area of both men and women (52, 68-70). CMV has been detected in cervical biopsies from four women with CMV cervicitis (59). However, CMV was not detected by PCR in urethral samples from 112 patients with urethritis (paper III).
Unlike CMV, HSV-1 and HSV-2, which all are known to cause severe infections in newborns, the role of EBV in such infections remains to be substantiated.

Cervicitis and urethritis in women (Paper V)

Cervicitis is the term for inflammation of the uterine cervix, but there is no generally accepted definition of this condition. The clinical signs mostly used for cervicitis in STI clinics in Scandinavia are visible mucopurulent secretion and easily induced bleeding of the cervical portio (187). The commonly used microscopical criterion for cervicitis in STI clinics is >30 PMNL per HPF in a stained cervical smear. An increased number of inflammatory cells in a vaginal wet smear is also used as a sign of inflammation, with a ratio PMNL:epithelial cells >1 interpreted as cervicitis (97).

The most most commonly used definition of microscopic urethritis in women is ≥5 PMNL per high power field (HPF) in ≥5 fields in 1000x magnification (121).

The aim of paper V was to evaluate the association of the clinical signs of cervical inflammation ─ mucopurulent discharge and friability ─ and the microscopical signs of inflammation (above-mentioned) with a positive SDA test for *Chlamydia trachomatis*. Our results showed a statistically significant correlation ($p<0.05$) with *C. trachomatis* for mucopurulent discharge in the cervical portio, easily induced bleeding from the same locus and for more PMNL than epithelial cells in the vaginal wet smear. However, correlation was not shown between positivity for *C. trachomatis* and microscopical cervicitis or microscopical urethritis in stained smears (Table 2, paper V).

In paper V the definition of microscopical cervicitis was >30 PMNL in a stained smear from the cervical orifice. The definition of microscopical urethritis was ≥5 PMNL in the urethral smear. If the number of PMNL exceeded the number of epithelial cells in the vaginal wet smear, it was regarded as "positive". Inflammatory cells in the vaginal wet smear may originate from both cervical and vaginal infections, and we preferred to regard a "positive" wet smear as a sign of "unspecific genital inflammation" rather than cervicitis and/or vaginitis. The definitions employed in our study are the ones used in many STI clinics in Scandinavia.

Comparison with other studies is complicated by varying diagnostic methods for *C. trachomatis* and differences in the prevalence of chlamydia and other STI in the studied populations. The literature on the association of various definitions of cervicitis and urethritis in women and chlamydia infection is inconsistent (188-190).

According to the STI guidelines from CDC in 2010, cervicitis is characterized by mucopurulent discharge in the uterine cervix and/or easily induced endocervical bleeding. More than 10 PMNL/HPF on microscopic examination of a vaginal wet smear is said to be associated with chlamydia infection of the cervix. However, according to the CDC, an increased number of PMNL on an endocervical Gram stain has not been standardised in the diagnosis of cervicitis. Urethral smears are not mentioned in connection with chlamydia infection in women in the CDC guidelines (191).

Marazzo et al. reported that inflammation in the endocervix or the urethra is associated with higher organism load and confers a higher likelihood of a positive diagnostic test for *C. trachomatis* (192). It is most likely that cases with a low bacterial load and discrete inflammatory signs were not diagnosed with *C. trachomatis*, when less sensitive diagnostic methods were used. This might contribute to the positive correlation between an increased number of PMNL in urethral and cervical smears and *C. trachomatis* in some older studies (189, 193-195).
However, also in two recent studies of female STI patients a correlation between ≥5 PMNL in the urethral smear and chlamydia detected by more sensitive NAAT methods was observed (196, 197). Of our female patients with chlamydial infection, only 19.0% (8/42) had ≥5 PMNL in their urethral smears compared to 47.5% (19/40) in the study by Falk et al. in 2005. Of the chlamydia-negative women, 14.3% (6/42) in paper V compared to 25.4% (104/409) in Falk et al had microscopic urethritis. This notable difference could be due to inter- and intra-observer variation in the interpretation of microscopical smears, previously shown by Smith et al. (198). In a study from 2003 of ten experienced microscopists, inter-observer consistency was found in 97% of negative slides (<5 PMNL), 68% of low-grade slides (5-19 PMNL) and

94% of high-grade slides (>20 PMNL). Intra-observer concordance was 96% for negative smears, 75% for low-grade and 89% for highly positive smears (198). Among our patients only three women had urethral smears with >9 PMNL per HPF: thus the majority (11/14) of the smears assessed as urethritis were in the "low-grade" range, shown to be more difficult to estimate consistently compared to both oneself and other microscopists.

Differences in the instruments and the sampling procedure could also be of importance. For the endocervical stained smears and the vaginal wet smears, the same sampling device was used in our study as in the studies by Moi et al. and Falk et al. (196, 197). However, these above-mentioned authors both used a blunt curette for the urethral smears, whereas we used a 1 µl plastic loop. This might have affected the outcome of the stained urethral smears. Nevertheless, since the aim of our study was to evaluate the value of different criteria of urethritis and cervicitis used in our clinical practice, all specimens were collected according to the standard procedure in the clinic.

The selection of patients in our study is described in "Material and methods" as well as in paper V. The included patients all attended due to chlamydia partner notification, whereas the women described by Falk et al. were consecutive female STI patients attending for various reasons. It could be that patients attending due to partner tracing caught the infection more recently than unselected STI patients. If inflammatory signs evolve gradually from the time of transmission, fewer patients attending due to partner tracing would have high-grade inflammatory smears. However, in a more recent study by Falk et al., comprising female patients attending due to chlamydia partner notification, 42% (15/36) of chlamydia-infected women had ≥5 PMNL in their urethral smears and 28% (10/36) had >9 PMNL per HPF (196).

In clinical practice microscopy of urethral and endocervical smears is used to estimate the probability of STI in individual patients. The sensitivity of these smears to predict N gonorrhoeae is >95% in symptomatic men but lower in women and asymptomatic patients (40-60%) (199). The incidence of *N. gonorrhoeae* in Swedish women was 2.3 cases/100 000 women in 2010 (80). In

Swedish STI clinics, with a very low incidence of *N. gonorrhoeae* (<1%) in unselected patients, the smears are often utilized as part of the decision-making whether to give antibiotic treatment before the result of the chlamydia test is available. The sampling and examination of smears from the endocervix, vagina and urethra in STI patients take time and resources for a clinic. Nevertheless, it is important to perform a vaginal wet smear in female patients with symptoms or signs suggestive of candida, bacterial vaginosis or *Trichomonas vaginalis* infection. On suspicion of gonorrhoeae, endocervical and urethral stained smears are justified, since the specificity of visible gram negative diplococci for *N. gonorrhoeae* infection is 95-100% and the sensitivity of the diagnostic culture methods for Ng is 80-90% in women (1).

The results of paper V may not be representative of women tested in other clinical settings or in countries with a different panorama of STI. In low-prevalence populations and low-resource settings, presumptive diagnostic criteria may be used for selective screening. This is not the case in STI clinics with known high prevalences of *C. trachomatis.* However, routine sampling of smears from the cervix and the female urethra in unselected STI patients needs further evaluation.

CONCLUSIONS

Papers I-II

Herpes simplex type 2 infection was common in both STI patients and pregnant women. The estimated seroprevalence in pregnant women in Sweden (paper II) was not higher than in contemporary reports of pregnant women from other Nordic countries. HSV-2 infection is often asymptomatic or gives non-specific symptoms. HSV-2 antibody testing should be performed generously in patients with recurring, non-specific genital symptoms, if the results of PCR tests for HSV are negative.

Paper III

Epstein-Barr virus was significantly more common in urethral samples from patients with microscopical urethritis than in asymptomatic controls without an increased number of PMNL in the urethral smear. Correlation between EBV and urethritis has not been demonstrated earlier, but a possible causality between EBV and urethritis is still to be shown.

Paper IV

The detection of each of the viruses cytomegalovirus and Epstein-Barr virus in >10 % of cervical samples from young women supports sexual transmission of these viruses. The occurrence of EBV in >10% of the women emphasises the need for further investigation of congenital transmission of EBV.

Paper V

A significant correlation with a positive test for *C. trachomatis* was shown for mucopurulent discharge in the cervical portio, easily induced bleeding from the same locus and for more polymorph nuclear leucocytes (PMNL) than epithelial cells in the vaginal wet smear. However, correlation was not demonstrated between >30 PMNL in a stained smear from the cervix or ≥5 PMNL in a stained smear from the urethra and a positive test for *C. trachomatis* in female STI patients. Hence, routine sampling for microscopy from the cervix and the urethra in unselected female STI patients is questionable.

FUTURE PROSPECTS

The panorama of infectious diseases varies between populations and time periods. Since the HSV-2 seroprevalence is correlated to sexual behaviour, it would be of importance to repeatedly follow the age-related seroprevalence of HSV-2 in the general population as well as in selected groups, such as STI patients and pregnant women. Changes in HSV-2 seroprevalence reflect sexual behaviour and might be used to estimate the long-term effect of preventive strategies. There is a strong correlation between HSV-2 and HIV and, especially in developing countries, the HSV-2 seroprevalence may contribute to comprehension of the STI/HIV epidemiology and to guiding preventive projects. Data on the epidemiology of HSV-2 infections also have implications for vaccine development.

For improved understanding of the interaction of HSV-1 and HSV-2 infection, a long-term prospective study of first episodes of genital herpes with PCR as well as serological analysis at inclusion and at intervals further on would be of value.

In paper III a correlation between the detection of EBV in urethral samples and microscopical urethritis was demonstrated, but a possible causal connection is still to be shown. Since EBV establishes latency, and thereby life-long infection, a curing treatment is hard to obtain, but an effective medical drug would otherwise be helpful to evaluate causality of EBV in urethritis. To more certainly distinguish active replication of EBV from passive latency would increase the probability of EBV as a causative agent in urethritis.

In addition to its association with male urethritis, EBV was detected in the uterine cervix of young women (paper IV). Further support for EBV as a sexually transmitted agent would be gained if EBV were found in the genital area of sexual partners to those EBV positive individuals, especially if the same strains of EBV were found within sexual couples.

Further studies of non-specific urethritis in men are needed to elucidate the etiology, the implications of an increased number of PMNL in urethral smear,

the risk of complications and indications for treatment and partner notification. Since health care resources are limited, and in consideration of our patients' psycho-sexual well-being, asymptomatic men with non-infectious urethritis should ideally not be identified or at least distinguished from the ones with a treatable infection. Moreover, in the era of rising antibiotic resistance as a threat to public health, it is of utmost importance to reconsider our indications for antibiotic treatment.

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