PhD thesis

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Syphilis and HIV co-infection

Epidemiology, treatment and molecular typing of
Treponema pallidum

Copenhagen, January 2015

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Contents

Preface

The work presented in this thesis was carried out at the Department of Infectious Diseases, Rigshospitalet and at STI Research and Development at the Department of Microbiology and Infection Control, Statens Serum Institut from 2011–2014.

Firstly, I would like to thank my supervisors Terese Katzenstein, Jan Gerstoft and Jørgen Skov Jensen. My interest in HIV research started years ago when I was a research scholar at the Department of Infectious Diseases at Rigshospitalet under the supervision of Terese Katzenstein and Jan Gerstoft. Based on the increasing rates of syphilis in Denmark we conceived the idea of a PhD project on syphilis and HIV co-infection. Though this PhD project started out rather inertly it became a reality thanks to the continued support of Terese Katzenstein and Jan Gerstoft. In 2012 the PhD project really accelerated when I was very warmly welcomed by Jørgen Skov Jensen at Statens Serum Institut and became part of the STI Research and Development group. Without the help and hard work of this very enthusiastic group of people study III of the current thesis would never have been a reality. Especially I would like to single out the work by Christina Nørgaard, Susanne Cramer Johansson, Carola Hilby, Elvira Chapka and Lene Bertelsen and the academic discussions with Peter Ahrens, Maria Frølund and Jørgen Skov Jensen. Also, at Statens Serum Institut I met Susan Cowan and Steen Hoffmann who shared the interest in the fascinating organism *Treponema pallidum* and who helped provide and interpret data. Also, thanks to my fellow PhD students at the PhD office at the Department of Infectious Diseases at Rigshospitalet for a very helpful and fun work environment and thanks to Dorthe Hass and Margrethe Lüneborg-Nielsen from the AIDS Laboratory at Rigshospitalet for years of good collaboration.

Secondly, I must acknowledge the tremendous support that I have received from my family. My parents, Ulla and José for their support, encouragement and practical help which I am aware of is more than one could ever expect. Also, I would like to thank Julia, Erik, Jan, Annette, Caroline and Frederikke for taking so good care of Cornelia when I have been engaged in work. Finally, I dedicate this thesis to Andreas who has made it possible for me to pursue my professional as well as personal ambitions.

Copenhagen, January 2015

Kirsten Salado-Rasmussen

Papers

This PhD thesis is based on the following papers:

I: Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000–2010. **Kirsten Salado-Rasmussen**, Terese Lea Katzenstein, Jan Gerstoft, Susan Alice Cowan, Troels Bygum Knudsen, Lars Mathiesen, Steen Hoffmann, Niels Obel. Sex Transm Infect. 2013 Aug; 89(5):372-6.

II: Serological response to treatment of syphilis with doxycycline compared to penicillin in HIV-infected individuals. **Kirsten Salado-Rasmussen**, Steen Hoffmann, Susan Cowan, Jørgen Skov Jensen, Thomas Benfield, Jan Gerstoft, Terese Lea Katzenstein. *Submitted to Acta Dermato-Venereologica.*

III: Molecular typing of *Treponema pallidum* in Denmark: A nationwide study of syphilis. **Kirsten Salado-Rasmussen**, Susan Cowan, Jan Gerstoft, Helle Kiellberg Larsen, Steen Hoffmann, Troels Bygum Knudsen, Terese Lea Katzenstein, Jørgen Skov Jensen. *Submitted to Acta Dermato-Venereologica.*

Objectives

The objective of this PhD thesis was to describe the interactions of syphilis and HIV in Denmark from 2000 to 2013. The PhD thesis consists of three studies with specific objectives:

I: To estimate the risk of HIV acquisition in patients diagnosed with syphilis and to estimate the risk of reinfection with syphilis.

II: To assess whether doxycycline is equipotent to penicillin when treating HIV-infected patients for syphilis.

III: To perform strain typing of *Treponema pallidum* in patients diagnosed with syphilis by PCR testing and to link strain types with epidemiological data, including HIV status.

Abbreviations

AIDS	Acquired immune deficiency syndrome
AF-M	Anti-flagellum IgM
AF-G	Anti-flagellum IgG
arp	Acidic repeat protein
bp	Base pair
cART	Combination antiretroviral treatment
CD4	Cluster of differentiation 4
CDC	Centers for Disease Control and Prevention
CNS	Central nervous system
CI	Confidence interval
CPR number	Central person registration number
CSF	Cerebrospinal fluid
DHCS	Danish HIV Cohort Study
FTA-ABS	Fluorescent treponemal antibody absorption test
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IM	Intramuscular
IRR	Incidence rate ratio
MSM	Men who have sex with men
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RPR	Rapid plasma reagin
SSI	Statens Serum Institut
STD	Sexually transmitted disease
T. pallidum	*Treponema pallidum*
tpr	*T. pallidum* repeat
WR	Wassermann's reaction

Introduction

Syphilis is a sexually transmitted disease caused by *Treponema pallidum*. Syphilis was endemic in Europe between the 15th and the 20th century and in the 19th century 10–20% of the population in Europe and America was thought to be infected (1). After it became known that syphilis was easily treated with penicillin the disease almost disappeared in Western countries before it surprisingly reemerged in the late 1990s (2). In Western countries, syphilis is now mainly encountered among men who have sex with men (MSM) (3). By contrast, in developing countries the disease represents an extensive problem (4;5). Above all, syphilis is a concern especially to pregnant women due to the increased risk of spontaneous abortion, stillbirth and congenital malformations of the newborns (6-8).

Denmark reached its syphilis nadir in 1999 with 22 cases of newly acquired syphilis (9;10). Increasing rates were then observed throughout a decade and peaked in 2011 with 434 cases (11). Whereas only 33% of the cases from 1994–2002 were among MSM, MSM represented 78% of the cases in 2003–2004 (12). Also in contrast to earlier, where most syphilis patients reported being infected in Eastern Europe, Africa and Asia (10), the majority of the patients now report acquiring syphilis in Denmark (13). In 2010 screening of pregnant women was re-introduced in Denmark (14) after the screening had been discontinued as a general screening in 1998 (10).

Syphilis and HIV co-infection

Syphilis and HIV are strongly linked with one another. The proportion of patients with concurrent HIV at the time of syphilis diagnosis has been substantial since the reemergence of the disease in Denmark (11;13;15) and peaked in 2008 where 58% of MSM diagnosed with syphilis had concurrent HIV (16). By contrast, only 32% of MSM diagnosed with syphilis in 2013 had concurrent HIV – leaving 68% susceptible to infection with HIV (13). In **study I** of the current thesis, it was documented that almost 10% of Danish men diagnosed with syphilis acquired HIV infection within five years after the diagnosis of syphilis (17). The marked increase in syphilis among MSM has mainly been attributed to increased sexual risk behavior in response to the improved effect of antiretroviral therapy on HIV (18;19). However, increasing use of illicit drugs may also have contributed to the increasing rates of syphilis (20). A recent Danish study (21) found that the rate of unsafe sex was increasing in HIV-infected MSM – despite this, the number of new HIV infections did not increase, indicating that cART was the reason for the decreased risk of transmission of HIV despite increased practice of unsafe sex. Grassly *et al* (22) proposed an alternative explanation of the increasing rates of syphilis; they suggested that the increase in syphilis was due to endogenous oscillation in disease incidence predicted by the natural dynamics of the infection. However, this theory on herd immunity has been questioned (23).

Several studies have corroborated the negative effect of syphilis on HIV infection; during syphilis infection viral load increases and CD4 cell counts decreases transiently (24;25). Also, of particular public health concern is the increased risk of transmission and acquisition of HIV during syphilis infection (26;27). Likewise, HIV has an impact on the clinical course of syphilis infection. The Syphilis and HIV study (28;29) included patients with and without HIV. Surprisingly, this multicenter, prospective study found that HIV had only a minor impact on the clinical manifestations of primary and secondary syphilis; HIV-infected patients with primary syphilis tended to present with more genital ulcers, and genital ulcers were more frequently present in HIV-infected patients with secondary syphilis compared to HIV-uninfected patients (29). Likewise, a recent study found that HIV co-infection had an impact only on the serological response in patients with primary syphilis and a CD4 cell count <500 cells/µL (30).

As a consequence of the high rates of syphilis among HIV-infected patients, an annual screening has been implemented at the departments of infectious diseases in Denmark, where patients with HIV are seen at an outpatient basis at intended intervals of 6 or 12 months.

Treponema pallidum, the syphilis spirochete

T. pallidum subspecies *pallidum*, hereafter referred to as *T. pallidum,* is a spirochete. The spirochete family is characterized by a unique cell architecture with an outer membrane, a cell membrane and flagellar filaments in the periplasm (31). Other examples of spirochetes are the non-venereal treponemes *T. pallidum* subspecies *pertenue*, *T. pallidum* subspecies *endemicum* and *T. carateum* causing yaws, bejel (endemic syphilis) and pinta, respectively (32). The spirochete family also includes *Borrelia burgdorferi* and *Leptospira interrogans*.

T. pallidum is an obligate human parasite and its absolute dependence on a mammalian host for continuous viability has made it a difficult organism to study. The organism must be propagated by passage in rabbits, consequently it cannot be manipulated genetically. The genome (Nichols strain) is a circular chromosome of 1 138 006 base pairs (bp), making it one of the smaller prokaryotic genomes (33). In the absence of virulence factors such as toxins or secreted effector molecules, motility is crucial for the infectiveness and *T. pallidum* is extremely invasive and invades the skin or mucosa shortly after contact (34). The hematogenous dissemination occurs before the chancre even appears and *T. pallidum* often penetrates the blood-brain barrier during early infection (34). The clinical manifestations during infection are caused by the inflammatory response elicited by the bacteria and bacterial constituents such as lipoproteins (34). Many theories have been proposed to explain how *T. pallidum* persists in the infected host and evades the host immune system. Strategies proposed are the extremely low metabolic rate and the antigenic variation of the outer membrane proteins of *T. pallidum* (35). It is well-established that *T.*

pallidum elicits both a local and a systemic innate and adaptive immune response during early infection (36). In HIV-infected patients, cytokines have been shown to fluctuate with infection and treatment of syphilis (37). A recent study of ours investigating patients with HIV and hepatitis C virus (HCV) co-infection proposed that the reason for undetectable HCV RNA during syphilis infection was a *T. pallidum* induced cytokine secretion hindering HCV replication (38).

Transmission of syphilis

The main route of syphilis transmission is sexual contact; however, syphilis can also be transmitted through blood, congenitally and through non-sexual contact with syphilitic lesions on skin or mucosa. The main risk of transmission occurs when mucocutaneous lesions are present, which is usually within the first year of infection (34). In the late latent stage, syphilis is not transmitted sexually (39). Patients diagnosed with syphilis should be screened for HIV and likewise, all patients who have HIV should be screened for syphilis (39;40). Sexual contacts to patients with syphilis should also be offered screening for syphilis (39). Vertical transmission of *T. pallidum* has major implications in developing countries. The risk of congenital syphilis depends on the time interval from acquisition of the infection to pregnancy and ranges from 30–80% (34;41). Based on the findings of spirochetes in the placenta and the umbilical cord, transplacental infection is most likely the major route of fetal infection (42;43).

The clinical course of syphilis

Syphilis has been named 'The Great Imitator' due to its highly variable symptoms and multiple clinical stages. To make the diagnosis even more challenging the stages can be overlapping. From its site of primary infection, bacteria disseminate systemically through the blood to potentially any organ. The ulcer, most typically known as a chancre, appears at the inoculation site nine to 90 days after acquisition of the infection and heals spontaneously within three to eight weeks (34). In a study from the Sing Sing Prison where volunteers were inoculated intracutaneously with 10 or 100 spirochetes the mean incubation period was 24 days (44). Untreated, the disease can progress to the secondary stage with systemic manifestations that include fever, lymphadenopathy, typical erythematous skin rashes (characteristically on palms, soles and trunk) and mucocutaneous lesions about six weeks after the primary chancre. Hepatitis, periostitis, arthritis, and glomerulonephritis is also seen in the secondary stage (39). The secondary syphilitic symptoms can occur in several different phases and between these phases the patient is in the latent stage with no clinical symptoms. During the first year of infection, about 25% of the asymptomatic patients will experience relapse to the secondary stage, hereafter relapses are far less common. For this reason the latent, asymptomatic stage is divided into an early latent stage (<1 year) and a late latent stage (>1 year or unknown duration). The term 'early syphilis' is often used and includes the primary, secondary and early

latent stages of syphilis. If left untreated the tertiary stage develops 5 to 30 years after transmission in approximately 30% of infected patients (45;46). The tertiary stage is characterized by gummatous lesions, cardiovascular syphilis and central nervous system (CNS) affection. The type of neurological manifestations depend on whether the predominant involvement is meningovascular (strokes, meningomyelitis) or parenchymatous (tabes dorsalis, general paresis of the insane) (34). Of note, neurosyphilis can occur in all stages of syphilis and includes cranial nerve dysfunction, meningitis, stroke, altered mental status and auditory or ophthalmic abnormalities (40). Neurosyphilis in early stages primarily affects meninges and cerebral or spinal cord vasculature, whereas late forms affect the spinal cord or brain parenchyma. It should be noted that cerebrospinal fluid (CSF) abnormalities are common in early syphilis, even in the absence of neurological symptoms (40).

Diagnosis of syphilis

T. pallidum cannot be continuously cultivated *in vitro.* As a consequence, the use of direct diagnostic methods is limited. For over a century dark field microscopy of lesion exudate or tissue has been used, however, microscopy requires highly trained personnel and false-positive results can occur because *T. pallidum* is morphologically indistinguishable from commensal treponemes that can be present in body fluids (47). The mainstay for diagnosis of syphilis remains serologic testing although it should be noted that serologic tests cannot distinguish between syphilis and other treponematoses which can present a problem in regions where more treponemal infections are frequent (48;49). Since the 1990s polymerase chain reaction (PCR)-based methods for detection of *T. pallidum* have been developed (50;51) and are now routinely available in high-income countries. The targets are conserved regions of the *T. pallidum* genome. It is crucial that the target is highly specific to distinguish *T. pallidum* from other spirochetes (47). However, the success rate of the diagnostic PCR depends on the samples matrix (e.g., blood, blood fractions, CSF or lesion exudate) as well as disease stage (52;53). Where microscopy and PCR are feasible diagnostic measures in the primary, ulcerative stage of syphilis, later stages, and above all the latent stages, are diagnosed by serological testing. Accordingly, over two thirds of the Danish patients are diagnosed by serology (13).

In Denmark, serological testing for syphilis antibodies was centralized at Statens Serum Institut (SSI) until 2011. Serological tests for syphilis fall into two categories, non-treponemal and treponemal tests. Two non-treponemal tests were used for the studies included in the current thesis: Wassermann's reaction (WR) is a complement fixation test, and the rapid plasma reagin (RPR) titre was determined by agglutination. Furthermore, three treponemal tests were used: anti-flagellum IgM (AF-M) was determined by a capture

ELISA, anti-flagellum IgG (AF-G) was determined by an indirect ELISA and the fluorescent treponemal antibody absorption test (FTA-ABS) was done by immunofluorescence microscopy.

In general, the non-treponemal tests are sensitive but not highly specific. False-positive test results can occur due to viral infections, including HIV and HCV infection (54), drug addiction, malignancy, autoimmune diseases and probably also pregnancy (49). Therefore, patients with reactive non-treponemal tests should have confirmatory treponemal tests done to confirm the diagnosis. False-negative results caused by antibody in excess are called the prozone phenomenon (49). This reaction can occur during all stages of syphilis, but is most pronounced in secondary syphilis where antibody levels are at the highest level, and in the RPR test, which is dependent on the formation of an antigen-antibody lattice. The phenomenon has been associated with pregnancy and neurosyphilis (55).

The interpretation of serological test results can cause considerable confusion among clinicians, and available tests and treatment algorithms differ from country to country (56) (and now also from hospital to hospital in Denmark). In general, non-treponemal test titers decline after treatment, whereas treponemal IgG test titers will remain reactive for life. A 4-fold decline (or more) in non-treponemal titers is regarded as an appropriate serologic response, however in some patients non-treponemal antibodies can persist for a long time and these patients are referred to as 'serofast' which means that their non-treponemal test titers do not become non-reactive after an initial satisfactory response (40). Patients with a sustained 4-fold increase in non-treponemal titers combined with persisting or recurring symptoms of syphilis are considered as having a reinfection or experiencing treatment failure (40).

In 2009, PCR testing of material from ulcers was introduced in Denmark at SSI (57). Samples are tested for the presence of *T. pallidum* using two real-time diagnostic PCR assays with internal amplification controls, amplifying segments of the *flaA* (58) and *polA* (59) genes of *T. pallidum*.

The diagnosis of neurosyphilis can be a diagnostic challenge as no definitive criteria exist. When neurologic affection is suspected to be related to syphilis, the diagnosis is supported by laboratory testing of the CSF. The CSF is tested for the presence of antibodies, protein and cell count. However, it is of utmost importance to be aware that normal CSF parameters at the time of lumbar puncture do not rule out CNS infection because treponemes which recently accessed the CNS may not yet have elicited an inflammatory response (34).

Treatment of syphilis
Penicillin is the recommended treatment for syphilis in Europe (39) and the United States (40). Penicillin is administered parenterally in the form of benzathine penicillin G (a single dose of 2.4 million units IM for

early syphilis and three doses at 1-week intervals for late latent syphilis). Procaine penicillin is used as a second-line option (one dose of 600 000 units IM once daily for 10 days for early syphilis and 21 days for late latent syphilis). As a treatment alternative, doxycycline is used (100 mg orally twice daily for 14 days for early syphilis and for 30 days for late latent syphilis) (39). Azithromycin is also used as a treatment alternative and has been proposed as a useful agent in developing countries where penicillin injections may be problematic (60). However, macrolide resistance is an increasing problem in the United States (61-64) and Shanghai (65), limiting its usefulness. During pregnancy, parenteral penicillin G is the only treatment with documented effect. In the case of penicillin allergy, the Centers for Disease Control and Prevention (CDC) recommend desensitization and subsequent treatment with penicillin (40).

In Denmark, primary, secondary and latent stage syphilis is treated with penicillin or doxycycline according to local guidelines. At the Department of Infectious Diseases at Rigshospitalet in Copenhagen patients are mainly treated with doxycycline, whereas patients at the Department of Infectious Diseases at Hvidovre Hospital and the STD clinic at Bispebjerg Hospital, likewise in the Copenhagen area, are mainly treated with benzathine penicillin G.

As many as one-quarter of patients treated for syphilis will experience the Jarisch-Herxheimer reaction (66), characterized by fever, arthralgia, myalgia and headache within the first 24 hours after initiation of therapy (40). The immunologic reaction is thought to be caused by the massive degradation of treponemes shortly after the initiation of therapy.

To monitor the treatment response, patients treated for syphilis should be followed up with serological testing and clinical assessments. The 2014 European Guideline on the Management of Syphilis recommends that patients treated for early syphilis are followed up at 1, 3, 6 and 12 months (39). Most often the non-treponemal tests are used as they most accurately reflect disease activity (40).

Management of syphilis in HIV-infected patients

Diagnosis and management of syphilis in patients with concurrent HIV is in general similar to that in patients without HIV (39). Nonetheless, since a case report documented clinical treatment failure in 1987 in a patient with concurrent HIV (67), the optimal antibiotic regimen for co-infected patients has been debated (68). Rapid progression to more advanced stages and a higher risk of treatment failure and neurological complications has been reported for co-infected patients (69-71). Further, serologically defined treatment failure has been documented to be more common in patients with primary and secondary syphilis and concurrent HIV (28), e.g., extremely low or high titers are more often seen among these patients (40). But in general, serologic tests are accurate and reliable also when testing HIV co-

infected patients (39). The availability of cART has had a substantial impact by reversing (part of) the immunosuppression and today many HIV-infected patients may have a restored immune system, resulting in reduced rates of serologic failure (72).

Because HIV-infected patients might be at increased risk of neurologic complications (40) the need for lumbar puncture and its timing continues to be debated. Current guidelines recommend that HIV-infected patients have lumbar puncture done in two situations: 1) neurological symptoms at time of diagnosis or after treatment, 2) patients who fail to respond to therapy (69).

HIV-infected patients need closer follow-up after treatment of syphilis and the CDC recommend clinical and serological evaluation for treatment failure at 3, 6, 9, 12 and 24 months after therapy (40).

Molecular typing of *T. pallidum*

In general, when using molecular typing as an epidemiological tool, it is important to study more variable parts of the genome (47). If a sample is tested positive in a diagnostic *T. pallidum* PCR, the sample can be subjected to molecular typing. In line with the performance of diagnostic PCRs, the success rate of typing PCRs depends on the sample matrix (73;74). In the primary stage, treponemes circulate in the blood and should thus be detectable in blood samples. However, if the equivalent of 50–100 µL patient blood is subjected to a PCR that detects 5–10 target copies per reaction, then at least 100–1000 treponemal organisms per mL should be present in the blood sample taken, making it inherently difficult to use blood because the bacterial load is usually lower than this (47). Conversely, treponemes seem to be caught in the capillary beds of the skin and therefore ear scrapings may have high enough bacterial concentrations for detection by PCR (75).

In 1998, Pillay *et al* developed a subtyping system to distinguish different strains of *T. pallidum.* The method is based on heterogeneity of two genes: determination of the number of 60-bp repeats within the *acidic repeat protein (arp)* gene, and restriction fragment length polymorphism (RFLP) analysis of sequence differences in the *T. pallidum repeat (tpr)* subfamily II genes *(tprE, tprG* and *tprJ)* (76). This 2-component CDC subtype was recently supplemented with sequence analysis of a variable region of the *tp0548* gene (77). By combining the CDC subtype and the *tp0548* sequence type the discriminatory capacity was markedly improved. The new 3-component type was designated *strain type* by Marra *et al* (77) to distinguish it from the 2-component subtype.

Of note, the PCR products resulting from the amplification of the *arp* and *tpr* genes are long which is thought to compromise the sensitivity of the assays. Furthermore, the *arp* and *tpr* genes are not very specific targets because they are also present in other treponemes. However, because molecular typing is

performed on samples that are confirmed positive in diagnostic *T. pallidum* specific PCR assays this usually does not present a problem (47).

Setting and data sources

Denmark has a population of 5.6 million people (78) with an estimated HIV prevalence in the adult population of 0.07% (79). The number of persons with newly diagnosed HIV, and the proportion of MSM among these, has been stable for almost 10 years with approximately 200–250 new cases per year (80). Five hundred undiagnosed HIV-infected MSM are estimated to reside in Denmark, an estimate that has remained stable over the last 10 years (81). A recent Danish study concluded that the stable incidence of new HIV diagnoses among MSM despite increased engagement in unsafe sex was a consequence of the use of cART (21).

The Danish health care system is tax-funded, including free cART. Treatment of HIV is restricted to specialized departments and patients with syphilis are seen at specialized departments at hospitals or STD clinics. All individuals in Denmark are assigned a unique 10-digit central person registration (CPR) number at birth or upon immigration. The CPR number can be used to link data from different registers upon approval from the Danish Data Protection Agency. Two nationwide registers were used for **study I** and **study III** of the current thesis. The nationwide design is a major strength because the data are analyzed at a population-level, including all patients and not just a subgroup. However, the quality of the studies depends on the validity of the data in the registers (82) and misclassification will always be of concern.

The Danish national syphilis registration system

Primary, secondary and early latent syphilis are notifiable diseases according to Danish legislation and the notification is the responsibility of the treating physician. The cases are registered in the Danish national syphilis registration system – a nationwide database established in 1993, based at the Department of Infectious Disease Epidemiology at SSI. In addition to the cases notified, the register also includes patients identified with syphilis by positive serologic or PCR testing. The notification information includes sex, ethnicity, sexual orientation, mode and place of acquisition, as well as HIV status (12). Data from the Danish national syphilis registration system was used for **study I** and **study III**.

The Danish HIV Cohort Study

The Danish HIV Cohort Study (DHCS) is a nationwide, prospective, population-based study of all Danish HIV-infected patients treated at Danish hospitals since 1 January 1995. The data are updated yearly using standardized forms and the CPR number is used to avoid multiple registrations. To assure high data quality a data assurance program has been developed. The data include demographic data, mode of acquisition, AIDS-defining events, height, weight, blood pressure, lipid status, smoking status, cART, CD4 cell counts and HIV RNA (83). Data from DHCS were used for **study I**.

Methodological considerations

The studies included in the current thesis were all observational studies. Observational studies can be longitudinal or cross-sectional. In longitudinal studies it is possible to estimate incidence rates and due to the time factor it is possible to establish cause-effect relationships and large populations can be studied for long periods at a relatively low cost. Cross-sectional studies can demonstrate associations but conclusions on causal relationships should not be drawn. On the other hand, cross-sectional studies are less resource demanding. In general, observational studies have some limitations compared to clinical trials (84). Confounding is a common problem, but if a confounder is recognized it can be adjusted for. However, not all confounders are always recognized and the number of confounders that can be controlled for is also limited. Bias, on the contrary can occur in both observational studies and clinical trials, but are often minimized in clinical trials by randomization and double-blinding (85). However, in clinical trials the study population is highly selected and may not be representative of the population of interest.

Paper I: Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000–2010

Brief study outline

Syphilis is a marker of sexual risk behavior and is of interest in patients with HIV – as a marker of unsafe sex in this population. Syphilis is also of interest in patients without HIV – to assess the risk of subsequent HIV infection. We used two nationwide registers to estimate the 5-year risk of HIV or second syphilis infection, and to determine incidence rate ratios (IRR).

From the Danish national syphilis registration system all Danish men >16 years of age diagnosed with early syphilis in the period 2000–2010 were identified. Subsequently data on HIV status were extracted from the DHCS. Kaplan-Meier analysis was used to estimate the 5-year risk of HIV or second syphilis infection, stratified by the diagnosis of syphilis before and after 1 January 2006. We used Cox regression analysis with the date of the second syphilis diagnosis introduced as the time-updated variable to determine IRR for risk of HIV diagnosis before and after a second diagnosis of syphilis. Likewise, Cox regression analysis with the date of HIV infection introduced as the time-updated variable was used to determine IRR for risk of a second syphilis infection.

During the study period the national criteria for initiation of cART were acute HIV infection, presence of an HIV-related disease, pregnancy, CD4 cell count <300 cells/µL until May 2008 and <350 cells/µL thereafter, and plasma HIV-RNA >100 000 copies/mL (until 2001).

Principal findings

During the 11-year study period, 1217 male patients diagnosed with early syphilis were included. After 5 years, 9.8% (95% CI 7.0–12.6%) of the population had been diagnosed with HIV. Patients with a second diagnosis of syphilis had a substantially higher risk of being diagnosed with HIV (IRR = 3.1, 95% CI 1.2–8.0). After 5 years, 14.8% (95% CI 12.1–17.4%) of the population had been diagnosed with a second episode of syphilis. HIV-infected patients had a substantially higher risk of a second syphilis diagnosis (IRR = 4.0, 95% CI 2.8–5.9). Of the HIV-infected patients diagnosed with syphilis, 33.7% had viral loads >1000 copies/mL and thereby at risk of also passing HIV to their partner.

Considerations

A major strength of the study was our ability to link two nationwide registers of patients diagnosed with syphilis or HIV, however, the study has some limitations. First, screening of HIV-infected patients as part of the routine testing of this group may result in underestimating the syphilis prevalence in low-risk groups. We assume that an unknown number of patients have been left out because low-risk groups have not been

screened. Second, our study included both MSM and heterosexual men which might have different risk behaviors and exposures. Third, our data analysis did not account for mortality and emigration because these data were not available for the HIV-uninfected population. Finally, a 'case of syphilis' was based on serological results only and not on revision of patient files, thus reinfection or resurgence of an incompletely treated infection could potentially have been misclassified as a case.

Conclusion and perspectives

We concluded that the high risk of syphilis or HIV infection in men diagnosed with one of these STDs indicated a high frequency of unsafe sex in the Danish MSM population. The risk of being diagnosed with HIV subsequent to syphilis decreased during the later part of the study. Similar trends where syphilis outbreaks have not had a substantial impact on HIV incidence have also been reported from the United States (86). A possible explanation of the stagnation of the HIV transmission despite the increase in syphilis diagnoses could be that cART has become more widely used in recent years with viral suppression as a consequence. As one third of the HIV-infected patients in this study had viral load >1000 copies/mL our conclusion supported the initiation of cART in all HIV-infected MSM to reduce transmission of HIV.

Paper II: Serological response to treatment of syphilis with doxycycline compared to penicillin in HIV-infected individuals

Brief study outline

Penicillin is the drug of choice when treating syphilis, whereas doxycycline is used as a second-line option (40). The aim of the study was to evaluate the serological response to treatment of primary, secondary, early and late latent syphilis with intramuscularly administered penicillin or orally administered doxycycline in patients co-infected with HIV.

In this retrospective study, patients ≥18 years of age diagnosed with syphilis between 1 May 2004 and 31 October 2009 were eligible. The patients were included from three hospitals in the Copenhagen area (two departments of infectious diseases and one STD clinic). These hospitals were chosen because the vast majority of HIV-infected patients from the Copenhagen area are seen at these departments and because the vast majority of syphilis cases in Denmark is diagnosed in the Copenhagen area (87). We excluded patients who received intravenous therapy, were diagnosed with neurosyphilis or where patient files lacked information on treatment for syphilis. An individual could contribute with more than one episode, provided that treatment and appropriate treatment response was documented in the patient file. Treatment for syphilis consisted of doxycycline (100 mg orally twice daily for 14 days for early syphilis, and for 30 days for late latent syphilis) or penicillin (a single dose of intramuscular 2.4 million units of benzathine penicillin G for early syphilis and three doses at 1-week intervals for late latent syphilis).

To capture all available serological data, we obtained the serological test results from SSI where all serologic testing for syphilis was centralized during the study period. We defined serological cure as a ≥4-fold decline in non-treponemal titers following treatment of syphilis. Further, we defined serological failure as a lack of a 4-fold decline. Serological test results were allocated to a specific follow-up visit (3, 6, 9 or 12 months) if the test was done 30 days before or 30 days after the relevant point in time. The last-observation-carried-forward principle was used to handle missing values of WR and RPR.

The X^2 or Fisher's exact test were used to compare independent proportions and the t test and the Mann-Whitney test were used for comparison of continuous variables. The Kruskal-Wallis test was used for comparison of titers between different syphilis stages.

Principal findings

From 1 May 2004 to 31 October 2009, 221 cases of syphilis were diagnosed among 172 HIV-infected individuals attending three hospitals in the Copenhagen area of Denmark. The patients were diagnosed with primary, secondary, early and late latent syphilis, no patients were diagnosed in the tertiary stage. In

total, 202 cases were treated with doxycycline or intramuscular penicillin and included in the study. Of these, 126 cases were evaluated at 12 months; 78 cases were treated with doxycycline and 48 cases were treated with penicillin. The two treatment groups were comparable except for proportion of patients with CD4 cell count ≤200 cells/µL and proportion on cART.

The serological outcome was assessed at 3, 6, 9, and 12 months following treatment of syphilis infection. No statistically significant differences were observed in treatment outcome between treatment groups at any point in time (all $p > 0.05$). One year after treatment, 20 cases of serological failure were observed with 12 cases (15%) and 8 cases (17%) in the doxycycline and penicillin group, respectively, resulting in a non-significant difference of 2% (95% CI, -1.08%–5.08%; $p > 0.05$).

The proportion of patients who reached serological cure was affected by syphilis stage: of the cases of primary and secondary syphilis, 100% and 89% reached serological cure at 12 months, respectively, and of the cases with early and late latent syphilis, 71% and 67% reached serological cure at 12 months, respectively ($p = 0.006$). On the contrary, the serological cure rate did not vary by CD4 cell count, HIV RNA or age (all $p > 0.05$). However, WR and RPR titers at time of diagnosis were significantly higher in patients with serological failure 12 months after treatment (WR: $p = 0.002$; RPR: $p < 0.000$).

Considerations

HIV-infected patients in Denmark are followed at highly specialized and centralized departments. We included patients from two departments of infectious diseases in the capital area of Denmark; these two departments follow two thirds of the total Danish HIV-infected population (88). Further, if these patients opted for treatment of syphilis at the STD clinic at Bispebjerg Hospital these episodes were also included. All patient files from the three clinics were revised to assure detailed demographic, clinical and behavioral data on all patients and to distinguish between relapse and reinfection. Further, to capture all serological data, results of serological tests were obtained from the national provider of serological syphilis testing at SSI.

As in all retrospective studies, this study was limited by its non-randomized design. The treatment given was based on local guidelines and the patients at the Department of Infectious Diseases at Rigshospitalet were mainly treated with doxycycline, whereas patients at the Department of Infectious Diseases at Hvidovre Hospital and the STD clinic at Bispebjerg Hospital were mainly treated with penicillin. Overall, the patients in the two treatment groups were comparable. However, the proportion of patients with CD4 cell count ≤200 cells/µL and proportion on cART were not distributed evenly between treatment groups. Further analyses showed that more HIV-infected individuals *not* receiving cART opted for treatment at the

STD clinic compared to individuals on cART. This explains the lower proportion of patients on cART in the group receiving penicillin (the preferred regimen at the STD clinic). Likewise, even though the median CD4 cell count was comparable between treatment groups, a higher proportion of patients with CD4 cell counts ≤200 cells/µL were treated with penicillin. Because of the above-mentioned differences in treatment groups the serologic failure rate in the penicillin group may be overestimated since lower CD4 cell counts have been associated with increased risk of treatment failure (89).

Conclusion and perspectives

In line with others we found comparable rates of serologic failure in patients treated with doxycycline and penicillin (24;90). Although our study was not randomized, it seems safe to conclude that doxycycline can be used as a treatment alternative to penicillin if patients or clinicians prefer an oral treatment of syphilis. Whether doxycycline is non-inferior to penicillin can only be definitively evaluated in a clinical trial. However, it seems unlikely that such a large and expensive trial is about to be carried out. Furthermore, in this study the endpoint was serological cure 12 months after treatment. One could speculate that some of the patients who do not reach serological cure are patients without an active infection. Clinicians may prefer to treat HIV-infected patients who are asymptomatic but screen positive by serologic testing. If a clinical trial should be carried out it would be of utmost importance to exclude patients without active infection.

Because the treatment of HIV-infected patients in the Danish setting is restricted to few centers and because the patients have close follow-ups, poor adherence with respect to syphilis treatment would most likely be recognized at a regular check-up. Before recommending doxycycline as a more widely used treatment option it should be assured that the clinical setup has the relevant measures to avoid lost-to-follow-up. If compliance is assumed to be a problem, the single-dose, intramuscular penicillin regimen will always be the preferred treatment. In this connection it should be remembered that doxycycline is an effective agent for treatment of multiple STDs (91). Taken together we concluded that doxycycline can be used as an efficient treatment option when treating an HIV-infected population for syphilis.

Paper III: Molecular typing of *Treponema pallidum* in Denmark: A nationwide study of syphilis

Brief study outline

Molecular epidemiology can be used for understanding the transmission of infectious diseases and thereby preventing and controlling epidemics. HIV and syphilis co-infection is common and because genital ulcers facilitate HIV transmission it is of pivotal importance to elucidate the epidemiologic determinants underlying the high rates of syphilis. This study aimed to determine the strain type diversity among all patients diagnosed in Denmark with syphilis by PCR testing of material from genital ulcers during a 4-year period.

The study was performed as a nationwide study of *T. pallidum* positive genital ulcer samples collected between May 2009 and December 2013. *T. pallidum* strain type was linked with epidemiologic data (e.g., HIV status) from the Danish national syphilis registration system. Molecular strain typing was based on characterization of three variable treponemal genes; *arp, tpr* and *tp0548.* Categorical data were compared using χ^2 test or Fisher's exact test, where appropriate.

Principal findings

During the 4-year period, 278 samples positive by *T. pallidum* PCR testing were obtained from 269 patients. The majority of the patients were men (94%) and most of the male patients were MSM (86%). Further, the majority (93%) of the patients reported acquiring syphilis in Denmark. Of the 278 positive samples, 71% were typeable with all three PCR assays. Among the fully typeable samples, 22 strain types were identified. The most common type was 14d/g, accounting for 54% followed by 14d/f (18%) and 14l/g (5%). The remaining strain types were rare. Nineteen percent of the patients were HIV-infected at time of diagnosis and all of these reported that they were MSM. The patients with concurrent HIV were diagnosed with nine different full strain types and we did not find a difference in strain type by HIV status (p = 0.197).

Considerations

Our study included all Danish patients with PCR-positive syphilis within the 4-year study period. By use of the Danish national syphilis registration system we had epidemiological data on all patients. It is a major strength that all PCR testing for syphilis in Denmark is centralized at SSI. Further, the study was based in a setting where all health-care services are publicly funded and this universal access probably results in fewer undiagnosed cases. We used the 3-component strain typing system and could demonstrate a high discriminatory capacity. However, when concluding on our results it is a major limitation that we were not

able to perform typing on samples from the two thirds of the patients in Denmark who were diagnosed by serological testing during the study period (13).

Our main interest was to investigate the strain types in HIV-infected patients to assess if these patients belonged to separate sexual networks. Therefore, it is a major limitation that the majority of the HIV-infected patients are diagnosed by serological testing (this group is screened yearly in contrast to low-prevalence groups).

Besides the distribution of HIV, our interest was to investigate if imported cases resulted in circulating strains. Again, the interpretation was limited by the unknown strain types in the patients diagnosed by serological testing. However, based on the available samples, the imported cases did not result in circulating clones. We reached a relatively high number of typeable samples and our success rate of 71% is comparable to others who have used the 3-component strain type system with success rates of 41%, 63% and 77%, respectively (92-94).

Conclusion and perspectives

We concluded that strain type 14d/g was the predominant strain type in Denmark, but that a high degree of heterogeneity exists. The majority of the patients had acquired syphilis in Denmark and the imported cases of syphilis did not result in circulating clones. Furthermore, HIV-infected patients were diagnosed with a wide spectrum of different strains.

Currently the method of choice for *T. pallidum* typing consists of three PCR assays which is time-consuming and not applicable for routine testing. Recently a sequenced-based molecular typing system was proposed (95) and compared to the traditional CDC typing system in a group of patients with two or more parallel samples (i.e. taken at the same time). However, in the majority of the patients they found discrepancies within the *arp* and *tpr* loci using the CDC typing system (95). This was rather surprising because under experimental conditions, Pillay *et al* (76) have shown that the CDC subtype was stable with repeated rabbit passages of the Nichols strain. Marra *et al* have also confirmed the stability of the strains by demonstrating that neither the Sea 81-4 nor the Chicago C strain changed with repeated rabbit passages (77). Whether this inconsistency is caused by differences between human infections and experimental infections of rabbits or is due to the fact that skin and blood represent two immunologically distinct compartments has yet to be explored. Nonetheless, sequence-based typing systems for *T. pallidum* look promising (95-97).

Conclusion and perspectives for future research

The purpose of the current thesis was to improve the understanding of the highly complex epidemiologic relationship between HIV and syphilis. Given that syphilis facilitates transmission and acquisition of HIV and because the two infections have similar modes of transmission, increasing rates of HIV were expected to follow the increasing rates of syphilis. However, such an increased spread of HIV has not been observed. **Study I** of the current thesis demonstrated that syphilis and HIV co-infection was common in Denmark and that the majority of the HIV-infected syphilis patients were on cART. The use of cART, resulting in lower viral loads, might explain why the HIV incidence has not increased despite the assumption that the increasing rates of syphilis is a marker of higher rates of unsafe sex. In line with this, a recent Danish study (21) concluded that the increased use of cART explained the discordance in the rates of new syphilis and HIV infections. **Study I** suggested that the use of cART should be even more used as one third of the HIV-infected patients diagnosed with syphilis (indicating unsafe sex) had viral loads >1000 copies/mL. The rates of HIV co-infection among MSM who are diagnosed with syphilis have been declining in Denmark. One possible mechanism could be that MSM with syphilis and HIV co-infection have unprotected sex with HIV-uninfected MSM, but only transmit syphilis because they are efficiently treated for their HIV infection (98). From 2008 to 2012, 65% (range, 54%–76%) of all new HIV-infected patients in Denmark were on cART within a year of diagnosis (88).

In **study III** we investigated whether HIV-infected patients with syphilis belonged to separate sexual networks; however, we did not find evidence of the assumption of serosorting. On the contrary, we observed that HIV-infected patients were diagnosed with a wide range of strain types. From **study I** we knew that HIV-infected men had a substantially higher risk of a second diagnosis of syphilis compared to the HIV-uninfected syphilis patients. Whether this higher risk of syphilis in the HIV-infected population reflects that HIV-infected patients have unsafe sex based on serosorting (though no verified evidence of this in **study III**) or whether a core group with high-risk behavior is responsible for the increased risk in the HIV-infected population is unknown. For example, in **study III** a few medium-sized clusters consisted of MSM only, indicating localized transmission networks. Rather than a general increase in sexual risk behavior among all MSM, the re-establishment of a risk-taking core group of MSM may have enabled a higher level of endemicity, causing continuous syphilis circulation (99).

Study III provided new insights on the epidemiology of syphilis in the Danish population using molecular typing. However, to fully use the potential of the molecular typing as an epidemiological tool, a study including both patients diagnosed by serological testing as well as PCR testing would be highly relevant

although technically difficult. By inclusion of all patients it could be definitively investigated how the HIV-infected population interact with the HIV-uninfected population.

Study II demonstrated that the majority of the HIV-infected patients were diagnosed in the secondary and early latent stage. Diagnoses in the latent stages reflect that the annual syphilis screening of all HIV-infected patients is justified and even more frequent screening might result in earlier diagnoses and less transmission. Also, as long as it is not fully elucidated how syphilis spreads to low-prevalence groups, it seems prudent that syphilis screening was re-introduced in the general screening of pregnant women. Future studies should continue to focus on timely diagnosis of both HIV and syphilis. Moreover, **study II** demonstrated that syphilis was easily treated with both penicillin and doxycycline and that clinical treatment failure was not a common problem. Further, with the increased use of cART, the clinical manifestations of syphilis in HIV-infected patients might differ less from the HIV-uninfected patients in the future. Future studies focusing on the clinical manifestations of syphilis in HIV-infected patients in the cART era would be highly appreciated.

In conclusion, the rate of syphilis has stabilized in recent years but it is still too early to conclude whether syphilis is under control in Denmark. A spread to low-risk groups is of concern, especially if these patients are seen by clinicians who are not familiar with the symptomatology of syphilis. However, given the efficient treatment options, the targeted screening of HIV-infected patients, pregnant women and persons attending community-based testing (100) and STD clinics, control of the infection seems within reach. Avoiding new HIV infections is the major challenge and here cART may play a prominent role.

Summary

The studies included in this PhD thesis examined the interactions of syphilis, which is caused by *Treponema pallidum*, and HIV. Syphilis reemerged worldwide in the late 1990s and hereafter increasing rates of early syphilis were also reported in Denmark. The proportion of patients with concurrent HIV has been substantial, ranging from one third to almost two thirds of patients diagnosed with syphilis some years. Given that syphilis facilitates transmission and acquisition of HIV the two sexually transmitted diseases are of major public health concern. Further, syphilis has a negative impact on HIV infection, resulting in increasing viral loads and decreasing CD4 cell counts during syphilis infection. Likewise, HIV has an impact on the clinical course of syphilis; patients with concurrent HIV are thought to be at increased risk of neurological complications and treatment failure.

Almost ten percent of Danish men with syphilis acquired HIV infection within 5 years after they were diagnosed with syphilis during an 11-year study period. Interestingly, the risk of HIV declined during the later part of the period. Moreover, HIV-infected men had a substantial increased risk of re-infection with syphilis compared to HIV-uninfected men. As one third of the HIV-infected patients had viral loads >1000 copies/mL, our conclusion supported the initiation of cART in more HIV-infected MSM to reduce HIV transmission. During a 5-year study period, including the majority of HIV-infected patients from the Copenhagen area, we observed that syphilis was diagnosed in the primary, secondary, early and late latent stage. These patients were treated with either doxycycline or penicillin and the rate of treatment failure was similar in the two groups, indicating that doxycycline can be used as a treatment alternative – at least in an HIV-infected population. During a 4-year study period, the *T. pallidum* strain type distribution was investigated among patients diagnosed by PCR testing of material from genital lesions. In total, 22 strain types were identified. HIV-infected patients were diagnosed with nine different strains types and a difference by HIV status was not observed indicating that HIV-infected patients did not belong to separate sexual networks.

In conclusion, concurrent HIV remains common in patients diagnosed with syphilis in Denmark, both in those diagnosed by serological testing and PCR testing. Although the rate of syphilis has stabilized in recent years, a spread to low-risk groups is of concern, especially due to the complex symptomatology of syphilis. However, given the efficient treatment options and the targeted screening of pregnant women and persons at higher risk of syphilis, control of the infection seems within reach. Avoiding new HIV infections is the major challenge and here cART may play a prominent role.

Dansk resume

Studierne som indgår i denne ph.d.-afhandling undersøger samspillet mellem syfilis, der er forårsaget af *Treponema pallidum,* og HIV. Efter at have været en sjælden sygdom i mange år blev der i slutningen af 1990'erne rapporteret om en stigende forekomst af syfilis i mange vestlige lande, herunder Danmark. En betragtelig andel af syfilispatienter har samtidig HIV – i nogle år har en tredjedel været co-inficerede og i andre år har det været op til to tredjedele. Eftersom at syfilis faciliterer transmissionen af HIV er sammenhængen mellem disse to seksuelt overførte sygdomme af stor interesse for folkesundheden. Ved syfilisinfektion påvirkes HIV-infektionen uhensigtsmæssigt med stigende *viral load* og faldende CD4 celletal. På samme måde har samtidig HIV-infektion en uhensigtsmæssig effekt på infektion med syfilis da HIV-smittede patienter menes at have en større risiko for neurologiske komplikationer samt behandlingssvigt.

Vores studie viste at op mod 10 procent af danske mænd, der fik stillet diagnosen syfilis, fik konstateret HIV indenfor en periode på 5 år fra syfilisdiagnosen. Bemærkelsesværdigt nok faldt risikoen for at få HIV i den sidste halvdel af den 11-årige observationsperiode. Studiet viste endvidere, at HIV-smittede mænd havde en højere risiko for at få syfilis flere gange. Eftersom at en tredjedel af de HIV-smittede patienter havde *viral load* over 1000 kopier/mL konkluderede studiet, at flere HIV-smittede mænd burde tilbydes behandling med antiretroviral medicin for at mindske risikoen for transmission af HIV. I en 5-årig periode undersøgte vi størstedelen af HIV-patienter med syfilis fra Københavnsområdet. Patienterne blev diagnosticeret med syfilis i det primære, sekundære samt tidlige eller sene latente stadie. Patienterne blev behandlet med enten intramuskulær penicillin eller med doxycyclin som tabletbehandling, og det serologiske respons på behandlingen blev evalueret efter 12 måneder. Der var ingen forskel i serologisk respons mellem de to grupper, hvilket indikerer, at doxycyclin kan bruges som en ligeværdig behandling. I løbet af en 4-årig periode undersøgte vi distributionen af *T. pallidum* subtyper blandt alle patienter i Danmark, der var diagnosticeret ved hjælp af PCR på sårmateriale. Toogtyve forskellige subtyper blev påvist, og HIV-smittede patienter blev diagnosticeret med ni forskellige subtyper. Ved at opdele patienterne efter HIV-status undersøgte vi, om der var sammenhæng mellem HIV-status og subtype, men kunne ikke påvise en sådan sammenhæng.

HIV-infektion ses stadig hyppigt blandt patienter, der diagnosticeres med syfilis i Danmark, både blandt patienter diagnosticeret med serologiske tests samt patienter diagnosticeret med PCR. Grundet de effektive behandlingsmuligheder samt en målrettet screening af gravide og grupper med højere forekomst, forventes syfilis fremover at kunne kontrolleres i Danmark. Den største udfordring er derfor at undgå nye HIV-infektioner og her spiller antiretroviral behandling formentlig en fremtrædende rolle.

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Appendixes

Epidemiology

ORIGINAL ARTICLE

Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000–2010

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ABSTRACT

Objectives Risk of subsequent diagnosis of HIV in persons diagnosed with newly acquired syphilis, and syphilis in HIV-infected persons, are of interest as these infections are markers of unsafe sex.

Methods From a nationwide register, all Danish men aged >16 years diagnosed with syphilis in the period 2000–2010 (n=1217) were identified, and subsequently data on HIV status was extracted from the Danish HIV Cohort Study. We used Kaplan–Meier analysis to estimate the 5-year risk of HIV and second syphilis infection, and Cox regression to determine incidence rate ratios (IRR).

Results The 5-year risk of HIV diagnosis was 9.8% (95% CI 7.0% to 12.6%). Those with a second diagnosis of syphilis had a higher risk of being diagnosed with HIV (IRR=3.1, 95% CI 1.2 to 8.0). The 5-year risk for a second diagnosis of syphilis was 14.8% (95% CI 12.1% to 17.4%) and HIV-infected persons had a higher risk of a second syphilis diagnosis (IRR=4.0, 95% CI 2.8 to 5.9). Sixty-five percent of the persons were men having sex with men (MSM). Thirty-four percent of the HIV-infected persons had viral load >1000 copies/ml at time of syphilis diagnosis.

Conclusions The substantial risks of syphilis and HIV infection in men diagnosed with one of these sexually transmitted diseases indicate a high frequency of unsafe sex in the Danish MSM population. As one-third of the HIV-infected persons diagnosed with syphilis had high viral loads, our data support initiation of antiretroviral therapy in all HIV-infected MSM to reduce HIV transmission.

INTRODUCTION

Since 1999, the number of newly reported cases of syphilis in Denmark has increased dramatically. In Denmark, newly acquired syphilis, that is, primary and secondary syphilis, is notified to Statens Serum Institut (the National Institute for Health Data and Diseases Control).[1] Furthermore, the serological diagnosis of syphilis is centralised at Statens Serum Institut, and all persons notified and/or diagnosed with syphilis are registered in a nationwide database. In 2011, a total of 434 cases were diagnosed with newly acquired syphilis. The vast majority of the persons were men who had been infected in Denmark.[1] By contrast, only 22 cases were diagnosed in 1999, of whom the majority were infected in Eastern Europe, Africa and Asia.[2] In 2003, an outbreak of the disease among men who have sex with men (MSM) constituted 78% of the notified cases, whereas only 33% of the cases notified from 1994 to 2002 were MSM.[3] The proportion of MSM coinfected with HIV and syphilis has varied over the years, peaking in 2008 with 58% of the MSM being diagnosed with syphilis also being infected with HIV.[4] By contrast, 62% of the MSM diagnosed with syphilis in 2011 were HIV-negative—that is, susceptible to HIV—at the time of the diagnosis of syphilis.[1] As a consequence of the high rates of syphilis among HIV-infected persons, syphilis screening has been implemented at the specialised medical centres where these patients are seen at intended intervals of 12 weeks.

Increasing rates of syphilis have also been seen throughout Europe, North America and Australia, and the majority of the new diagnoses were found among MSM.[5] A possible change in behaviour may be attributed to factors such as loss of fear of contracting HIV due to improved outcome after highly active antiretroviral therapy (HAART).[6] Furthermore, the reduced transmission of HIV during successful HAART[7] may have led to a more risky sexual behaviour.

Since syphilis infection facilitates acquisition and transmission of HIV,[8] and because an increase in risky sexual behaviour has been reported,[9] concerns about a potential increase in HIV transmission have been raised—especially among MSM. We used two nationwide registers to describe the risk of HIV and syphilis among Danish men from 1 January 2000 to 1 January 2011. These infections are of interest as they are markers of sexual risk behaviour. We estimated the risk of HIV acquisition in persons who had been diagnosed with syphilis. Furthermore we estimated the risk of reinfection with syphilis.

METHODS

We used the unique 10-digit Central Person Registration number (CPR number) assigned to all individuals in Denmark at birth or upon immigration, to link data from the following registers: the Danish National Syphilis Registration System and the Danish HIV Cohort Study.

Setting

Denmark has a population of 5.6 million people with an estimated HIV prevalence in the adult

population of 0.07%.[10] [11] Approximately 44% of the HIV-infected population reported MSM contact as route of infection, and 74% reported Denmark as the country of origin.[11] The number of persons with newly diagnosed HIV has been stable over the last 10 years as well as the number of persons infected by homosexual transmission.[12] Five hundred undiagnosed HIV-infected MSM are estimated to reside in Denmark, an estimate that has been stable over the last 10 years.[13] Denmark's tax-funded healthcare system provides antiretroviral treatment free of charge to all residents. In Denmark, treatment of HIV infection is restricted to eight specialised medical centres, where patients are seen on an outpatient basis at intended intervals of 12 weeks. Syphilis screening at least once a year has been implemented as part of the routine testing in connection with these outpatient visits. During the study period, the national criteria for HAART initiation were any of the following: acute HIV infection, presence of a HIV-related disease, pregnancy, CD4 cell count <300 cells/µl until May 2008 and <350 cells/µl thereafter, and plasma HIV-RNA>100 000 copies/ml (until 2001). The National Board of Health recommends that patients tested for syphilis be offered HIV testing.

Data sources

All persons in Denmark diagnosed with newly acquired syphilis, that is, primary and secondary syphilis are registered in the Danish National Syphilis Registration System which was established in 1993. According to Danish law, newly acquired syphilis is a notifiable disease and the notification to the register is done by the treating physician. Data collected include route of infection, country of infection and result of prior HIV testing. Patients may be registered with more than one episode of syphilis. In addition to the cases notified to the Danish National Syphilis Registration System, the register also includes persons indentified with syphilis by positive serology or PCR. To assure anonymity, only the last six numbers of the 10-digit CPR number are registered in the Danish National Syphilis Registration System. To obtain the full CPR number, the reporting forms were matched with CPR numbers from persons with positive serology. In case of multiple options, the persons were matched according to ethnicity and geographical data. The procedure was approved by the Danish Data Protection Agency.

The Danish HIV Cohort Study, which has been described in detail elsewhere,[14] is a nationwide, prospective, population-based study of all Danish HIV-infected persons treated at Danish hospitals since 1 January 1995. The data is updated yearly and includes demographics, route of infection, CD4 cell counts, viral loads and antiretroviral treatment. The unique 10-digit CPR number is used to avoid multiple registrations and to track individuals in the Danish HIV Cohort Study. The study was approved by the Danish Data Protection Agency.

Study population

All male patients aged >16 years registered in the Danish National Syphilis Registration System with a diagnosis of newly acquired syphilis after 1 January 2000 were included. Date of study inclusion was the date of the first syphilis diagnosis after 1 January 2000. The study period was from 1 January 2000 to 1 January 2011.

Statistical analyses

Based on data from the Danish National Syphilis Registration System, the yearly number of men diagnosed with syphilis was calculated. From the Danish HIV Cohort Study, we obtained data on HIV status, and the yearly number of men diagnosed

with syphilis was stratified according to HIV status. For persons infected with HIV at the time of syphilis diagnosis, information on date of HIV diagnosis, start of antiretroviral treatment and viral load was extracted. HIV-RNA at date of first syphilis episode after HIV diagnosis was defined as the HIV-RNA closest to the date of syphilis diagnosis. We calculated the fraction of HIV-infected persons diagnosed with syphilis who had a viral load <1000 copies/ml. Our objective was to estimate the fraction with suppressed viral load, therefore, persons with missing HIV-RNA were allocated to the group with >1000 copies/ml. For the population diagnosed with syphilis, but not with HIV at the time of syphilis diagnosis, the time to HIV diagnosis was determined. In these analyses, the time from first diagnosis of syphilis within the study period to date of HIV diagnosis or 1 January 2011, whichever came first, was calculated. Time-to-event curves from Kaplan–Meier tables were constructed and stratified by the diagnosis of syphilis before and after 1 January 2006, respectively. We used Cox regression analysis with the date of the second syphilis diagnosis introduced as the time-updated variable to determine the incidence rate ratio (IRR) for risk of HIV diagnosis before and after a second diagnosis of syphilis. Likewise, the time to a second syphilis diagnosis was determined for the total population and Kaplan–Meier tables were stratified by syphilis diagnosis before and after 1 January 2006. We used Cox regression analysis with the date of HIV infection introduced as the time-updated variable to determine IRR for risk of a second syphilis infection.

The study was approved by the Danish Data Protection Agency. SPSS V.15.0 (SPSS Inc, Chicago, Illinois, USA) was used for data analysis.

Syphilis testing methods

The serological diagnosis of syphilis is centralised at Statens Serum Institut.[15] Sera samples from patients were examined for the presence of syphilis antibodies. Two non-treponemal tests were used; Wassermann's reaction was done with a complement fixation technique, and rapid plasma reagin was determined by agglutination. Furthermore, treponemal tests were used on seroreactive samples; Antiflagel IgG was determined by a capture ELISA and IgM by a direct ELISA. The fluorescent treponemal antibody absorption test was done by immunofluorescence microscopy. If an individual had tested seropositive for syphilis several times, the serology was carefully evaluated and compared with the reporting forms (mandatory for the treating physician). The results were regarded as being diagnostic of a new infection if a substantial increase in syphilis antibodies was evident, preceded by a decrease in antibody titre documenting cure of the prior episode. A few patients with genital chankres were diagnosed by PCR testing of material from the lesions.

RESULTS

During the 11-year study period from 1 January 2000 to 1 January 2011, a total of 1536 episodes of syphilis were diagnosed among 1361 persons, including 144 women, leaving 1217 men in the present study. In our study population, the median age was 37.8 years (table 1). The yearly number of men diagnosed with syphilis increased substantially in the study period irrespective of HIV infection, from 38 in 2000 to 344 in 2010 (figure 1). Sixty-five percent of the men with syphilis were notified as MSM on the syphilis reporting form, 14% were notified as heterosexual and finally 21% of the reporting forms were lacking information regarding the route of transmission.

Epidemiology

Table 1 Danish men diagnosed with newly acquired syphilis in Denmark in the period 2000–2010

Total study population	
Number of patients	1217
Age (median and IQR)	37.8 (30.4–45.7)
Number of patients diagnosed with syphilis more than once in the study period	133
Number of patients diagnosed with HIV prior to diagnosis of syphilis (%)	359 (29.5)
Age (median and IQR)	40.4 (35.1–46.9)
Caucasian race (%)	320 (89.1)
Time from HIV diagnosis to diagnosis of syphilis (years, median and IQR)	4.7 (1.8–10.7)
Number of patients diagnosed with syphilis more than once in the study period	79
On HAART (%)	242 (67.4)
<1000 HIV-RNA copies/ml (%)	238 (66.3)
Time from start of HAART to date of diagnosis of syphilis (years, median and IQR)	3.4 (0.6–6.9)
Route of HIV infection	
MSM (%)	323 (90.0)
Intravenous drug use (%)	2 (0.6)
Heterosexual (%)	20 (5.6)
Other/unknown (%)	14 (3.9)
Number of patients not diagnosed with HIV prior to diagnosis of syphilis (%)	858 (70.5)
Number of patients diagnosed with HIV after diagnosis of syphilis	64

HAART, highly active antiretroviral therapy; MSM, men having sex with men.

Men diagnosed with HIV prior to diagnosis of syphilis

A total of 359 men diagnosed with syphilis were diagnosed with HIV prior to diagnosis of syphilis, the majority of whom reported MSM as route of HIV infection (table 1). Sixty-six percent of the HIV-infected population had a viral load <1000 HIV-RNA copies/ml at the time of diagnosis of syphilis, and thereby, 33.7% of the HIV-infected population diagnosed with syphilis had viral loads >1000 HIV-RNA copies/ml. Data on viral load at the time of syphilis diagnosis were missing from 13 patients, and in these cases, the viral load was defined as >1000 HIV-RNA copies/ml. Other characteristics are described in table 1.
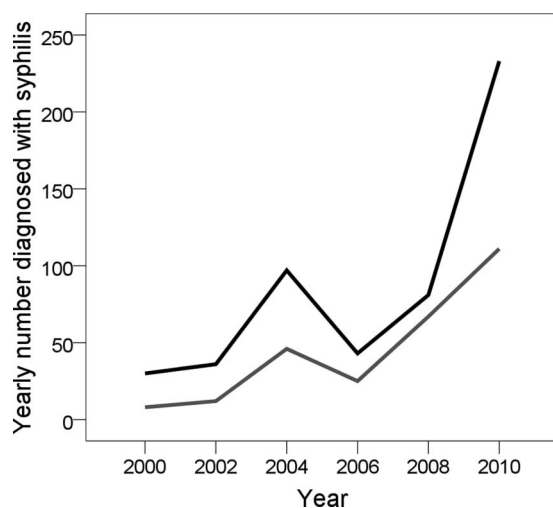
Men not diagnosed with HIV prior to diagnosis of syphilis— risk of HIV acquisition

At the time of diagnosis of syphilis, 858 men were not diagnosed with HIV. From this cohort, 64 men subsequently acquired HIV within the study period. After 5 years, 9.8% (95% CI 7.0 to 12.6%) of the population had been diagnosed with HIV. Figure 2 illustrates time from first syphilis diagnosis to HIV diagnosis stratified on men being diagnosed with syphilis before and after 1 January 2006, respectively. In a Cox model with the date of second diagnosis of syphilis introduced as a time-updated variable, the risk of being diagnosed with HIV was substantially higher after the second diagnosis of syphilis (IRR=3.1, 95% CI 1.2 to 8.0), that is, men with minimum two episodes of syphilis had a higher risk of being diagnosed with HIV compared with men with only one episode of syphilis within the study period.
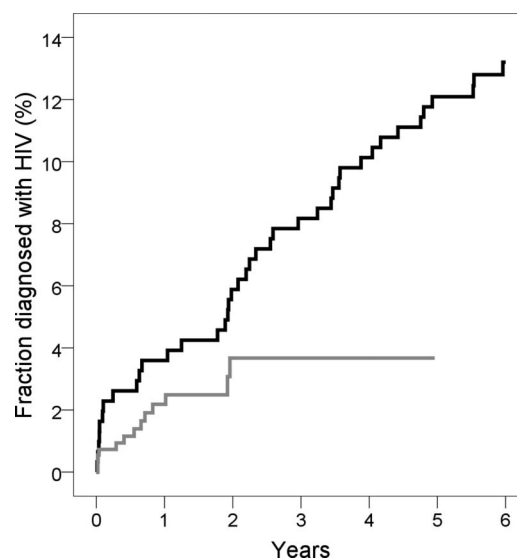
Risk of reinfection with syphilis

After 5 years, 14.8% (95% CI 12.1 to 17.4%) of the population had been diagnosed with a second episode of syphilis. Figure 3 illustrates the time from the first diagnosis of syphilis to the second episode within the study period before and after 1 January 2006, respectively. In a Cox model with the date of HIV infection introduced as a time-updated variable, HIV-infected persons had a higher risk of being reinfected with syphilis compared with syphilis patients not diagnosed with HIV within the study period (IRR=4.0, 95% CI 2.8 to 5.9).

DISCUSSION

In a nationwide cohort of Danish men diagnosed with newly acquired syphilis, we observed a very high risk of both prior and subsequent diagnoses of HIV and of reinfection with syphilis during an 11-year follow-up period. A major strength of the study is our ability to link two nationwide registers of persons diagnosed with syphilis and HIV.

During the last decade, Denmark has experienced a substantial increase in the number of persons diagnosed with syphilis, the majority of whom were men. From our data, the dominant profile of the epidemic was MSM. This is in line with the

Figure 1 Number of men, newly infected with syphilis per calendar year in Denmark from 1 January 2000 to 1 January 2011, stratified by HIV status: HIV-negative (black line) and HIV-positive (grey line).

Figure 2 Time from first diagnosis of syphilis to HIV diagnosis in 858 men not diagnosed with HIV prior to syphilis diagnosis from 1 January 2000 to 1 January 2011 in Denmark. Stratified on diagnosis of syphilis before (black line, n=306) and after (grey line, n=552) 1 January 2006.
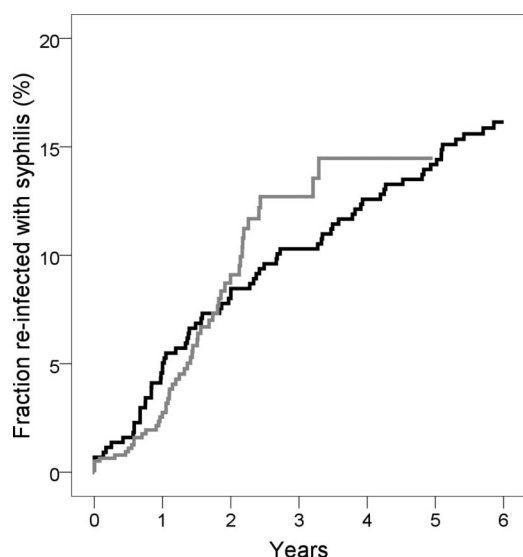
Figure 3 Time from first diagnosis of syphilis to second diagnosis of syphilis in 1217 men diagnosed with syphilis from 1 January 2000 to 1 January 2011 in Denmark. Stratified on diagnosis of syphilis before (black line, n=437) and after (grey line, n=780) 1 January 2006.

findings from a number of studies which indicate that the resurgence of syphilis is geographically widespread and outbreaks have been seen throughout Europe, North America and New Zealand. Overall, the affected population was MSM.[16–19] The marked increase in syphilis among MSM has mainly been attributed to behavioural factors, that is, a more risky sexual behaviour in the wake of the impressive impacts of antiretroviral therapy on HIV progression rates.[20 21] Supporting this assumption is the rise in other sexually transmitted diseases among MSM, for example, *Chlamydia trachomatis* lymphogranuloma venereum variant and gonorrhoea.[22] Whether the increase is due to a general increase in sexual risk behaviour among MSM or due to the establishment of a core group with high-risk behaviour is not known.[23] Our finding that HIV-infected persons had a four times greater risk of acquiring a second case of syphilis compared with persons diagnosed with syphilis without HIV could support the existence of such a core group with a high-risk behaviour. But unsafe sex among all MSM in Denmark seems to be increasingly common.[9]

An alternative explanation has also been put forward by Grassly *et al*[24] who suggested that the increase in syphilis was mainly due to endogenous oscillation in disease incidence predicted by the natural dynamics of the infection. They found support for this theory in the different dynamics of syphilis and gonorrhoea incidences. These conclusions have, however, been questioned.[25]

Thirty percent of the persons diagnosed with syphilis were diagnosed with HIV prior to being diagnosed with syphilis. The majority of these persons were under HAART treatment but only two-thirds of the HIV-infected persons had HIV-RNA levels below 1000 copies/ml. This figure is lower than that observed in 2004 in the national database for all HIV-infected persons.[26] As previously mentioned, the HIV-population had a higher risk of a second syphilis diagnosis, which may indicate a more risky sexual behaviour. As one-third of the HIV-infected persons diagnosed with syphilis had high viral loads, our data supports initiation of antiretroviral therapy in all HIV-infected MSM to reduce HIV transmission

The aim of the current study was to describe the pattern of syphilis among men in Denmark, and also to investigate whether being diagnosed with syphilis was predictive for subsequent HIV acquisition. Almost 10% of the population diagnosed with syphilis was subsequently diagnosed with HIV within 5 years. Interestingly, the risk of being diagnosed with HIV subsequent to receiving a syphilis diagnosis decreased during the latter part of the study period. Similar trends where outbreaks of syphilis have not had a substantial impact on HIV incidence have been observed in North America.[27] A possible explanation of the stagnation of the HIV transmission despite the increase in syphilis diagnoses could be that antiretroviral treatment has become more widely used in recent years with viral suppression as a consequence. Even without taking into account the decreasing HIV transmission risk, the finding could be explained by the increase in the number of persons diagnosed with syphilis during the study period.

By contrast to the risk of being diagnosed with HIV, the risk of being diagnosed for the second time with syphilis did not decrease from the first to the second part of the study period. This is in line with the increase in syphilis incidence that was observed especially in the latter part of the study period.

We found a substantially higher risk of being diagnosed with HIV after a second diagnosis of syphilis indicating that syphilis is a marker for sexual risk behaviour. This is in line with a study among high-risk HIV-uninfected MSM in Peru where newly acquired syphilis (and herpes simplex virus type 2), as well as sex with a casual partner, were found to be associated with incident HIV infection.[28]

The present study combines data from nationwide registers and provides a well-founded estimate about the current syphilis epidemic among men in Denmark. However, some limitations to our study must be addressed. First, a possible bias is caused by selective syphilis screening of HIV-infected persons as part of the routine testing of this group, thus underestimating the syphilis prevalence in low-risk groups. Therefore, an unknown number of cases of syphilis have evidently been left out since low-risk groups have not been screened. Second, our study includes both MSM and heterosexual men who have different risk behaviours and exposures. Even though information on route of transmission is supposed to be stated on the syphilis reporting forms, this data was rather incomplete since information on this variable was missing in 21% of the forms. We expect that the actual fraction of MSM may be significantly higher than the 65% who reported MSM as route of infection on the syphilis reporting form. Third, our data analysis did not account for mortality and emigration since this data was not available for the HIV-uninfected population. This could potentially have an impact on our risk estimates because of the competing risk of fatality making the actual risk higher. Another limitation of the study lies in the definition of a 'case of syphilis' as it requires interpretation to distinguish between a reinfection and resurgence of an incompletely treated infection.

In conclusion, our study demonstrates that men diagnosed with syphilis have a high risk of a subsequent HIV diagnosis, and men coinfected with HIV and syphilis have an increased risk of a second syphilis diagnosis. Furthermore, the study underlines that a substantial fraction of the HIV-infected men with confirmed HIV viremia are diagnosed with syphilis, thereby indicating that unsafe sex is prevalent in the Danish HIV-infected MSM population with the concomitant risk of transmitting HIV. This finding supports the thesis that offering antiretroviral therapy to all HIV-infected MSM could potentially reduce HIV transmission.

Epidemiology

Finally, the high rates of syphilis and HIV among men who have already been in contact with the Danish healthcare system reflect a major missed opportunity for risk reduction in terms of postcounselling.

Key messages

▶ Danish men diagnosed with syphilis have a high risk of subsequent HIV diagnosis.
▶ Danish men coinfected with HIV and syphilis have a higher risk of being reinfected with syphilis.
▶ These patients are frequently in contact with the Danish healthcare system. Efforts must be made to reduce risk behaviour.

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STI

Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000–2010

Kirsten Salado-Rasmussen, Terese Lea Katzenstein, Jan Gerstoft, et al.

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Notes

Serological Response to Treatment of Syphilis with Doxycycline compared to Penicillin in HIV-Infected Individuals

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ABSTRACT

Background. The recommended treatment of syphilis remains benzathine penicillin G with doxycycline being used as a second-line agent. We assessed the serological response to treatment of syphilis with orally administered doxycycline or intramuscularly administered penicillin in patients with concurrent HIV infection.

Methods. We performed a retrospective study of all HIV-infected individuals diagnosed with primary, secondary, early or late latent syphilis attending three hospitals in Copenhagen, Denmark. During the study period from 2004 to 2009 serologic testing for syphilis was centralized at Statens Serum Institut. Serological cure was defined as a ≥4-fold decline in WR and RPR and serological failure was defined as a lack of a 4-fold decline in WR and RPR 12 months following therapy.

Results. In total, 202 cases were treated with doxycycline or intramuscular penicillin. Of these, 126 cases were evaluated at 12 months following therapy; 78 cases were treated with doxycycline and 48 cases were treated with penicillin. At 12 months, serological failure was observed in 12 cases (15%) treated with doxycycline and in 8 cases (17%) treated with penicillin ($p > 0.05$). The serological cure rate at 12 months was highest in patients with primary syphilis (100%), followed by patients with secondary (89%), early latent (71%) and late latent (67%) syphilis ($p = 0.006$).

Conclusion. The serological cure rates were the same in the doxycycline and the penicillin group. In conclusion, our study provides evidence for the use of doxycycline as a treatment option when treating a HIV-infected population for primary, secondary, early and late latent syphilis.

INTRODUCTION

Since its introduction in the 1940s, penicillin has been used for treatment of syphilis. Penicillin G, administered parenterally, is the recommended first-line treatment for all stages of syphilis in Europe and the United states, however the preparation, dosage and length of treatment depend on disease stage and whether there is central nervous system (CNS) involvement (1;2). In the 1950s, successful use of tetracycline derivatives for treatment of syphilis was reported (3;4). Doxycycline, a tetracycline derivative with increased bioavailability and fewer side effects, was then introduced. An uncontrolled observational study from 1979 evaluated the serologic response to doxycycline treatment of syphilis during the course of a year and found excellent response rates for patients with primary (100%), secondary and early latent syphilis (90%) (5). In a more recent retrospective case-control study, doxycycline was compared to benzathine penicillin G in doses recommended by the Centers for Disease Control and Prevention (CDC) and appeared to be an effective agent for treatment of early syphilis (6). Azithromycin has also been used as a treatment alternative but very high rates of the resistance-conferring A2058G mutation have been reported from the United States and Shanghai (7-9), limiting its usefulness.

Syphilis and HIV coinfection is common in Denmark (10). Since a case report documented neurologic relapse after treatment with benzathine penicillin G in an HIV-infected individual (11), the management of syphilis in patients with concurrent HIV has been debated. Although many controversies exist on the impact of HIV on the clinical course of syphilis (12), serologically defined treatment failure has been documented to be more common in patients with primary and secondary syphilis and concurrent HIV (13). However, a recent study found that HIV coinfection had an impact only on the serological response in patients with primary syphilis and a CD4 cell count of <500 cells/μL (14). Today many HIV-infected individuals are treated with combination antiretroviral therapy (cART) and may have a restored immune system, resulting in reduced rates of serologic failure (15). The CDC recommends that HIV-infected individuals are evaluated clinically and serologically for treatment failure at 3, 6, 9, 12 and 24 months after therapy (2).

The aim of our study was to compare the serological response to treatment of primary, secondary, early or late latent syphilis with intramuscularly administered penicillin or orally administered doxycycline in patients with concurrent HIV.

METHODS

Patients

The study was performed as a retrospective study of HIV-infected individuals ≥18 years of age diagnosed with syphilis between 1 May 2004 and 31 October 2009. All HIV-infected individuals attending the Department of Infectious Diseases at Copenhagen University Hospital, Rigshospitalet, the Department of

Infectious Diseases at Copenhagen University Hospital, Hvidovre and the sexually transmitted disease (STD) clinic at the Department of Dermato-venereology at Copenhagen University Hospital, Bispebjerg were included. These hospitals were chosen because the vast majority of HIV-infected individuals from the capital area of Copenhagen are seen at these departments and because the vast majority of syphilis cases in Denmark are diagnosed in the Copenhagen area (16). We excluded patients who received intravenous antibiotics, were diagnosed with neurosyphilis or lacked information on therapy. An individual could contribute with more than one episode, provided that treatment and appropriate treatment response was documented in the patient files.

Antibiotic treatment

Therapy consisted of doxycycline (100 mg orally twice daily for 14 days for early syphilis, i.e., primary, secondary and early latent stages, and for 30 days for late latent syphilis) or penicillin (a single dose of intramuscular 2.4 million units of benzathine penicillin G for early syphilis and three doses each at 1-week intervals for late latent syphilis). In the beginning of the study period 15 patients were treated with intramuscular procaine penicillin (one dose of 600 000 units once daily for 10 days), and these cases were grouped with the benzathine penicillin G treated cases.

Definitions of syphilis stages

Disease stage was based on clinical examination, patient history and result of serological tests. Patients were classified as having primary syphilis (i.e., ulcer), secondary syphilis (i.e., seroreactivity with clinical manifestations such as skin rash or mucocutaneous lesions), early latent syphilis (i.e., seroreactivity, no clinical manifestations and known duration of less than a year), late latent syphilis (seroreactivity, no clinical manifestations and known duration of more than a year or unknown duration) or tertiary syphilis (i.e., seroreactivity with cardiac or gummatous lesions). CNS involvement can occur during all stages of syphilis, and neurosyphilis was defined as seroreactivity in the cerebrospinal fluid (CSF) or elevated CSF cell count combined with unexplained neurological manifestations consistent with neurosyphilis (1;2). Most classifications were done when the patient was seen at the clinic and only a few cases were classified retrospectively.

Data collection

Sociodemographic information (age, sex and ethnicity), mode of acquisition (e.g., men who have sex with men (MSM) status), syphilis disease stage, CNS symptoms, treatment of syphilis, country of acquisition, history of previous syphilis infection, HIV RNA, CD4 cell counts, cART, hepatitis B virus status (presence of hepatitis B surface antigen), hepatitis C virus status (presence of hepatitis C antibody) and information on concurrent STDs was extracted from the patient files.

Laboratory tests

To capture all available serological data, we obtained the serological test results from Statens Serum Institut where all serologic testing of syphilis was centralized during the study period. Two non-treponemal tests were used: Wassermann's reaction (WR) was done with a complement fixation technique, and rapid plasma reagin (RPR) was determined by agglutination. Furthermore, three treponemal tests were used: anti-flagellum IgM (AF-M) was determined by a capture ELISA, anti-flagellum IgG (AF-G) was determined by an indirect ELISA and the fluorescent treponemal antibody absorption test (FTA-ABS) was done by immunofluorescence microscopy.

Definition of outcomes

Serological cure was defined as a ≥4-fold decline in non-treponemal titers following therapy. Serological failure was defined as a lack of a 4-fold decline. We allocated serological test results to a specific follow-up visit (at 3, 6, 9 or 12 months post-therapy) if the test was between 30 days before and 30 days after the relevant point in time. Furthermore, the last-observation-carried-forward principle was used to handle missing values of WR and RPR, e.g., if a patient had no available tests at 12 months but had reached serological cure at 9 months the patient was classified as serologically cured at 9 months.

Data analysis

Descriptive statistics was used to characterize the study population. Sex, ethnicity, MSM status, country of acquisition, history of syphilis, hepatitis B and C virus status and cART were evaluated as dichotomous variables. WR and RPR were evaluated as discrete variables. CD4 cell count was evaluated as a continuous variable and as a categorical variable (CD4 cell count ≤200 cells/μL or >200 cells/μL). HIV RNA was evaluated both as a logarithm base 10-transformed continuous variable and as a categorical variable (≤200 copies/mL, >200 copies/mL and <100 000 copies/mL or ≥100 000 copies/mL).

Where appropriate, the X^2 or Fisher's exact test were used to compare independent proportions (multivariable models were not used because the number of patients with treatment failure was small). For comparison of continuous variables the t test and the Mann-Whitney test were used for normal distributed and non-normal distributed variables, respectively. The Kruskal-Wallis test was used for comparison of titers between different syphilis stages. Differences with $p < 0.05$ (2-sided) were considered statistically significant. Data analysis was done using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA). Exemption for review by the ethics committee system and for obtaining informed consent was obtained from the Committee on Biomedical Research Ethics for the Capital Region of Denmark.

RESULTS

Clinical and baseline characteristics

From 1 May 2004 to 31 October 2009, a total of 221 cases of syphilis were diagnosed in 172 HIV-infected individuals attending three hospitals in the Copenhagen area of Denmark (138 individuals contributed with a single episode, 48 with two episodes, 21 with three episodes, 1 with four episodes and 2 with five episodes). Characteristics of the individuals are shown in Table 1. The patients were diagnosed with primary, secondary, early and late latent syphilis. No cases of tertiary syphilis were seen in these patients. Patients with neurosyphilis (14 individuals) were excluded from the analysis of serological response to treatment (Figure 1). In total, 202 cases were treated with doxycycline or intramuscular penicillin. Of these, 126 cases were evaluated at 12 months; 78 cases were treated with doxycycline and 48 cases were treated with penicillin. Table 2 summarizes the demographical and clinical characteristics of the doxycycline treatment group and the penicillin treatment group. No statistically significant differences between treatment groups were observed, except for CD4 cell count ≤200 cells/μL which was less common and proportion on cART which was higher for the doxycycline treated group (Table 2).

Serological outcome

Serological outcome was assessed at 3, 6, 9, and 12 months following therapy and no statistically significant differences were observed between treatment groups at any point in time (all $p > 0.05$). At 12 months, the point in time often used to evaluate serological cure rates, 20 cases of serological failure were observed (Table 3). All these patients had reactive treponemal tests which confirmed the diagnosis of syphilis. In the doxycycline group, 12 cases (15%) of serological failure were observed. In the penicillin group, 8 cases (17%) of serological failure were observed, resulting in a non-significant difference of 2% (95% confidence interval, -1.08%–5.08%; $p > 0.05$). Among these 20 cases, two individuals contributed with more than one episode of serological failure. The first individual contributed with three episodes (one episode was treated with procaine penicillin, one episode with benzathine penicillin G and one episode with doxycycline). The other individual contributed with two episodes and was diagnosed with relapse due to malaise and persisting seroreactivity one year after treatment of early latent syphilis. This patient had been treated with doxycycline for 14 days, followed by a documented 4-fold decrease in non-treponemal titers. Hereafter titers stabilized but did not reverse and the patient received retreatment with doxycycline for 30 days but remained seroreactive hereafter. The patient was an 80-year old man with nine previous episodes of syphilis and reinfection could not be ruled out completely. In all other cases of serological failure the patients did not receive retreatment and no indications of reinfection were found.

When comparing WR and RPR titers between treatment groups at time of diagnosis and 3, 6, 9 and 12 months post treatment no statistically significant differences were observed (all $p > 0.05$) (Table 2). As a consequence, further analyses were conducted using combined data from the doxycycline and penicillin groups. At the time of diagnosis, there was a statistically significant difference in WR and RPR between syphilis stages (WR: $p = 0.017$; RPR: $p = 0.02$). From the descriptive statistics it was evident that the highest titers were observed during the secondary stage (WR: 11; RPR: 128) compared to the primary stage (WR: 7; RPR: 32), the early latent stage (WR: 9; RPR: 64) and the late latent stage (WR: 8; RPR: 64). Among the cases with primary and secondary syphilis, 100% and 89% reached serological cure at 12 months, respectively, and of the cases with early and late latent syphilis it was 71% and 67%, respectively ($p = 0.006$). The serological cure rate in our patients did not vary by CD4 cell count, HIV RNA or age (all $p > 0.05$). However, WR and RPR titers at the time of diagnosis were significantly higher in patients with serological failure 12 months after treatment (WR: $p = 0.002$; RPR: $p < 0.000$).

DISCUSSION

We assessed the serological treatment response in 202 cases of syphilis in HIV-infected individuals; 127 cases were treated with doxycycline and 75 cases were treated with intramuscular penicillin. Current treatment guidelines in Europe and the United States recommend penicillin as the preferred treatment option, whereas doxycycline is reserved to patients allergic to penicillin (1;2). Our findings suggest that doxycycline can be used as an efficient alternative, at least in an HIV-infected population with close serological and clinical follow-up.

In a recent study comparing single dose with multiple doses of benzathine penicillin G for treatment of syphilis in HIV-infected individuals, 91% of the included patients exhibited serologic cure by 13 months and 97% by two years (17). In addition, a systematic review found a failure rate of 1%–22% among HIV-infected individuals (18). In our study, serologic cure rates of 85% and 83% for doxycycline and penicillin, respectively, fall within this range and furthermore no difference in serological outcome was observed between treatment groups. Moreover, a previous study from our group with the primary objective to assess the effect of syphilis and HIV coinfection on viral load and CD4 cell count found comparable response rates to treatment with penicillin and doxycycline (19).

A study by Sena *et al* (20) demonstrated that persisting non-treponemal titers were common among patients with early syphilis and that retreatment only had a minor effect on serofast patients. In keeping with this, one patient in our study was diagnosed with relapse and the patient did not serorevert despite retreatment. Another study showed that patients experiencing their first infection were more likely to serorevert than patients with repeated infection (21). In line with these findings the patient with relapse in

our study had nine former episodes of syphilis. A more recent large study in a HIV-uninfected population assessed factors associated with serological response in patients with early syphilis and found that serological cure at six months after treatment was associated with younger age and fewer sex partners, but surprisingly not with a history of syphilis (22). Accordingly, whether persistent seroreactivity represents low-level infection or is caused by variability in the host response remains controversial.

We found a higher rate of serological cure in earlier stages of syphilis. The highest rates of serological cure at 12 months were seen in the primary and secondary stages. Previous studies have also reported slower serologic responses in late stages of syphilis (14;21;23). Likewise, a study similar to ours comparing doxycycline and penicillin, corroborated that HIV-infected patients with secondary syphilis were more likely to achieve serological cure than patients in other stages (24). CD4 cell count has also been shown to have an effect on serological treatment outcome; a CD4 cell count of <200 cells/µL at the time of syphilis diagnosis has been associated with an increased risk of serologic failure (15). Nevertheless, in our study we did not demonstrate such an association.

The strengths of the current study include the close follow-up and the detailed demographic, clinical and behavioral data on all patients. Further, to capture all serological data, results of serological tests were obtained from the national provider of serological syphilis testing. The samples were not batched but all analyses were performed at the same laboratory. All reactive non-treponemal tests were confirmed using treponemal tests thereby minimizing the risk of false positive test results. Of note, the use of cART appears to decrease the prevalence of biologic false positive tests in HIV-infected individuals (25). Also, all patient files were revised, enabling us to confirm that seroreactive tests were clinically perceived as a case and to distinguish between relapse and reinfection. Finally, in contrast to others our study also included patients with late latent stage syphilis.

This study is limited by the retrospective non-randomized design. The treatment given was based on local guidelines and the patients at the Department of Infectious Diseases at Rigshospitalet were mainly treated with doxycycline, whereas patients at the Department of Infectious Diseases at Hvidovre Hospital and the STD clinic at Bispebjerg Hospital were mainly treated with penicillin. However, the patients in the two treatment groups were comparable except for proportion on cART and CD4 cell count. The patients in this study were all attending either of the two departments of infectious diseases for treatment or monitoring of their HIV-infection, however, for treatment of syphilis, some of these patients opted for the STD clinic. More HIV-infected individuals not receiving cART opted for treatment at the STD clinic compared to individuals on cART. This explains the lower proportion of patients on cART in the group receiving penicillin (the preferred regimen at the STD clinic). Likewise, even though the median CD4 cell count was comparable

between treatment groups, a higher proportion of patients with CD4 cell counts ≤200 cells/µL were treated with penicillin. The similar response rates in the treatment groups might be caused by the higher proportion of patients with low CD4 cell counts in the penicillin group because low CD4 cell counts have been associated with increased risk of treatment failure and delayed treatment response in HIV-infected individuals (26). Confounding by indication is a risk when doing a retrospective study of treatment options but our treatment groups were overall comparable. Finally, the vast majority of the patients in this study were MSM and whether our results are generalizable to other populations is unknown.

Systematic reviews have concluded that guidelines for treatment of HIV-infected individuals are based on very few objective data (18;27). Whether doxycycline is equipotent to penicillin can only be definitively evaluated in a randomized clinical trial. However, with the well-functioning treatments with penicillin such a study might not counteract the costs of a large clinical trial. Regardless of its observational design, our study provides evidence for the use of doxycycline as an acceptable treatment option for patients with close follow-up, who prefer oral treatment for syphilis. A study similar to ours, comparing doxycycline and penicillin in HIV-infected individuals supports this assumption (24), although their response rates to both penicillin and doxycycline were lower than in our study and lower than previously reported (6).

In addition, doxycycline is an effective agent for treatment of multiple STDs (27). Importantly, all our patients had concurrent HIV but the results may be generalizable to patients without HIV although the requirement of multiple days of treatment may present a problem in a population less used to daily medication. Taken together, our study supports the use of doxycycline as an efficient treatment option for syphilis when treating an HIV-infected population with close follow-up.

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TABLES

Table 1. Main characteristics of the study population; 172 HIV-infected individuals contributed with 221 cases of syphilis

Characteristic	Individuals (n=172)	Cases (n=221)
Age, median (range)	40 (20–78)	40 (20–83)
Female	2 (1)	2 (1)
Male	170 (99)	219 (99)
MSM	160 (94)	207 (95)
Danish citizen	132 (77)	175 (80)
Syphilis stage		
Primary	14 (8)	21 (10)
Secondary	102 (60)	130 (59)
Early latent	23 (13)	31 (14)
Late latent	31 (18)	36 (16)
Relapse	0 (0)	1 (0)
Unknown	2 (1)	2 (1)
Neurosyphilis	12 (7)	14 (6)
Syphilis treatment		
Doxycycline	101 (59)	129 (59)
Penicillin intramuscularly	58 (34)	76 (34)
Penicillin intravenously	4 (2)	6 (3)
Ceftriaxone	6 (3)	7 (3)
Unknown	3 (2)	3 (1)
Infected in Denmark	104 (60)	127 (57)
History of syphilis	59 (34)	108 (49)
CD4 cell count, cells/μL		
Median (IQR)	460 (336–639)	450 (338–630)
≤200	14 (8)	17 (8)
>200	104 (61)	129 (58)
Unknown	54 (31)	75 (34)
HIV RNA, $\log_{10}$ copies/mL		

Mean (SD)	2.24 (1.66)	2.18 (1.65)
HIV RNA, copies/mL		
≤200	77 (45)	99 (45)
>200–100 000	30 (18)	36 (16)
≥100 000	11 (6)	13 (6)
Unknown	54 (31)	73 (33)
Cart	117 (68)	153 (69)
Coinfections		
Hepatitis B virus infection	17 (10)	27 (12)
Hepatitis C virus infection	15 (9)	22 (10)
Gonorrhea	4 (2)	5 (2)
Chlamydia	4 (2)	6 (3)

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: MSM, men who have sex with men; IQR, interquartile range; SD, standard deviation; cART, combination antiretroviral therapy.

Table 2. Comparison of the doxycycline and the penicillin treatment group (n=202 cases)

Characteristic	Doxycycline (n=127)	Penicillin (n=75)	*p*-value
Age, median (range)	40 (20–83)	39 (24–61)	NS
Female	1 (1)	1 (1)	NS
Male	126 (99)	74 (99)	NS
MSM	121 (96)	70 (95)	NS
Danish citizen	106 (83)	54 (72)	NS
Syphilis stage			
Primary	12 (9)	8 (11)	NS
Secondary	75 (59)	42 (56)	NS
Early latent	18 (14)	10 (13)	NS
Late latent	21 (17)	13 (17)	NS
Relapse	1 (1)	0 (0)	NS
Unknown	0 (0)	2 (3)	NS
Infected in Denmark	76 (60)	39 (72)	NS
History of syphilis	66 (52)	33 (44)	NS
CD4 cell count, cells/µL			
Median (IQR)	480 (340–630)	430 (270–638)	NS
≤200	7 (6)	9 (12)	0.03
>200	84 (66)	34 (45)	0.03
Unknown	36 (28)	32 (43)	0.045
HIV RNA, $\log_{10}$ copies/mL			
Mean (SD)	2.11 (1.58)	2.34 (1.80)	NS
HIV RNA, copies/mL			
≤200	61 (48)	28 (37)	NS
>200–100 000	22 (17)	11 (15)	NS
≥100 000	6 (5)	6 (8)	NS
Unknown	38 (30)	30 (40)	NS
cART	96 (76)	44 (59)	0.009
Coinfections			
Hepatitis B virus infection	19 (15)	6 (8)	NS

15

Hepatitis C virus infection	15 (12)	5 (7)	NS
Gonorrhea	1 (1)	2 (3)	NS
Chlamydia	4 (3)	3 (4)	NS
Median WR titer at time of diagnosis	10	11	NS
Median RPR titer at time of diagnosis	64	64	NS

Data are presented as No. (%) unless otherwise indicated.

P-values compare the doxycycline treatment group with the penicillin treatment group using X^2 or Fisher's exact test for categorical variables and the Mann-Whitney test or t test for continuous variables.

Abbreviations: NS, not statistically significant; MSM, men who have sex with men; IQR, interquartile range; SD, standard deviation; cART, combination antiretroviral therapy; WR, Wassermann's reaction; RPR, rapid plasma reagin.

Table 3. Serological outcomes 3, 6, 9 and 12 months after treatment (n=202 cases). Serological cure was defined as a ≥4-fold decline in WR and RPR. Serological failure was defined as a lack of a 4-fold decline in WR and RPR
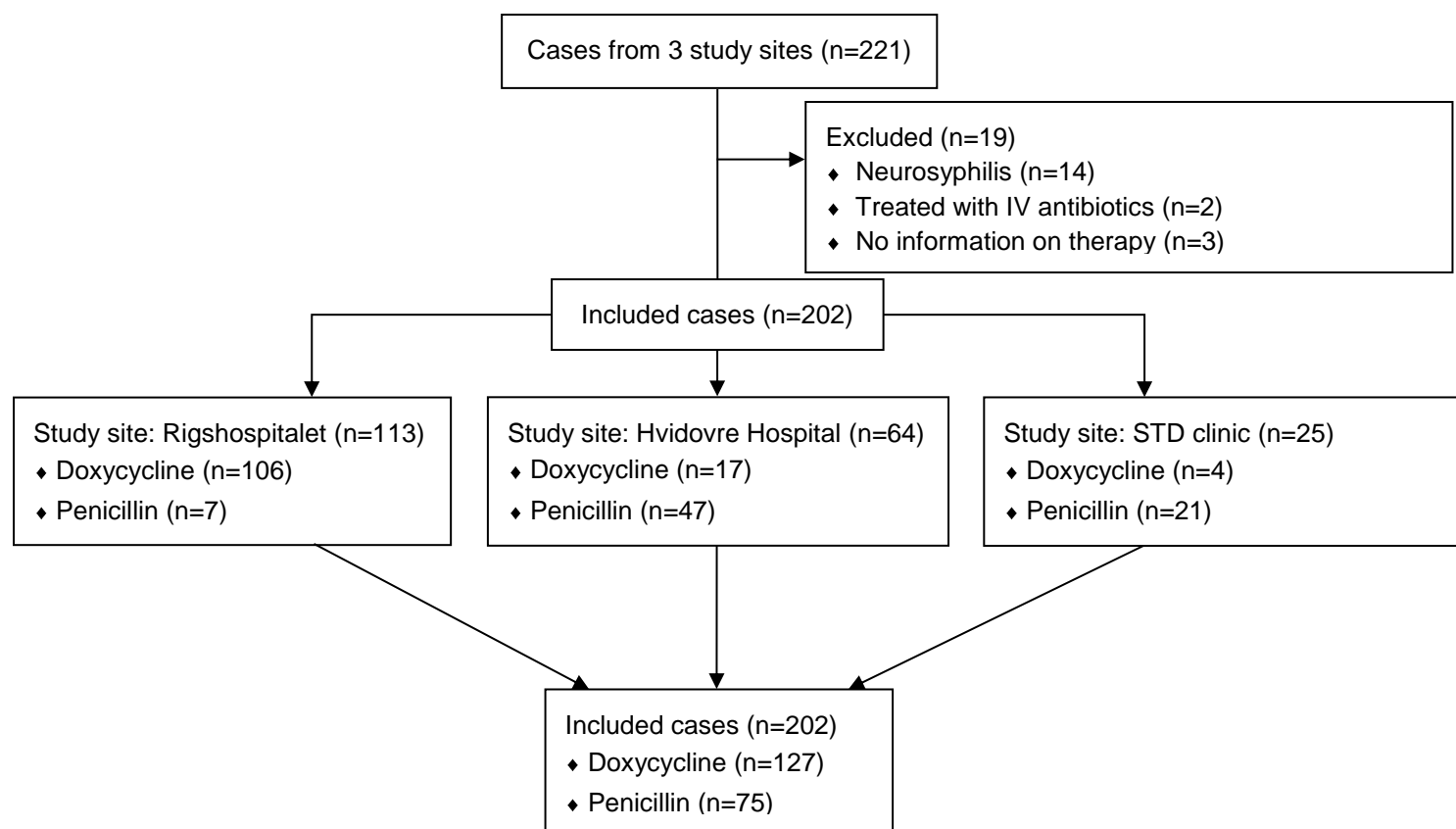
Serological Outcome	3 months		6 months		9 months		12 months	
	Doxy (n = 89)	PC (n = 58)	Doxy (n = 74)	PC (n = 45)	Doxy (n = 68)	PC (n = 39)	Doxy (n = 78)	PC (n = 48)
Cure	20 (22)	12 (21)	37 (50)	28 (62)	52 (76)	31 (79)	66 (85)	40 (83)
Failure	69 (78)	46 (79)	37 (50)	17 (38)	16 (24)	8 (21)	12 (15)	8 (17)

Data are presented as No. (%).

n Indicates number of cases with available serologic test results at each point in time.

Abbreviations: Doxy, doxycycline; PC, penicillin.

Figure 1. Flow diagram of inclusion of cases for comparison of serological treatment outcomes

Cases from 3 study sites (n=221)

Excluded (n=19)
- Neurosyphilis (n=14)
- Treated with IV antibiotics (n=2)
- No information on therapy (n=3)

Included cases (n=202)

Study site: Rigshospitalet (n=113)
- Doxycycline (n=106)
- Penicillin (n=7)

Study site: Hvidovre Hospital (n=64)
- Doxycycline (n=17)
- Penicillin (n=47)

Study site: STD clinic (n=25)
- Doxycycline (n=4)
- Penicillin (n=21)

Included cases (n=202)
- Doxycycline (n=127)
- Penicillin (n=75)

Abbreviations: IV, intravenous.

Molecular Typing of *Treponema pallidum* in Denmark: A Nationwide Study of Syphilis

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Keywords: *Treponema pallidum*; syphilis; HIV.

Running title: Molecular Typing of Syphilis in Denmark

ABSTRACT

Background. Syphilis, caused by *Treponema pallidum*, is increasingly frequent in Denmark and coinfection with HIV is common. This study aimed to determine the strain type diversity among patients diagnosed with syphilis by polymerase chain reaction (PCR) testing during a 4-year period in Denmark.

Methods. The study was performed as a nationwide study of *T. pallidum* positive genital ulcer specimens collected between May 2009 and December 2013. Epidemiological data on all patients were obtained from the Danish national syphilis registration system. Molecular strain typing was based on characterization of three variable treponemal genes, *arp, tpr* and *tp0548*.

Results. A total of 278 specimens from 269 patients were positive by *T. pallidum* PCR testing and 197 specimens were fully typeable with the 3-component strain typing system. Among the fully typeable specimens, 22 strain types were identified, with one type, 14d/g, accounting for 54% followed by 14d/f (18%) and 14l/g (5%). The majority (93%) of the patients reported acquiring syphilis in Denmark and the predominant profile was men having sex with men (86%). Among patients with concurrent HIV, nine full strain types were identified and we did not find a difference in strain type by HIV status (p = 0.197).

Conclusions. Strain type 14d/g is the predominant strain type in Denmark but a high degree of heterogeneity exists. The majority of the patients had acquired syphilis in Denmark and the imported cases of syphilis did not result in circulating clones. Furthermore, HIV-infected patients were diagnosed with a wide spectrum of different strain types.

INTRODUCTION

Treponema pallidum, the causative agent of syphilis, is a major cause of sexually transmitted infections. Although syphilis is mostly encountered among men who have sex with men (MSM) in western countries, the disease represents an extensive problem in the developing world [1]. During the last decade, Denmark has witnessed increasing rates of infectious syphilis, from almost eradicated in 1994 with 34 cases [2] to 352 cases in 2013 [3]. According to Danish law, all cases are registered in a nationwide database. Most reported cases in Denmark are among MSM, many of whom have concurrent human immunodeficiency virus (HIV) infection [3]. In an earlier study we demonstrated that almost 10% of Danish men with syphilis were diagnosed with HIV within the subsequent five years [4]. Of particular public health concern is the increased risk of transmission and acquisition of HIV during syphilis infection [5].

Molecular typing is a useful tool for understanding epidemics and molecular epidemiology can be used for preventing and controlling infectious diseases. Since 1998, *T. pallidum* has been characterized with the Center for Disease Control and Prevention's (CDC) molecular typing method based on two target genes, i.e. determination of the number of 60-base-pair (bp) repeats within the acidic repeat protein (*arp*) gene, and restriction fragment length polymorphism (RFLP) analysis of sequence differences in the *T. pallidum* repeat (*tpr*) subfamily II genes (*tprE, tprG and tprJ*) [6]. Recently, this typing system was supplemented with sequence analysis of a variable region of the *tp0548* gene [7]. The discriminatory capacity was markedly refined by combining the CDC subtype and the *tp0548* sequence type now yielding a strain type, using the nomenclature proposed by Marra *et al* [7].

Macrolide resistance has been investigated in several countries where azithromycin is used as a treatment alternative, showing rates ranging from very low in southern Africa [8] to very high rates of the resistance conferring A2058G mutation in the United States and Ireland [9-12] and 100% resistance in Shanghai [13]. In Denmark syphilis is treated with penicillin or doxycycline, and to date there have been no documented reports of treatment failures.

T. pallidum subtype diversity has been investigated in several countries [14-18]. Nevertheless, no data exist on the diversity of strain types at a national level. As genital ulcers facilitate HIV transmission it is of pivotal importance to elucidate the epidemiologic determinants underlying the high rates of syphilis. Our study aimed to determine the strain types among all Danish patients with syphilis diagnosed by polymerase chain reaction (PCR) testing of material from genital ulcers since the implementation of this method in Denmark in 2009, and four years ahead. Moreover, we linked the *T. pallidum* strain type with epidemiologic data (e.g. HIV status) from a nationwide database on syphilis.

MATERIALS AND METHODS

Setting

Denmark has a population of 5.6 million people [19] with an estimated HIV prevalence of 0.07% [20] in the adult population. The Danish health care system is tax-funded and patients suspected of having syphilis are primarily seen at specialized departments at hospitals or sexually transmitted disease (STD) clinics.

Specimens

PCR testing for *T. pallidum* is centralized at Statens Serum Institut where the method was implemented in 2009. The specimens in this study were taken for diagnostic purposes from patients with genital ulcers. We included 278 *T. pallidum* PCR positive ulcer specimens from 269 patients diagnosed with syphilis during May 2009–December 2013 in Denmark. Additional 50 specimens were *T. pallidum* PCR positive but due to insufficient material these were not subjected to strain typing and were excluded from the present study. The 3-component strain type was obtained by combining the *arp* size, the *tpr* RFLP pattern and the *tp0548* sequence type (for example 14d/f i.e., 14 *arp* repeats, the *tpr* E, G and J pattern "d" and the *tp0548* sequence type "f") [6, 7] (see Supplementary Material for details).

Data sources

Under Danish law, newly acquired syphilis, i.e. primary, secondary syphilis and early latent syphilis, are notifiable and the notification is done by the treating physician. The patients are registered in the Danish national syphilis registration system which is based at Statens Serum Institut and was established in 1993. The collected data include mode of infection, country of infection and result of prior HIV testing. The unique 10-digit central person registry (CPR) number assigned to all individuals in Denmark at birth or upon immigration was used to link data from the Danish national syphilis registration system to patient specimens. Exemption for review by the ethics committee system and for obtaining informed consent was obtained from the Committee on Biomedical Research Ethics for the Capital Region.

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher's exact test, where appropriate. The Mann-Whitney test was used for comparison of the bacterial load between typeable and non-typeable specimens. Differences with $p < .05$ (two-sided) were considered statistically significant. Data analysis was done using SPSS version 16.0 (SPSS Inc., Chicago, Il, USA).

RESULTS

From May 2009 through December 2013, 278 specimens positive by *T. pallidum* PCR were obtained from 269 patients (seven patients returned with >1 episode of syphilis during the study period). Most of the patients were men (94%), and when self-reported sexual orientation was stated, 206/239 (86%) of the men were MSM. The median age was 36 years (range, 15–71 years); other characteristics of the patients are summarized in Table 1. Of the 278 specimens, 197 (71%) were fully strain-typed by the *arp*, *tpr* and *tp0548* assays and 63 (23%) specimens were partially typeable with minimum one of the three assays (Table 2). Eighteen (6%) of the specimens were negative in all three assays. We found a statistically significant lower bacterial load in the non-typeable specimens ($p < 0.001$).

***T. pallidum* strain distribution**

A total of seven *arp* sizes (6, 7, 8, 11, 14, 15 and 16) and eight *tpr* RFLP patterns (b, d, e, f, j, k, l and p) were identified. Sequence analysis of the *tp0548* gene revealed four sequence types (c, d, f and g). By combining the three typing systems, we could establish 22 strain types. The most common strain type was 14d/g (54%) followed by 14d/f (18%) and 14l/g (5%). The remaining strain types were rare (Table 3).

Characteristics of patients with fully typeable specimens

Several strain types were detected in only one patient, indicating that they were imported cases. For example, only one patient had strain type 14d/c and this patient reported being infected in Pakistan. Further, only one patient had strain type 16d/d and this patient reported being infected in Brazil. Also, only two patients had strain type 6b/g and both reported being infected outside Denmark, in Thailand and Greenland, respectively. However, the majority of the patients reported being infected in Denmark and overall the clustering of the patients confirmed this. Among the patients reporting being infected in Denmark, 16 full strain types were identified (Table 3). Moreover, among the patients with full strain types, 10 of the clusters/singletons were constituted of MSM exclusively (Table 3). Women were represented in five different clusters. For example, in one cluster including three patients infected with strain type 14b/f, a transmission chain could be clearly demonstrated as two women were diagnosed with this uncommon strain type only a few weeks apart in the same region of Denmark.

Syphilis and HIV coinfection

Information on HIV status was available from 256/269 (95%) patients. Of these patients, 48/256 (19%) were HIV-infected at time of syphilis diagnosis. Only one was female (and non-Danish). Of the patients with full strain types, information on HIV status was available from 185/197 (94%) and 35/185 (19%) had concomitant HIV infection. The patients with concurrent HIV at time of syphilis diagnosis were all MSM and

presented nine different full strain types (Table 3). We did not find a difference in strain type by HIV status when analyzing specimens with full strain type (p = 0.197). In comparison, when looking at clusters containing only few patients, both HIV-infected and HIV-uninfected MSM were represented, indicating shared sexual networks (Table 3). For example strain type 6d/g was diagnosed in two MSM with different HIV status in the Copenhagen area in the same month.

Reinfection

Full strain type was identified in four patients with more than one episode of syphilis within the study period. One patient with concurrent HIV returned with a second syphilis episode after five months, both episodes with the uncommon 14f/g strain type, but the latter episode was acquired in Great Britain. Another patient had fully typeable specimens from three episodes of syphilis with 6-month-intervals; the very common strain type 14d/g was identified during the two first episodes while strain type 14l/g was identified during the latter episode. This patient had four episodes of primary syphilis within the study period. Moreover, a patient with concurrent HIV had two episodes of syphilis with an interval of two years; both infections were with the very common 14d/g strain type. Yet another patient had two episodes of syphilis with an interval of two years; again both infections were with the very common 14d/g strain type and the patient reported acquiring the infection in Denmark at both occasions.

Temporal distribution of strain types

Our specimens were collected over four years and the distribution of strains was investigated from 2010 to 2013 (Figure 1). For purpose of analysis, the specimens were divided into two groups of 24 months (2010–2011, 2012–2013). No significant change in the strain type distribution was identified over the two periods (p = 0.183).

DISCUSSION

The population-based design allowed us to study *T. pallidum* at a national level by linking strain type with epidemiologic data from patients with PCR-positive syphilis during a 4-year period. Of the 278 cases of infectious syphilis diagnosed by amplification of *T. pallidum* DNA in genital ulcer specimens, 71% of the specimens were fully typeable with both the *arp, tpr* and *tp0548* assays.

In this population 22 different strain types were identified. Others have suggested that settings where syphilis is endemic have higher levels of strain diversity [8, 18, 21] and the strain diversity in this study was surprisingly high. However, with the use of the 3-component strain typing system we did expect to demonstrate additional diversity. When applying the 2-component subtyping system to our specimens we

could only demonstrate 14 different subtypes which is in line with the discriminatory capacity demonstrated by Marra *et al* [7].

The most common strain type was 14d/g followed by 14d/f. Previous studies have shown that 14d is the predominate subtype in various geographic regions [6-8, 11, 14, 15, 22, 23] and 14d/g may be a more virulent or transmissible strain. It is interesting to note that the not-previously described strain type 6b/g was found in two patients who reported infection outside Denmark, i.e. Greenland and Thailand, respectively. In addition, the distribution of the patients who reported acquisition of syphilis outside Denmark showed that these imported cases did not result in circulating clones. However, since patients diagnosed with syphilis by serologic testing were not included in the study, we cannot rule out that more patients have become infected with imported strains. Of note, many of the uncommon strain types in this study have also been identified as subtypes in Scotland [22] indicating possible links across Europe. Finally, we identified three strain types that to our knowledge have not previously been reported (6b/g, 6d/g and 7f/g).

The majority of the patients were MSM and a few medium-sized clusters consisted of only MSM, indicating localized transmission networks. The marked increase in syphilis among MSM has mainly been attributed to increased sexual risk behavior in response to the improved effect of antiretroviral therapy on HIV [24, 25]. Rather than a general increase in sexual risk behavior among all MSM, the re-establishment of a risk-taking core group of MSM may have enabled a higher level of endemicity, causing continuous syphilis circulation [26]. In a previous study we investigated the risk of reinfection with syphilis and found that HIV-infected men had a markedly higher risk of reinfection with syphilis relative to HIV-uninfected men [4], indicating that the risk behavior that resulted in an HIV infection was continued after seroconversion. In the present study we did not find an association between HIV status and strain type, suggesting that HIV serosorting, i.e. trying to establish knowledge about HIV status concordance before practicing unprotected sex, was not practiced to any large degree. A study from London, UK also found no association between HIV status and either full *T. pallidum* subtype or type according to the *tp0548* sequence alone [23], further supporting this theory. Declining rates of HIV serosorting could also explain the declining rates of HIV coinfection among MSM who are diagnosed with syphilis; MSM who are syphilis and HIV coinfected have unprotected sex with HIV-uninfected MSM, but only transmit syphilis because they are effectively treated for their HIV infection [27].

The proportion of concurrent HIV in our study was lower than the 32% recently reported among MSM diagnosed with syphilis in Denmark [3]. This is probably reflecting that patients with HIV are screened for

syphilis yearly. Consequently, patients with concurrent HIV are overrepresented among patients diagnosed by serological testing compared to the patients diagnosed by PCR testing of material from genital lesions comprising this study.

Our data did not provide evidence of occurrence of treatment failures in Denmark, and even though several patients had more than one episode of syphilis with the very common 14d/g strain type, we assume each new chancre represents a reinfection and not a treatment failure. However, because patients are also diagnosed by serologic testing and because patients would not necessarily present with genital ulcers in case of relapse due to treatment failure, we cannot definitively conclude that treatment failures do not occur in Denmark.

We did not detect a shift in the strain type distribution during the study. However, four years is a relatively short period when investigating a temporal distribution.

The strengths of our study are the nationwide design, including all Danish patients with PCR-positive syphilis within the study period, and the epidemiological data on all patients. Further, the study was based in a setting where all health-care services are publicly funded and this universal access is probably resulting in fewer undiagnosed cases. Moreover, many studies investigating *T. pallidum* have used only two typing methods. Since the 3-component strain type system was introduced, the discriminatory capacity has been markedly improved and we applied this enhanced typing system.

This study has certain limitations. Firstly, the study only included patients diagnosed with PCR-positive genital lesions. Consequently, as two thirds of Danish syphilis patients are diagnosed by serological testing at later stages [28] our results might be biased. This is accentuated by the fact that more HIV-infected patients are diagnosed with syphilis by serological testing at their yearly screening. However, our patient characteristics (e.g. age and sexual orientation) were very similar to Danish syphilis patients diagnosed through an 11-year period in a study including both patients diagnosed by serologic and PCR testing [4]. Certainly, when investigating transmission chains from imported cases, inclusion of all syphilis patients, and not just the minority diagnosed by PCR testing, would have been preferable. Secondly, a relative high number of specimens were not fully typeable. This could be caused by DNA degradation during storage of the specimens. The specimens were collected during a 4-year period, resulting in some specimens having been stored for up to three years. Others have encountered the same problem, and Muller *et al* demonstrated that only 85% of *T. pallidum*-positive specimens were positive after re-testing stored specimens [8]. In our study the non-typeable specimens had significantly lower bacterial load in the

diagnostic PCR and our success rate above 70% is comparable to others using the 3-component system with success rates of 41%, 63% and 77%, respectively [23, 15, 22].

The current method of choice for *T. pallidum* typing, consisting of three components, is time-consuming and is not applicable for routine testing. A recent study compared the CDC typing system with sequenced-based molecular typing in a group of patients with two or more parallel specimens (i.e. taken at the same time). The study found differences in treponemal genotypes detected in whole blood and swab specimens, suggesting important differences between compartments. In the majority of the patients they found discrepancies within the *arp* and *tpr* loci using the CDC typing system [29]. However, under experimental conditions, Pillay *et al* showed that the CDC subtype was stable with repeated rabbit passages of the Nichols strain [6]. This was confirmed by Marra *et al*, who additionally demonstrated that neither the Sea 81-4 nor the Chicago C strain changed with repeated rabbit passages [7]. Whether this inconsistency is caused by differences between human infections and experimental infections of rabbits or is due to the fact that skin and blood represent two immunologically distinct compartments has yet to be explored. Further studies on advanced high-throughput technologies are highly needed before molecular typing can be implemented in routine STD surveillance and a sequenced-based typing system with promising results has recently been proposed [16, 29, 30].

To our knowledge there have been no other studies investigating the *T. pallidum* strain type diversity at a national level. The molecular typing methods combined with epidemiological data shed some light on the current syphilis endemicity in Denmark. However, the picture remains complex, especially the interaction with HIV infection. In summary, the strain diversity was surprisingly high in Denmark and HIV-infected patients were diagnosed with a wide spectrum of different strain types.

Potential conflicts of interest.

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TABLES

Table 1. Main characteristics of 269 patients diagnosed with syphilis by PCR testing in Denmark in the period 2009–2013

Characteristic	n	%
Female	15/269	6
Male	254/269	94
MSM	206/239[a]	86
HIV+	48/256[a]	19
Danish citizen	235/260[a]	90
Infected in Denmark	207/222[a]	93

Abbreviation: MSM, men who have sex with men.

[a] Patients with missing data are excluded from denominator.

Table 2. Proportion of typeable specimens in 278 *T. pallidum* positive specimens using the *arp, tpr* and *tp0548* assays

| Assay	N	%
Arp	238	86
Tpr	223	80
tp0548	241	87

Table 3. Strain type distribution of 197 *T. pallidum* positive specimens typeable by the *arp, tpr* and *tp0548* assays and number of HIV-infected patients, number of MSM based on self-reported sexual orientation and number of patients infected in Denmark in each strain type

	Strain type	HIV	MSM	Denmark
6b/g	2 (1.0)	0 (0)	1 (50)	0 (0)
6d/g	2 (1.0)	1 (50)	2 (100)	2 (100)
7f/g	1 (0.5)	0 (0)	1 (100)	1 (100)
8d/f	1 (0.5)	0 (0)	1 (100)	1 (100)
14b/f	3 (1.5)	0 (0)	1 (33)	3 (100)
14b/g	7 (3.6)	2 (29)	7 (100)	6 (86)$^{M=1}$
14d/c	1 (0.5)	0 (0)	0 (0)	0 (0)
14d/d	1 (0.5)	$^{M=1}$	$^{M=1}$	$^{M=1}$
14d/f	35 (17.8)	4 (11)$^{M=2}$	25 (71)$^{M=1}$	23 (66)$^{M=8}$
14d/g	107 (54.3)	18 (17)$^{M=6}$	87 (81)$^{M=8}$	87 (81)$^{M=18}$
14e/g	4 (2.0)	0 (0)	2 (50)$^{M=1}$	3 (75)$^{M=1}$
14f/f	3 (1.5)	0 (0)	2 (67)	3 (100)
14f/g	7 (3.6)	3 (43)$^{M=1}$	7 (100)	6 (86)
14j/g	3 (1.5)	0 (0)$^{M=1}$	2 (67)	2 (67)$^{M=1}$
14k/f	1 (0.5)	1 (100)	1 (100)	1 (100)
14k/g	2 (1.0)	2 (100)	2 (100)	2 (100)
14l/c	1 (0.5)	$^{M=1}$	$^{M=1}$	$^{M=1}$
14l/f	1 (0.5)	1 (100)	1 (100)	1 (100)
14l/g	10 (5.1)	3 (30)	10 (100)	6 (60)$^{M=3}$
14p/g	3 (1.5)	0 (0)	3 (100)	3 (100)
15d/d	1 (0.5)	0 (0)	$^{M=1}$	$^{M=1}$
16d/d	1 (0.5)	0 (0)	0 (0)	0 (0)

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: MSM, men who have sex with men; M, number of patients with missing data.

Figure 1. *Treponema pallidum* **strain type distribution in the periods 2010–2011 and 2012–2013.**
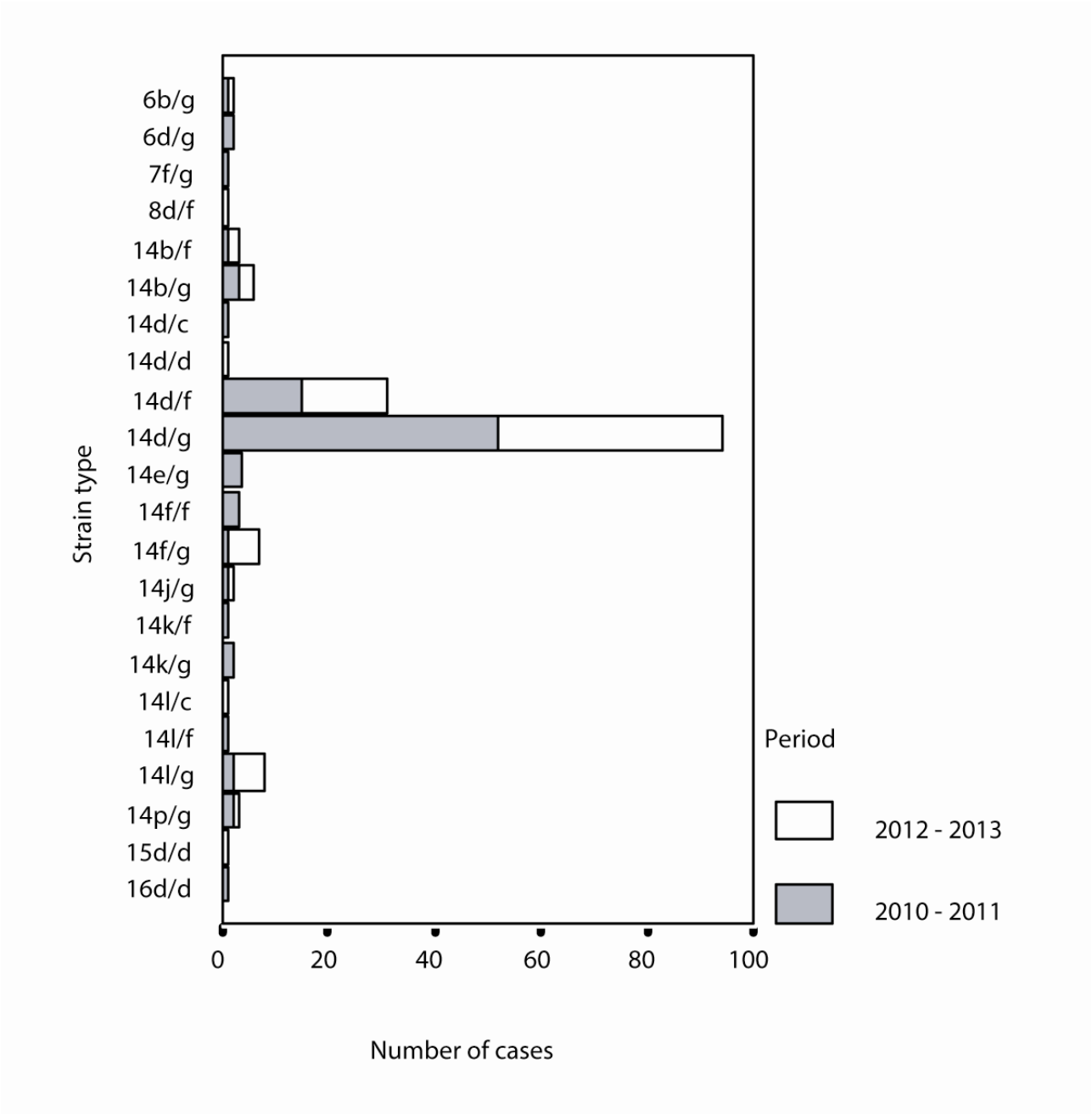
SUPPLEMENTARY MATERIAL

Laboratory methods

Specimens were collected in UTM tubes and DNA was extracted using BT Chelex 100 Resin (Bio-Rad Laboratories Inc., CA, USA).The specimens were tested for the presence of *T. pallidum* using two real-time diagnostic PCR assays with internal amplification controls, amplifying segments of the *flaA* [1] and *polA* [2] genes of *T. pallidum*. Subsequently, the specimens were stored at –20˚C until they were subjected to strain typing. PCR was used to determine the number of 60-bp repeats within the *arp* gene as previously described [3], but with primer modifications as described by Katz *et al* [4]. In brief, five microliters of DNA was amplified in a 100 µL reaction, using 2 U Taq DNA polymerase (Platinum Taq, Invitrogen), 2.5 mmol/L dUTP, 20 µM primers, 50 mmol/L MgCl₂ and 10X PCR Rxn buffer (Invitrogen). We used the primers ARP N1 (forward) and ARP N2 (reverse) [4]. Cycling conditions, including a touchdown step, consisted of an initial cycle of 94˚C for 2 minutes, followed by 10 cycles of 94˚C for 30 seconds; 74˚C for 45 seconds decreasing 1˚C per cycle, and 72˚C for 90 seconds. The next step consisted of 35 cycles of 94˚C for 30 seconds; 64˚C for 45 seconds, and 72˚C for 90 seconds. Final extension was achieved at 72˚C for 10 minutes. The amplification yielded different amplicon sizes depending on the different number of 60-bp repeats within the *arp* gene. The number of 60-bp repeats was estimated using plasmids with a known number of repeats as reference, kindly provided by Bess Charmie Godornes from the University of Washington, Seattle, USA. Further, specimens were compared to the Nichols strain of *T. pallidum* with the known amplicon size of 1155 bp, corresponding to 14 repeats.

A nested PCR was used to analyze *tpr*. In brief, ten microliters of DNA was amplified in a 100 µL reaction, using 2 U Taq DNA polymerase (Platinum Taq, Invitrogen), 1.25 mmol/L dNTP, 20 µM primers, 50 mmol/L MgCl₂ and 10X PCR Rxn buffer (Invitrogen). A first-round was performed using primers 5'CAGGTTTTGCCGTTAAGC3' (forward) and 5'AATCAAGGGAGAATACCGTC3' (reverse) as previously described [5]. We used the following cycling conditions, including a touchdown step: an initial cycle of 95˚C for 2 minutes was followed by 10 cycles of 95˚C for 15 seconds; 70˚C for 15 seconds decreasing 1˚C per cycle, and 72˚C for 2 minutes. The next step consisted of 40 cycles of 95˚C for 15 seconds; 60˚C for 15 seconds, and 72˚C for 2 minutes. Final extension was achieved at 72˚C for 7 minutes. A second-round was performed using primers 5'CTGTTATGGGGCCTA CC3' (forward) and 5' CTCTATGAGACTGGCTGAAA3' (reverse). One microliter template was amplified in a 50 µL reaction, using 1 U Immolase, 1.25 mmol/L dNTP, 20 µM primers, 50 mmol/L MgCl₂ and Immolase buffer. The following cycling conditions were used for the second round: an initial cycle of 95˚C for 10 minutes was followed by 15 cycles of 94˚C for 30 seconds; 50˚C for 15 seconds, and 72˚C for 90 seconds. Amplicons from the second PCR were digested with

the restriction endonuclease MseI. The digestion products were separated by gel electrophoresis and the patterns were compared with published data [3, 6].

Sequence analysis of an 84-bp region of the *tp0548* gene was performed as described previously by Marra *et al* [5] with some modifications. In brief, five microliters of DNA was amplified in a 100 µL reaction, using 2 U Taq DNA polymerase (Platinum Taq, Invitrogen), 1.25 mmol/L dNTP, 20 µM primers, 50 mmol/L $MgCl_2$ and 10X PCR Rxn buffer (Invitrogen). We used the primers 5'GCGTGGTGGTGAGTTCTTCT3' (forward) and 5'CGTTTCGGTGTGTGAGTCAT3' (reverse). An initial cycle of 94˚C for 2 minutes was followed by a touchdown step, consisting of 10 cycles of 94˚C for 15 seconds; 65 ˚C for 15 seconds decreasing 1˚C per cycle, and 72˚C for 30 seconds. The next step consisted of 35 cycles of 94˚C for 15 seconds; 55˚C for 15 seconds, and 72˚C for 30 seconds. Final extension was achieved at 72˚C for 10 minutes. The PCR products were sequenced using standard methods. *T. pallidum* positive controls and negative water were used in all PCR assays. Specimens that initially gave negative results were repeated with the relevant assay.

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