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Syphilis and HIV co-infection in Dublin; strategies to enhance diagnosis, investigation and management

By

Eavan G. Muldoon, MBBChBAO (Hons) BMed.Sc., MRCPI

Department of Genitourinary Medicine and Infectious Diseases,

St James's Hospital, Dublin.

A thesis submitted to Trinity College,

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Under the supervision of Prof. Fiona Mulcahy

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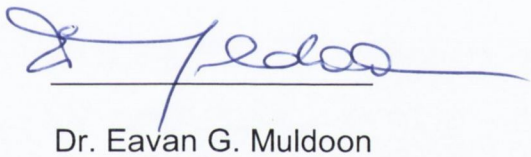

Dr. Eavan G. Muldoon

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Syphilis; consequences and implications in delayed diagnosis. Five cases of secondary syphilis presenting with ocular symptoms.
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Sex Transm Infect. 2010 Dec;86(7):512-3. Epub 2010 Nov 8

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Azithromycin Resistance in *Treponema pallidum* in Dublin, Ireland- Little Change in Six Years.
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50th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 2010

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Muldoon, E. Mulcahy, F.
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Fitzgerald, M. Owens, M. Courtney, G. Squance, S. Lyons, F. Clarke, S. Keating, S. O'Hora, A. Hickey, L. **Muldoon, E.** Mulcahy, F.

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HIV and syphilis Co-Infection; Emerging Trends Post Epidemic.

Muldoon, E. G. Kelleher, M. Squance, S. Lyons, F. Mulcahy, F. Bergin, C

16th Conference on Retroviruses and Opportunistic Infections, February 2009.

Abbreviations

ART	Anti-retroviral therapy
BASSH	British Association for Sexual Health and HIV
CDC	Centers for Disease Control
CI	Confidence Interval
CNS	Central nervous system
CROI	Conference on Retroviruses and Opportunistic Infections
CSF	Cerebrospinal fluid
ddNTP	dideoxynucleotide triphosphate
DFA-TP	Direct fluorescent antibody testing
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetc acid
EIA	Enzyme Immunoassay
FAM	6-carboxyfluoroscein dye
FTA-ABS	Fluorescent treponemal antibody absorbed
GMHS	Gay Men's Health Service
GP	General Practitioner
GUIDE	Genitourinary Medicine and Infectious Diseases
GUM	Genitourinary Medicine
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
hpf	High power field
HSE	Health Service Executive
HSV	Herpes Simplex Virus
IC	Internal control
ID	Infectious Diseases
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JH	Jarish-Herxheimer
kDa	Kilo Dalton
LGBT	Lesbian, gay, bisexual & transgender
LP	Lumbar puncture

MHA-TP	Microhaemagglutination assay for *T. pallidum*
MS	Microsoft
MSM	Men who have sex with men
MU	Mega units
NFQ	Non-fluorescent quencher
NFW	Nuclease free water
ORF	Open reading frames
PCR	Polymerase chain reaction
PHV	Phocine Herpes Virus
polA	DNA polymerase I gene of *T. pallidum*
RCC	Red cell Count
RNA	Ribonucleic acid
RPM	Rotations per minute
RPR	Rapid plasma reagin
SAKA	Syphilis Awareness Knowledge and Action
SpR	Specialist Registrar
STD	Sexually transmitted disease
STI	Sexually transmitted infection
TPPA	*T. pallidum* particle agglutination
Tpr	*T. pallidum* repeat
TSS	Tuskegee Syphilis Study
UK	United Kingdom
US (USA)	United States (of America)
UV	Ultra violet
VA	Visual acuity
VDRL	Venereal disease research laboratory
VL	Viral load
WCC	White Cell Count
WHO	World Health Organisation

Summary

Following historically low levels of reported cases of syphilis in Dublin in the 1990s there was a large outbreak of syphilis reported in 2001. Numerous interventions were implemented and the rates again decreased in 2003. In 2008 a cluster of five patients with ocular syphilis presented in a short time period. This prompted a review of the number of cases of syphilis presenting to the Department of Genitourinary Medicine and Infectious Diseases in St. James's Hospital.

There has been a significant increase in the number of cases of syphilis diagnosed and treated since 2007. The proportional increase was 118% from 2007 to 2009. The mean age of patients presenting is 35 years and the vast majority are men who have sex with men. A worrying trend has been the increase in the number of patients co-infected with Human Immunodeficiency Virus (HIV). In the outbreak in Dublin in 2001-2003, 17.5% of cases of syphilis occurred in patients co-infected with HIV. During 2007- 2009, 28.7% of cases occurred in HIV positive individuals. Equally worrying is the high rate of re-infection of syphilis and the fact that the majority of cases of re-infection are occurring in HIV positive patients.

Paramount to controlling the spread of syphilis infection is identifying cases and providing quick, effective and tolerable therapy. Education of both patients at risk and practitioners in contact with such patients is invaluable. International guidelines on the management of syphilis differ due to the relative paucity of large clinical trials. This leads to varying practice amongst

Infectious Disease specialists. A survey of such specialists in Ireland demonstrates differing practice and the need for Irish national guidelines to standardise practice. Many patients are delayed in their diagnosis of secondary syphilis, and efforts to highlight it's resurgence to non-infectious disease specialists are required. Knowledge of syphilis transmission, among STI clinic attendees is poor particularly among certain groups and public education campaigns are needed.

The diagnosis of syphilis can be problematic given that serology, an indirect diagnostic method is often relied upon. The utility of syphilis IgM is reviewed and the use of newer molecular techniques such as polymerase chain reaction (PCR) is studied. Both of these methods are promising, particularly in cases of primary syphilis when darkground microscopy is not available.

In 2004, the A2058G mutation that is associated with azithromycin resistance was described. At that time, Dublin had the highest proportion of samples found to have azithromycin resistance (88%, n=17). The rates of *T. pallidum* resistance to azithromycin were unknown. Molecular sequencing was performed on isolates of *T. pallidum* from the GUIDE clinic. 93% were found to contain the A2058G mutation.

The role of lumbar puncture in the management of HIV positive patients with syphilis is controversial. The role of follow up LP in patients diagnosed and treated previously and those newly diagnosed with syphilis is studied and discussed.

I presume there are very few mortals in Europe who are not in danger of waking some morning and finding themselves syphilitic

James Joyce

Chapter 1 – Introduction

1.1 - General Introduction

Syphilis is a disease that has had a great effect on human history. Medical historians have speculated that many notable historical figures were infected with syphilis. Literary genii, Shakespeare[1], Wilde and Joyce[2] may have been infected. Joyce writing in 1906 stated "I presume there are very few mortals in Europe who are not in danger of waking some morning and finding themselves syphilitic" [2]. Composers Schubert, Beethoven and Schumann may also have been infected. Schumann upon his admission to an asylum stated "In 1831 I was syphilitic and treated with arsenic"[2].

The list could go on, but it has not just been those in the arts whose potential syphilis infection has been speculated upon, what of those in power and influence? Some have postulated that Abraham Lincoln the 16th president of the United States, whose term in office coincided with the American civil war and the end of slavery, may have been infected. There has even been speculation that Adolf Hitler was infected with syphilis, rumours at the time circulated that he may have been infected by a Jewish prostitute in Vienna in 1908. He seemed to take the disease seriously- writing in "Mien Kampf" in 1923 he dedicated thirteen pages to the subject, regularly referring to it as the "Jewish Disease". "Combating syphilis," he wrote, "should have been made to appear as the task of the nation. Not just one more task...Everything- future or ruin- depends upon the solution to this question". He believed that the disease was hereditary, despite the fact that German researchers Schauddin and Hoffmann had identified the treponeme as the causative agent in 1905[3]. Hitler also hired a renowned syphilologist, Dr Theodor Morell as his personal

physician. Others have however disputed the theory that Hitler had syphilis, opting instead to suggest bipolar affective disorder, amphetamine abuse or progressive post traumatic stress disorder as possible explanations for his bahaviour[4, 5]. The art of retrospective diagnosis is difficult [6] and can lead to much debate[7], particularly in the case of syphilis with it's protean manifestations.

Much debate has surrounded the origins of syphilis. Three main hypotheses have been debated; the Columbian hypothesis, that syphilis was a disease of the new world introduced to Europe after the return of Columbus, the pre-Columbian hypothesis, that syphilis was a mutation of a treponeme already present in Europe and a third argument that syphilis was transported from the old to the new world[8].

In an address to the Osler Club, London In April 1958, Prof J. F. D. Shrewsbury put forward the argument that syphilis was a mutation of treponemes that had previously caused milder disease such as sibbens in Scotland[9]. Sibbens would seem to be related clinically to yaws[10], it's name derives from the Gaelic for raspberry- suibbean due to it's clinical appearance. Lesions were first seen in the mouth, ulceration followed, the skin lesions, which were blue-red, dry and crusted often gave rise to a spongy substance, similar in appearance to a raspberry. It was seen more frequently in lower socioeconomic groups and was thought to be spread by sharing eating utensils. It was thought to have been introduced into Scotland by Cromwell's army and the last recorded case was in Banff in 1851. A similar disease was

described in Ireland in the 1820s and 30s and called "button scurvy" by Corrigan[11]. Proponents of this theory set forth arguments that authors of ancient times described genital lesions typical of syphilis, that ancient Greek writers described aneurysms typical of syphilitic aneurysms and that the bones from bodies discovered in prehistoric graves had changes suggestive of syphilis[9]. In addition, according to evidence from Columbus himself, his crew were in excellent health, which would argue against the sailors having been infected in Hispaniola.

The counter argument is that the findings described by ancient writers are suggestive of syphilis and that syphilis is only one of multiple differential diagnoses that may have been responsible. Harrison writing in 1959, also reasoned that if syphilis had been endemic prior to Columbus' return from the New World then surely lecherous royals such as King Henry II who was known for consorting with "low class ladies of easy virtue" would not have had eight children none of whom died in infancy and seven of whom survived to adulthood[9]. Doubt is also cast on Columbus' honesty, as he reported that the first voyage took thirty-three days when in fact it took seventy-one. There is also evidence that the disease was present in Hispaniola as was well recognised by the native population from the eye witness accounts of Oviedo and Las Casas[9]. Both were there before the end of the 15th century and wrote of their experiences, noting that the disease took a much milder form in the natives than the Spaniards. Las Casas wrote, " I took the trouble upon several occasions to interrogate the Indians of this island as to whether the disease was of great antiquity, and they answered "Yes, that it dated from a period

long before the advent of the Christians, the origin of it being beyond the memory of any man, and nobody can disbelieve this".

The use of bone changes to answer the conundrum of the origins of syphilis is addressed by Rotchschild[8]. Treponemal disease leaves an osseous signature, marked by periosteal reaction, tibial remodelling (sabre shin formation) and sometimes bony destruction or gummas[12]. Traditionally the problem arose that examining bones for periosteal reaction and distinguishing it from post mortem damage had poor reproducibility due to inter-observer variation. Rothschild developed a macroscopic technique with good reproducibility and based on this demonstrates that no remains from the pre-Columbian era have bony changes consistent with syphilis. In contrast examination of 536 skeletal remains from The Dominican Republic, an area where Columbus would have visited, demonstrated periosteal reaction consistent with treponemal disease in 6-14% of skeletons[13]. In the UK and Ireland, however cases from the 13th century AD show a high frequency of polyostotic periosteal reaction classic of yaws.

A commonly held view proposes that treponemal disease originated in Africa as yaws, passed through Asia to North America. In Asia a mutation occurred to produce bejel and this disease also passed into North America. In North America, a further mutation occurred to form syphilis. This may have occurred sometime between 2000 and 1800 years ago as this is when the first identified osteotype of syphilis has been identified[8]. It is curious that continental Europe stayed free of treponemal disease until the late 1400s.

The presence of yaws in Ireland and Britain is thought to be related to the Slave trade from West Africa[8].

Newer research based on the phylogenetics of syphilis proposes a different origin. This is based on the observations of a Canadian group working with an isolated Akwio tribe in Guyana. They observed that 5% of children were infected with a treponemal disease that appeared clinically to be a combination of syphilis and yaws[14, 15]. The hypothesis is that the older treponemal disease identified in the Aikwio tribe was spread easily through skin contact as the tribes people did not wear a large amount of clothing. With the arrival of the Europeans who were heavily clad, the disease had to adapt to continue transmission to new human hosts. The phylogenetic analysis sheds light on the relative order in which the *T. pallidum* subspecies emerged[16]. *T. pertenue* (which causes yaws) was found to occupy the basal part of the phylogenetic tree, thus the oldest of the pathogenic treponemes. *T. endemicum* (endemic syphilis or bejel) diverged from *T. pertenue* stains at a later date, and *T. pallidum* strains emerged most recently.

The actual designation syphilis originates in an ancient myth about a Shepard named Syphilis, which was re-interpreted in 1530 by Venetian physician and poet, Girolamo Fracastoro, in his epic poem "*Syphilis sive morbus gallicus*"[17]. Due to the associated rash and to differentiate syphilis from small pox it was also termed "the great pox". As human nature dictates many groups were anxious to blame anyone but themselves, resulting in syphilis being called "the most disowned infection in history"[18]. The Germans and English called it

"the French pox", the Russians "the Polish sickness", the Poles "the German sickness", the French "the Neapolitan sickness" and the Flemish, Dutch, Portuguese and North Africans "the Spanish or Castilian sickness"[8]. Whatever the origins of syphilis, there can be no mistaking the devastation that occurred during the epidemic in Europe, which started in the late 1400s. Some have maintained that these cases may have been new or misdiagnosed leprosy. This seems unlikely given that Milanese physicians were attributing some deaths to leprosy since 1452 and others to "the French disease" after 1503 [6].

This epidemic, like others was helped by war[1, 6]. In 1494, Charles VIII of France invaded Italy; Naples was held by a Spanish garrison who may have practiced an early form of biological warfare. According to anatomist, Gabriel Fallopius the defenders "drove their harlots and women out of the citadel, and especially the beautiful ones, whom they knew to be suffering from the infectious disease". Naples fell without a fight on February 22nd 1495, but in a few months Charles' army was a debauched diseased rabble[1]. When Charles' army dispersed in July 1495, the mercenaries are said to have ignited the European pandemic. Syphilis had arrived in India with Vasco de Gama in 1498 and in Japan by 1512.

The impact of the syphilis epidemic across Europe was huge. In Elizabethan England, London was particularly affected. The poor often opted for suicide rather than the painful treatment available, and workhouses could not fulfil their intended function as so many of the poor were disabled by the pox [6]. Records from St. Bartholomew's Hospital in London from 1549 show that 24%

of the patients were syphilitic, while in 1579 London physician William Clowes claimed that up to 75% of the patients had the French pox [1]. The treatment that was available during the Elizabethan era was based on the observation by Spanish physician Ruy Diaz de Isla in 1539 that high fever often arrested the progress of syphilis. Hot baths, mercury and a Spartan diet were the mainstay of treatment.

> Be a whore still: they love not that use thee;
> Give them diseases, leaving with thee their lust.
> Make use of thy salt hours: season the slaves
> For tubs and baths; bring down rose-cheeked youth
> To the tub-fast and the diet[19].
>
> William Shakespeare

Interestingly it has been postulated that the reason that late neurosyphilis was not described until the late 18th century is due to frequent epidemics in Europe which may have caused fever associated treponemal lysis[1].

A belief that syphilis and gonorrhoea were manifestations of the same disease persisted from the Elizabethan times for some centuries thereafter. This misconception was not helped by Scottish Surgeon John Hunter's somewhat brave and foolish experiment in 1767 when he inoculated himself with pus from a patient with gonorrhoea who also happened to be co-infected with syphilis. However, it has been argued that perhaps the fact that he inoculated

himself is medical fokelore[20], he did however describe a patient who developed a chancre following the inoculation of urethral pus.

By the turn of the 20th century it was estimated that between 5-20% of the population in Europe and the USA had syphilis or would be infected with syphilis during their lifetime[21].

In 1910 Ehrlich developed the organic salvansan, which claimed to clear a chancre or rash of syphilis, and subsequently was used in combination with mercury or bismuth. Julius Wagner Jauregg an Austrian psychiatrist was awarded the noble prize in Medicine in 1927[21], for the use of vivax malaria therapy in the treatment of syphilis, despite the fact that it had an associated mortality of 9%. Wagner-Jauregg's experiments were problematic, initially he used erysipelas to induce fever, but this treatment was complicated by infection of other patients. The vivax experiments themselves were complicated, in 1914 he accidentally inoculated 4 patients with falciparum malaria, 3 of whom died.

Syphilis had a significant impact on the morbidity and mortality until the discovery of penicillin. Autopsy data on patients with aortic aneurysms published in 1955[22] collected from 1892 to 1953, showed that syphilis was the aetiology in 53.7% of cases. Interestingly when the authors compared the first 100 cases, which were seen in the pre-antibiotic era to the last 100 cases from the antibiotic era the proportion of aneurysms attributed to syphilis infection fell from 77% to 49%.

Large-scale public health awareness programmes were put in place in the 1940s in an attempt to decrease the complications of late syphilis infection. In the United States, the federal government established "rapid treatment centers" in 1943[23] these were inpatient facilities providing 5 to 10 day arsenical therapy (image 1.1). With the advent of penicillin therapy, treatment became more successful and the incidence of congenital syphilis declined by 90% between 1941 and 1972. As complications decreased, the focus shifted to interrupting transmission and the modern concept of contract tracing evolved. In 1999 the Centers for Disease Control and Prevention (CDC) in the United States announced a campaign to eliminate syphilis transmission by 2005[24, 25].

These US public awareness programmes were in stark contrast to one of the most controversial episodes in the history of Syphilis. The U.S. Public Health Service Syphilis Study at Tuskegee, or the Tuskegee Syphilis Study (TSS) as it is more commonly known was a study of untreated syphilis in nearly 400 African American men from 1932-1972 who were observed to autopsy[26]. The withholding of effective penicillin therapy when available, and the way in which information was withheld from patients has been particularly criticised. A legacy of mistrust amongst African American patients is attributed to the TSS[27] leading to a presidential apology by President Bill Clinton in 1997[28].

Image 1.1- United States Public Health Campaign poster 1940's

(http://www.nlm.nih.gov/exhibition/iconographyofcontagion)

Unlike syphilis, the other treponemal diseases have seen a significant decline. It is unsure if the disease pinta still exists, cases were reported in the 1970s[16] and none had been reported again until 1999 when a case of an Austrian woman who had been living in Cuba, a previously endemic area, was reported[29]. Endemic syphilis or bejel was eradicated from Bosnia where it was once endemic[30] and one infected family has been reported in Turkey in the last forty years[31]. Yaws was the target of a global campaign supported by WHO and UNICEF in 46 countries during the years 1952 to 1964. More than 50 million treatments were administered during this time and as a result the prevalence fell by 95%[32]. Cases are still reported particularly in africa[33].

Some of the difficulty that arises in the genetic study of the treponemal diseases is that there is little genetic variability between the species. It is suggested that the most variation between the species pallidum and pertenue lies in the tpr gene family[16]. This gene family is made up of twelve genes and accounts for only 2% of the genome.

The discovery of *Treponema pallidum* as the causative agent of syphilis was first published in April 1905 by Schauddin and Hoffmann. They demonstrated spirocheta pallida as it was then known from the exudates of a papule from the right labia of a 25-year-old woman diagnosed with secondary syphilis who had not yet received any treatment. Fritz Schauddin was the Director of the Protozoon Laboratory of the Kaiserlichen Gesundheitsamtes and Erich Hoffman an honorary lecturer and researcher in the Berlin syphilis clinic. They demonstrated the organism using a modification of a Giemsa azur-eosin stain

and commented on the fact that the material needed to be fresh for examination. They also published two photographs of the spirochetes along with their work[3]. Schauddin died at the age of 34 in 1906 and never lived to see the implications of his work, Hoffmann lived until 1959 and continued to contribute to syphilis reseach[34].

Given the difficulty in culturing *T. pallidum,* the advent of genomic research opened potential research avenues. Molecular work using pulsed gel electrophoresis had shown *T. pallidum* to have a circular chromosome of 1,000 kilo base pairs, making it one of the smallest prokaryotic genomes[35]. The genome of the spirochete was sequenced by Fraser *et al*[36] in 1998. Their work demonstrated a relatively small genome at 1,138,006 base pairs and identified unique and common genes when they related it to another pathogenic spirochete, *Borrelia burgdorferi.* The genome of syphilis was found to be a circular chromosome, 1,138,006 base pairs with an average G and C content of 52.8%. It has a total of 1041 predicted open reading frames (ORFs) which represent 92.9% of it's total genomic DNA. The average size of the predicted proteins encoded for have values similar to those observed in other bacterial species. As syphilis is an obligate human parasite relying on it's host for multiple nutrients, 5% of it's ORFs encode 18 distinct transporters with predicted specificity for amino acids, carbohydrates and cations. Although the transport systems in syphilis have a similar specificity to those found in *B. burgdorferi* and *M. genitalium,* there are many differences, for example the transporter for glutamate/aspartate is most similar to mammalian glutamate transporters. Syphilis also lacks basic heat shock proteins, which may be the

reason for it's thermal sensitivity. Motility associated genes are highly conserved, in contrast to most bacteria which only have one core protein in periplasmic flagellar filaments, syphilis has 3 core proteins and a sheath protein. The virulence factors were thought to be a large family of duplicated genes (tpr A-L) that encoded putative membrane proteins that were reminiscent of a 32-member gene family encoding the outer membrane proteins in *H. pylori*. Many of the genes sequenced are unique to syphilis and of unknown function.

1.1.1 - Syphilis in Ireland

In Dublin Dr. Steeven's Hospital opened in 1733 to provide treatment for venereal disease with mercurial and arsenical compounds followed closely by clinics in Rainsfort Street (1755) and Kings Street (1758)[37]. The high prevalence of syphilis across Europe had an effect on the admissions of mental health institutions. In Sainte Anne asylum Paris, 30.5% of voluntary and 17.4% of involuntary male admissions between 1876 and 1914 were due to general paralysis of the insane; a manifestation of late neurosyphilis. The extent of the Irish problem was unclear; there was a suggestion that syphilis was not as prevalent in Ireland. Mortality data from 1905 recorded general paralysis of the insane in 26.4% of male and 7% of female asylum deaths in England but only 5.3% of male and 1.1% of female deaths in Ireland.

The first systemic study of the psychiatric implications of syphilis in Ireland in the nineteenth century was performed by Dr. William Saunders Hallaran in

Cork. His findings would seem to confirm that perhaps the prevalence of general paralysis of the insane was less prevalent in Ireland. He attempted to identify the causes of insanity in 1431 individuals admitted between 1798 and 1818, 3.7% of these were attributed to venereal disease[37].

In 1914, it was estimated that greater than 100,000 new cases of syphilis and 3 million persons had syphilis in Great Britain, giving an estimated prevalence of 7%[21]. Given that, in 1914, Ireland was still part of Great Britain, this may well have been the prevalence in Ireland too, although undoubtedly region differences did occur.

In 1978 a telephone survey of Irish general practitioners[38] was carried out to ascertain the frequency of sexually transmitted disease (STD) diagnosis in the preceding year. This was done due to concerns over under reporting of STD. 281 general practitioners were contacted and the response rate was 98.6%. At that time, only gonorrhoea and syphilis were identified individually in public health notification. All practitioners reported that they saw less than 10 cases of syphilis a year, and the estimated frequency of syphilis diagnosis was 7.7 per 100,000 per year. It was estimated that only 24.3% of syphilis cases were reported, although this was significantly higher than estimated reporting rates for gonorrhoea or non-specific venereal disease.

When men who have sex with men (MSM) were surveyed in the early 1980s in Dublin, Sweden, Australia and Finland, the percentage prevalence of prior

syphilis was 5% in Dublin MSM[39] significantly lower than that in the other communities surveyed.

The number of reported cases of syphilis in Ireland fell to their lowest level in ten years in 1999[40]. In the 1990s on average fifteen cases were reported each year, and all cases occurred in heterosexual patients[40, 41]. In 2001 a large outbreak was reported with a shift in the epidemiology of the infection[41]. The majority of patients were MSM and by 2003 when the numbers of new cases were falling and the outbreak declared over, 17.4% of cases had occurred in HIV positive patients and 4.5% of patients had received a concurrent diagnosis of HIV[42].

1.2 - Clinical Manifestations of Syphilis

Transmission of syphilis is by direct contact with an infectious individual. Transmission can occur by sexual contact, kissing or close contact with an active lesion, transplacentally in the case of congenital syphilis or by transfusion or accidental innoculation[43]. The vast majority of cases are transmitted sexually. The manifestations of syphilis are protean, hence the reason that syphilis has often been referred to as the "great pretender" or the "great impostor".

Infection is generally divided into [44]

1) Incubation period
2) Primary syphilis characterised by a primary chancre

3) Secondary syphilis
4) A period of sub-clinical infection or latent syphilis
5) Tertiary syphilis, which does not occur in every patient.

The patient is most infectious in the early stages of disease, particularly when the primary chancre, mucous patch or condyloma latum is present. An immunologically intact person cannot spread syphilis after 4 years of infection[44].

An infected patient may inoculate *T. pallidum* onto an area of the body that is touched. Wet nurses occasionally spread the disease in the past to the infants they fed and visa versa. In 1752 in Nerac in France 40 women and children in addition to several male contacts were infected as a result of transmission by wet nurses after one wet nurse developed a primary chancre on her nipple after feeding a congenitally infected infant[43]. Syphilis of the fingers "the physicians chancre" was most common among medical professionals prior to the universal precaution of wearing gloves[44]. Blood transfusion is now a very rare method of acquiring syphilis, as all blood donors are screened[45] and *T. pallidum* cannot survive longer than 24-48 hours under the conditions of blood bank storage[44].

1.2.1 - Incubation period

T. Pallidum penetrates the intact mucous membrane or gains access through abraded skin. Within hours to days, it enters the lymphatics or blood stream

and disseminates throughout the body. This occurs soon after contact, patients have previously been infected by blood transfusions from donors with negative serology who are in the incubation period. The incubation period is directly proportional to the size of the inoculum [46] and in rabbit models as few as four to eight spirochetes are needed to establish infection [44]. The median incubation period is three weeks, but can vary from 3 to 90 days.

1.2.2 - Primary Syphilis

Classically a primary chancre is described as a single painless papule (image 1.2) that occurs at the site of inoculation (image 1.3). It appears after the incubation period, erodes and becomes indurated. The base is usually smooth, and the borders raised and firm with a cartilaginous consistency. The ulcer has a clean appearance with no exudates (unless it is super-infected). It is usually round, however it may be oblong following tissue lines. There is little pain or bleeding even when the ulcer is scraped as may occur while obtaining samples for darkfield microscopy (see below). Multiple chancres can occur, particularly in the setting of HIV infection[47] [48]. Prior to the advent of darkground microscopy many physicians dedicated a lot of time and effort into describing the morphology of a chancre[49] (table 1.1). Atypical lesions are said to occur in 60% of patients [44], and many patients may have no primary lesion.

Image 1.2 – Primary Chancre- penile

Image 1.3 - Primary chancre at site of inoculation (oral cavity).

The appearance of the lesion depends on the size of the inoculum, the immune status of the patient and concurrent antibiotic usage. A small number of treponemes inoculated into volunteers produced only a papule, whereas a larger bacterial inoculum produced an ulcerative chancre[46]. Secondary infection is most common with oral and anal lesions. Regional lymphadenopathy of moderately enlarged, firm non-suppurative painless lymph nodes accompanies the primary lesion.

The pathological characteristics of the primary chancre are that of an intense infiltration of plasma cells and scattered histiocytes, concentric endothelial and fibroblastic proliferative thickening of small blood vessels and eventually obliterative endarteritis.

The differential diagnosis of a primary chancre includes genital herpes, chancroid and traumatic super-infected lesions. Other diagnoses that should be considered are early warts caused by human papilloma virus, granuloma inguinale, tuberculosis or atypical mycobacterial infections.

1. Chancre tends to be single rather than multiple
2. Relatively long incubation period
3. Painless if uncomplicated (chancre of finger an exception)
4. Induration of base of the lesion
5. Erosion of the surface rather than ulceration
6. Sharply defined border with a fine haemorrhagic line at the periphery.
7. Base clean with a faint greyish pellicle or a raw muscle colour
8. Serous exudate
9. Chancre indolent- runs a slow course 3-8 weeks
10. Associated painless lymphadenopathy
11. Lymphadenopathy more likely unilateral in the case of extra-genital lesions
12. On healing, a thin atrophic scar may remain.

Table 1.1 - Physical Characteristics of Primary Syphilis[49]

1.2.3 - Secondary Syphilis

Secondary syphilis or disseminated syphilis occurs when the spirochete multiplies and disseminates throughout the body. It lasts until a sufficient host response develops to control the organism. It begins 2 to 8 weeks after the appearance of the chancre, however, this is highly variable and the signs of primary and secondary infection may overlap. The manifestations of secondary syphilis are protean (table 1.2).

Manifestation	Percentage of Cases
Skin • Rash Macular Maculopapular Papular Pustular • Condyloma Lata • Generalised Lymphadenopathy	90
Mouth and Throat • Mucous patches • Erosions • Apthous ulceration	35
Genital Lesions • Chancre • Condyloma lata • Mucous patch	20
Constitutional Symptoms • Pyrexia, Malaise • Pharyngitis, lymphangitis • Anorexia, weight loss • Arthralgia • Alopecia	70
Central Nervous System • Asymptomatic • Symptomatic ▪ Headache ▪ Meningismus ▪ Meningitis ▪ Ocular ▪ Otitic ▪ Cranial nerve involvement	8-40 1-2
Renal • Glomerulonephritis • Nephrotic syndrome	Unusual
Gastrointestinal • Hepatitis • Intestinal wall invasion	Unusual

Table 1.2 - Clinical Manifestations of Secondary Syphilis[44]

The most commonly recognised signs of secondary syphilis are skin lesions. The rash of secondary syphilis may be macular, maculopapular, papular, pustular or a combination of the above. Vesicular lesions occur only in congenital syphilis. Skin lesions usually begin on the trunk and proximal extremities. Classically they are pink-red macular lesions 3-10mm in diameter. Any surface area can become involved (image 1.4).

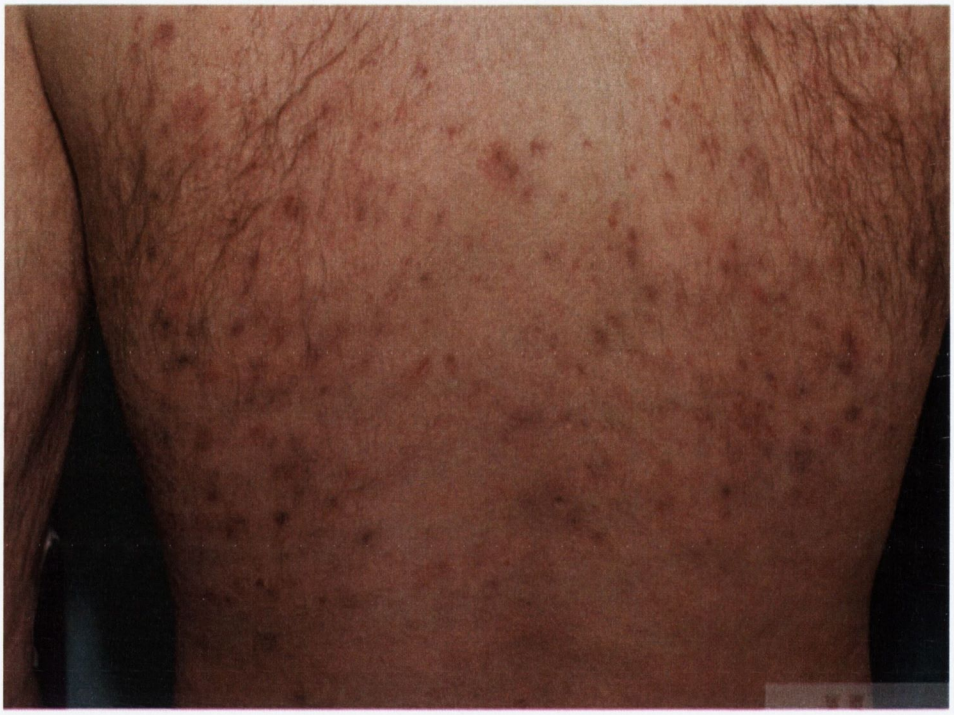


Image 1.4 - Rash of secondary syphilis

The rash can last from a few days to up to 8 weeks. It may evolve into papules and in some patients to pustules called pustular syphilids. All the rashes may be present at the same time and the rash can involve any body surface, including the palms and soles, which is highly suggestive of the diagnosis (image 1.5). Alopecia occurs when the hair follicles become involved (follicular syphilids).

Image 1.5 – Rash of secondary syphilis involving the palms

In warm moist areas such as the genital and perianal area, inner aspects of the thighs, skin under breasts, nasolabial folds, axillary area, antecubital fossae, and webs of fingers and toes the papules can coalesce and erode causing painless broad grey white highly infectious plaques called condylomata lata (image 1.6). These lesions may also develop on mucous membranes where they are called mucous patches. During relapses the skin lesions tend to be less florid and condylomata lata are common.

Image 1.6 - Condyloma lata

Constitutional symptoms as listed in table 1.2 are also common. Enlargement of the epitrochlear lymph nodes is a unique finding that should always suggest the diagnosis.

1.2.4 - Neurological syphilis

Neurosyphilis i.e. syphilitic infection of the central nervous system (CNS) can present at any time after infection. It can be classified into early and late disease. Early in the course of syphilis, most forms of neurosyphilis will involve the cerebrospinal fluid (CSF), meninges and vasculature, while later disease that occurs in tertiary syphilis tends to affect the brain parenchyma and spinal cord. In the pre-antibiotic era 25 to 33% of patients with syphilis had neurological involvement[50]; a third had asymptomatic neurosyphilis, a

third tabes dorsalis, 10% had paresis, 10% meningovascular disease and the remaining patients had a variety of other manifestations including symptomatic meningitis and cranial nerve abnormalities.

Currently neurosyphilis is most common in patients with human immunodeficiency virus (HIV) infection, and these patients almost exclusively have the early forms of neurosyphilis, often with concomitant eye disease.

Post neuro-invasion by treponemes, spontaneous resolution may occur in some cases without an inflammatory response. Asymptomatic neurosyphilis where persistent meningitis occurs in the absence of symptoms is a consequence of failure to clear organisms from the CSF. These patients are more likely to develop long-term neurological sequelae than those with normal CSF[50]. Symptomatic meningitis occurs more frequently in the first year after infection. Signs are similar to other forms of meningitis, however visual acuity may be impaired if there is eye involvement and cranial neuropathies can occur. Meningovascular syphilis may manifest as thrombosis, ischaemia and infarction. Late neurosyphilis manifested as tabes dorsalis and paresis is now uncommon. Although case reports show that sporadic cases do still occur[51].

The mechanism of clearance of organisms from the CSF is probably similar to the immune response peripherally, where opsonized organisms are cleared by activated macrophages. A study in non-human primates demonstrated increased CD4 cells and gamma interferon responses in the CSF as the bacteria were cleared form the CNS consistent with a "Th-1-type" cellular

immune response[52]. While it is still unclear whether human host response or organism characteristics determine the risk of developing symptoms following neuro-invasion, a rabbit model has suggested that there are particularly neuroinvasive strains of *T. pallidum*[53]. More recently the same group presented data on the strain type in 66 patients with syphilis between 2001 and 2007[54]. Three molecular methods were used to assign strain type and five strains were identified in the 66 patients. The strain type was seen to change significantly over time. Strain type was also significantly associated with neurosyphilis; none of the patients with neurosyphilis had stain type 11, while 54% of those infected with strain type 10 had neurosyphilis. Molepo *et al*[55] looked at molecular typing in patients with neurosyphilis and found type 14a to be the most common (53.8% of 13 patients).

1.2.4.1 - Ocular syphilis

Ocular syphilis is part of the spectrum of neurosyphilis. Ocular manifestations can occur at any stage of the disease. The presentation of ocular involvement is varied reflecting syphilis' nickname "the great impostor".

The most common presentation of syphilis in the eye is uveitis. This can occur as early as six weeks after primary infection. When uveitis occurs as part of secondary syphilis it often follows the resolution of other signs and symptoms. The most common manifestations in patients with uveitis who presented to a New York clinic over a five-year period were granulomatous iridiocyclitis, non-granulomatous iridiocyclitis, panuveitis, posterior uveitis and keratouveitis[56].

Anterior segment inflammation may be associated with a vitritis. Posterior uveitis is most commonly manifested as a chorioretinits. As the clinical manifestations of syphilitic uveitis are non-specific, the work up for patients presenting with an unexplained uveitis should include testing for syphilis.

Ocular involvement may be more severe in patients with HIV co-infection, is frequently bilateral and more frequently involves the posterior segment [57]. Syphilis is more common than cytomegalovirus as a cause of uveitis among patients on highly active anti-retroviral therapy (HAART).[57] Ocular syphilis can be difficult to diagnose and if left untreated can lead to irreversible visual loss.

1.2.5 - Tertiary syphilis

Late syphilis has now become, in the developed world at least, so uncommon that it's presentation is worthy of case reports[51]. Tertiary syphilis is divided into cardiovascular, neurological and gummatous disease[44]. Tertiary syphilis can occur up to 30 years after first infection. Many of the details regarding the progression to tertiary syphilis, are from the pre-antibiotic era. Professor Boeck of the University of Oslo, conducted the Oslo Syphilis study from 1890-1910[58]. Twenty eight percent of patients went on to develop clinical complications of late syphilis. 10% developed cardiovascular syphilis, but only if they acquired syphilis after the age of 15 years. 6.5% developed neurological symptoms and 16% gummatous or late benign syphilis. In 15% of men and 8% of women, syphilis was considered the primary cause of death. The study however has been criticised[59] as more than 40% of patients

received some form of treatment either in the form of potassium iodide or mercury. Similar results were reported from the infamous Tuskegee study[26].

1.2.5.1 - Late neurological syphilis

Many classify late neurosyphilis as asymptomatic and symptomatic, the latter subdivided into meningovascular and parenchymal disease[44], in reality there is often overlap clinically of symptomatic disease. Asymptomatic neurosyphilis is a diagnosis of patients who have no clinical signs or symptoms of neurological disease but who, on examination of their CSF have one or more abnormality (pleocytosis, raised protein count, positive rapid plasma reagin (RPR) or venereal disease research laboratory (VDRL) test). The incidence of asymptomatic neurosyphilis in untreated patients ranges from 8-40%[44]. These patients are treated to prevent progression to symptomatic neurosyphilis

Late symptomatic neurosyphilis can be divided into meningovascular and parenchymal disease. Pathologically meningovascular disease is characterised by an endarteritis obliterans and parenchymal disease by the destruction of nerve cells. Meningovascular disease can lead to a wide spectrum of clinical presentation, such as stroke to progressive neurological defects. Parenchymous disease includes general paresis and tabes dorsalis. Another distinct variant is syphilis otitis, which can lead to progressive deafness.

1.2.5.2 - Cardiovascular syphilis

The pathological findings in cardiovascular syphilis are again an endarteritis obliterans, but in this instance involving the vaso vasorum of the aorta. This results in the formation of a saccular or fusiform aneurysm[44]. The ascending aorta is most commonly affected and aortic regurgitation may result. The aneurysms rarely dissect and neurological involvement is common in patients with cardiovascular sequelae of syphilis infection.

1.2.5.3 - Gummatous Syphilis (late benign)

A gumma is a non specific granulomatous-like lesion that occurs in late syphilis infection[44]. They are most commonly found in the skin and musculoskeletal systems, but can occur in any organ. They cause local destruction and a therapeutic trial of penicillin causes a rapid and dramatic response.

1.3 - Diagnostics

Treponema pallidum subspecies *pallidum (T. pallidum)* is a microaerophilic bacterium that is 6-20 microns long and 0.10- 0.18 micron in width. As a result, it is too slender to be visualised by direct microscopy. The bacterium cannot be cultured[60] and naturally this complicates the diagnosis of syphilis. Darkfield microscopy, a specialised technique that utilises an oblique light to visualise the organism can be used in a limited number of cases. As a result the vast majority of cases are diagnosed by the use of serology; an indirect

diagnostic method. More recently, the use of polymerase chain reaction (PCR) has offered promising results as a potential new adjunct in the diagnosis of syphilis. Ultimately confirmation of clinically suspected cases of syphilis will depend on the stage of the disease.

1.3.1 - Darkfield Microscopy

Darkfield or darkground microscopy demonstrates mobile treponemes by the use of oblique light and without the need for staining. It was first described in 1909 by Coles who commented specifically on the motility of the organism[61]. On visualisation the treponemes have regular tight spirals with a coil wavelength of 1.1 μm and an amplitude of 0.2-0.3 μm. The organisms move via a forward and backward motion with rotation around the longitudinal axis[62] (image 1.7).

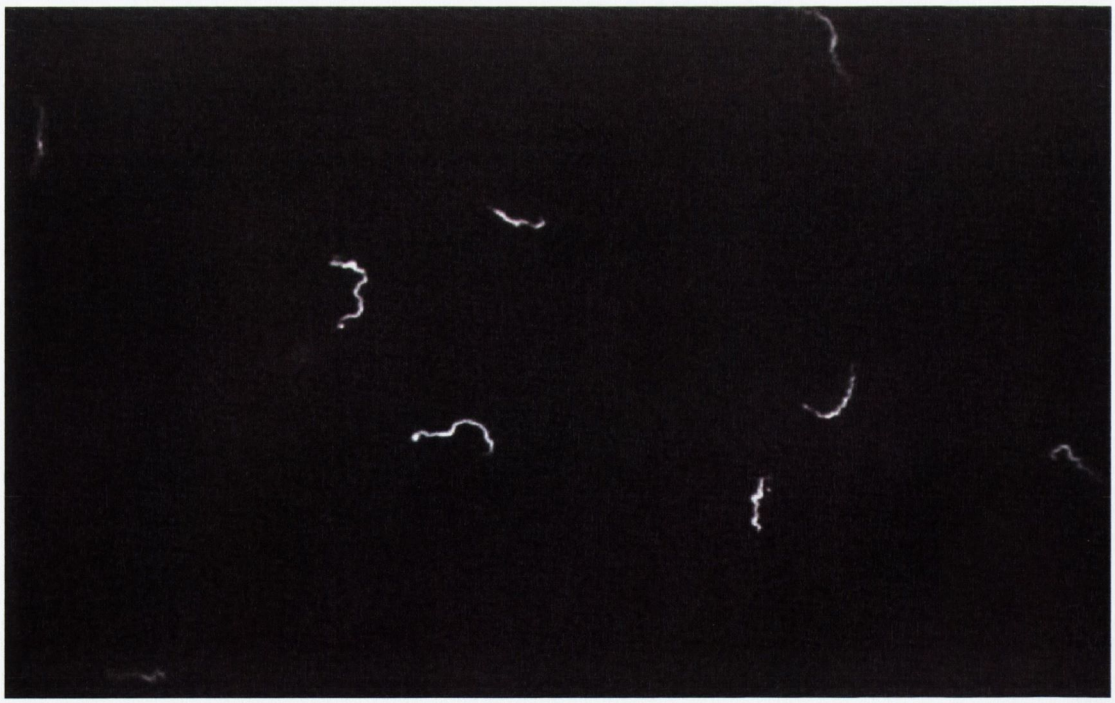


Image 1.7 – Positive Darkground Microscopy Sample

The use of darkfield microscopy is limited to patients who have demonstrable clinical lesions. It is most productive during primary, secondary infectious relapsing and early congenital syphilis. Moist lesions containing large numbers of treponemes such as primary chancres, condyloma lata or mucous patches are particularly amenable to darkfield mciroscopy[61]. It is not recommended for oral lesions[61, 63] as saprophytic spirochetes in the mouth may be difficult or impossible to differentiate from *T. pallidum*. For diagnosis, the organism must be viable and samples must be transported to the laboratory and analysed within 5-10 minutes of collection. Additionally darkfield microscopy needs to be performed by experienced personnel (table 1.3). Poor results from darkfield microscopy have been reported in some series and have been attributed to lack of experience of medical practitioners in performing tests[64]. In more recent series following the large outbreak of syphilis in the western world in the late 1990s- early 2000s darkfield microscopy has performed significantly better.[65]

A darkfield microscope has a double- or single reflecting dark-field condenser a x40 to x45 objective and a x90 to x100 objective with a funnel stop[61] and uses an oblique light to visualise the organism. The sensitivity of darkfield microscopy approaches 80%[61], when done by experienced personnel. A negative darkfield examination does not exclude the diagnosis of syphilis. There may be too few organisms, the lesion may be healing, or the test may be unsatisfactory as the result of debris such as air bubbles, bloods cells or tissue fragments.

Darkfield microscopy is generally only available within specialist sexual health centres. In an audit of laboratory diagnostic methods only 1% of microbiology laboratories surveyed and 34% of genitourinary medicine clinics in the UK performed darkground microscopy[66]. There are currently two laboratories performing darkground in the Republic of Ireland- St. James's Hospital and The Mater Misericordiæ University Hospital, both in Dublin (Dr Brendan Crowley- personal communication) and one private practitioner, Dr Derek Freedman (personal communication).

- Specimens for darkfield examination should consist of serous fluid that contains treponemes but should be free of erythrocytes, other organisms and tissue debris.
- Observe universal precautions as organisms must be viable
- Cleanse incrusted or obviously contaminated lesions (physiologic saline that does not contain anti-bacterial agents should be used)
- Use the minimum amount of liquid for cleaning
- Gently abrade the lesion
- Apply pressure until clear serum exudes
- Place a drop of serum onto a slide and cover with a cover slip
- Examine the specimen within 5-10 minutes while the organism is still motile.
- For cervical or vaginal lesions, clean with saline and abrade with gauze before collecting the sample with a bacteriological loop or Pasteur Pipette.

Table 1.3 - Collection of specimens for darkfield microscopy[61, 65]

1.3.2 - Fluorescent antibody testing

Direct fluorescent antibody testing (DFA-TP) is another direct diagnostic method that can be employed in the diagnosis of syphilis. Samples are collected in a manner described in table 1.3 for darkfield microscopy. It has the advantage that the organism can be identified even when smears cannot be examined immediately. It is specific for *T. pallidum* antigens and as a result it avoids the misidentification of other non- pathogenic treponemes[67], it cannot however, distinguish between pathogenic treponemes. Unfortunately, again this test is limited to centres where there is a fluorescence microscope equipped with a darkfield condenser and the standard set of filters and a trained and experienced technologist is available to read the test. A negative test does not exclude the diagnosis of syphilis, as the condition of the sample affects the test sensitivity. When fresh samples are used sensitivity approaches 100% [68].

1.3.3 - Serology

T. Pallidum cannot be cultured, and in the latent and late stages of disease there is no specimen source of the organism to perform darkfield microscopy or DFA-TP. As a result the majority of diagnoses are made using serological testing in lieu of direct microbiological culture.

Serological tests fall into two categories, non-treponemal tests and treponemal tests. Non-treponemal tests such as rapid plasma reagin (RPR) or

Venereal Disease Research Laboratory (VDRL) tests are used for screening. Positive tests are then confirmed with a treponemal test such as *Treponema pallidum* particle agglutination (TPPA) or fluorescent treponemal antibody absorbed (FTA-ABS). False positive reactions may occur with the non-treponemal tests in autoimmune conditions and various other infections, including HIV[61]. Unusual serologic responses may be seen in HIV-infected persons who have syphilis. Such responses include unusually high serologic titres, false-negative tests, and the delayed appearance of seroreactivity.

1.3.3.1 - Non-Treponemal Tests

Non-treponemal tests such as RPR or VDRL can be used either qualitatively as screening tests or quantitatively to follow response to treatment. All non-treponemal tests are based on an antigen containing measured amounts of cardiolipin, cholesterol and sufficient purified lecithin to produce standard reactivity. These tests measure immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies to lipoidal material released form damaged cells, lipoprotein like material and possibly cardiolipin released from treponemes. Anti-lipoidal antibodies are also produced in response to other treponemal infections and some non-treponemal infections. As a result, reactivity to non-treponemal tests does not confirm the diagnosis of syphilis.

In qualitative tests used for screening, undiluted serum is used to detect the presence or absence of antibody. In quantitative tests used to monitor the response to treatment, serial two fold dilutions are made until an endpoint is

reached. The results are reported in terms of the highest dilution in which the specimen is fully reactive. In RPR testing, carbon particles are added to the cardiolipin antigen suspension. These particles are not attached to the antigen but get trapped in the lattice created when the antibody-antigen complex forms and allows the test to be read macroscopically (image 1.8).

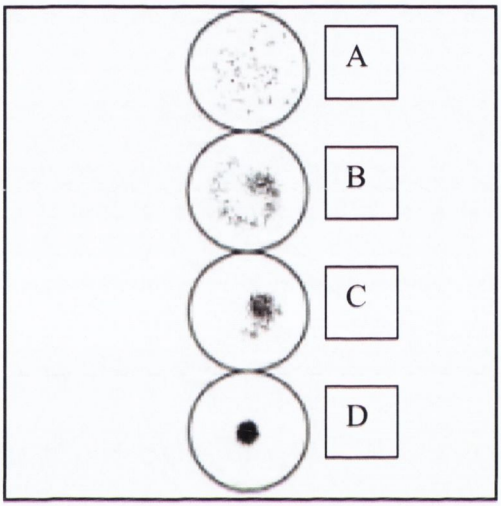


Image 1.8 – RPR test.

(A= reactive, B= weakly reactive, C= minimally reactive, D= non-reactive)

Quantitative tests are useful for providing a baseline from which change can be measured. A recent re-infection can be demonstrated by a fourfold rise in titre. All of the non-treponemal tests have a similar sensitivity and specificity, the RPR which is used in St. James's microbiology laboratory has a sensitivity of 73-86% depending on the stage of disease and a specificity of 98%[61].

The reactivity of tests can differ depending on variation in antigen preparation. For this reason it is recommended that sequential tests should be performed using the same laboratory method[69] and preferably by the same laboratory. It

is also recommended that the baseline quantitative result used to monitor treatment response is the one drawn on the day of initiation of therapy, as success of treatment is based on a fourfold decrease in dilution titre[61, 63].

Non-treponemal tests have the advantage of being inexpensive, widely available and convenient to perform, and as a result are ideally placed as a screening test. These tests do however lack sensitivity, particularly in very early and late syphilis. There is also the possibility of false negatives due to the prozone phenomenon (see below) and false positives due to other medical conditions and infections.

The incidence of false positives depends on the test performed and the population studied [70, 71]. False positives are generally classified into acute false positive reactions (those of less than 6 months duration) and chronic false positive reactions (those that persist for 6 months or more). Acute false positive tests have been associated with viral infections such as Epstein Barr and measles and with pregnancy or lab error [61, 62]. Chronic false positives have occurred with connective tissue diseases such as systemic lupus erythematosus, leprosy, malignancy, hepatitis C and HIV infection[71, 72]. Up to 10% of patients over 70 years of age may have a false positive result[44]. Women are more likely to have biological false positive results than men[71] and some series have reported an increased rate of false positive tests among drug users[73] while others have not [74].

1.3.3.1.1 - Prozone phenomenon

The prozone phenomenon is said to occur in 1-2% of patients with secondary syphilis[75]. The prozone reaction is an immunological event seen when performing tests such as the RPR, which rely on antibody to antigen interaction. The optimal ratio the "zone of equivalence" of antibody to antigen yields an insoluble precipitate that is visible and the test is read as positive. In the case of excess antibody or prozone, false negative tests can occur as the lattice does not form correctly and the carbon particles are not trapped. It can occur particularly in secondary syphilis, pregnancy or in the setting of HIV co-infection when antibody titres may be very high[44, 75, 76]. This may be due to anomalous B cell behaviour leading to a hyper-responsiveness to antigenic stimulation and excess antibody production in HIV positive patients[77].

1.3.3.2 - Treponemal Tests

The predictive value of the non-treponemal tests is increased when combined with a reactive treponemal test. These tests measure antibodies against specific *T. pallidum* antigens. They are used to confirm the reactivity in non-treponemal tests, they may also be used in the diagnosis of clinically suspected syphilis where the non-treponemal test is non reactive. They are technically more difficult to perform and are more expensive than the non-treponemal tests.

Treponemal tests are the fluorescent treponemal antibody absorption test (FTA-ABS), it's counterpart the FTA-ABS double screening, both which are based on adding a fluorosceine labelled anti-human immunoglobulin to a mixture of the patients serum and a suspension of killed treponemes[61] and the microhaemagglutination assay for antibodies to *T. pallidum* (MHA-TP) and the *T. pallidum* particle agglutination (TPPA) which use sensitised erythrocytes to detect the presence of treponemal antibody.

The TPPA is manufactured using gelatine particle carriers sensitized with purified pathogenic *T. pallidum.* The test is based on the principle that sensitised particles are agglutinated by the presence of antibodies to *T. pallidum* in human serum or plasma.

The treponemal tests cannot be used to monitor treatment. In 85% of treated patients test results remain active for years, if not a life time[61]. The false positive rate using the treponemal tests is reported to be 1%[78]. However when coupled with a reactive non-treponemal test the two tests together are highly specific. The haemagluttination tests give less false positive results than the FTA-ABS tests [61].

1.3.3.3 – Enzyme Immunoassays

Competitive enzyme immunoassays (EIA) for human immunoglobulin G (IgG) or IGM are a specific rapid and automated method for screening samples for syphilis. The assays are competitive assay for the screening of total anti-

treponemal antibodies. The reaction is based on the competition between these antibodies and a human anti *T. pallidum* IgG peroxidase labelled conjugate for specific sites from Nichols strain, which is used to coat the wells on microtitre plates[79]. The sensitivity of EIA in detecting all stages of syphilis is reported at between 94.5-100% [79, 80].

Traditionally the non-treponemal tests were used for the screening of samples for cost reasons. Patients with a reactive non-treponemal test would then go on to have a treponemal test done to confirm the diagnosis. However, more recently this practice has been brought into question. The CDC in the United States reported in 2008 on the reversal of the traditional methods in four New York laboratories[81]. These laboratories had been using automated treponemal tests, such as enzyme immunoassay (EIAs) to screen large volumes of samples. They reported that 3664 (3%) of the specimens would have not been identified using traditional testing algorithms. This method of screening, i.e. using an automated EIA and then testing reactive samples for RPR and TPPA is the method currently employed in St. James's Hospital Microbiology department.

1.3.3.4 – Polymerase Chain Reaction

More recently use of molecular tests such as polymerase chain reaction (PCR) have demonstrated *T. pallidum* DNA within tissue samples[82, 83]. This technique may be particularly useful in the diagnosis of early syphilis, when the serological tests may not be positive.

In 2001 Liu *et al* [84] developed primers designed on 2 unique characteristics of the *polA* gene of *T. pallidum.* The primers were tested on specimens containing *T. pallidum* subspecies *pallidum* (Nichols strain), subspecies *pertenue* and *endemicum* along with other non pathogenic treponema, *T. denticola, T. phagedenis* and *T. refringens*, related spirochetes *B. burgdorferi* and leptospira species and an additional 59 species of bacteria and viruses including pathogens that cause ulcerating genital disease such as herpes simplex virus (HSV) and *Haemophilus ducreyi.* Two sets of primers were developed that gave rise to amplicons of 377 or 395bp form the *polA* gene of *T. pallidum*. The 2 sets of primers showed similar sensitivity and specificity and amplified DNA only from the 3 subspecies of *T. pallidum*. Sensitivity testing using known concentrations of organisms demonstrated a detection limit of 10 organisms, which was increased to 1 organism per assay when a fluorescence detecting method was used. The assay was also used on 112 genitourinary ulcer specimens and was shown to have a sensitivity of 95.8% and a specificity of 95.7% when compared to a previously reported multiplex PCR assay (Roche Molecular).

Using these primers, Leslie *et al* [82] developed a real time PCR assay with a sensitivity and specificity of 80.39% and 98.40% respectively when compared to serology for the detection of early syphilis. This was carried out in response to a marked increase in the number of locally acquired syphilis infections in Victoria Australia, most of the patients at the time were men who have sex with men (MSM) and a disproportionate number were HIV positive. The aim was to develop a robust sensitive and specific real time PCR assay for the

detection of syphilis DNA. The target was the *polA* gene of *T. pallidum*. A TaqMan real-time PCR assay was used which targeted a 67bp sequence of the *polA* gene. A BLAST search of the 67bp fragment showed no similar sequences in the GeneBank database. The assay has a detection limit of 35 organisms per swab (1.75 target copies per reaction). 660 specimens from 590 patients were tested yielding 55 positive samples from 51 patients. There was 95% agreement with serology in the 301 patients with adequate serological follow up. All of the samples tested were genital, mouth or skin swabs and CSF. Blood was not tested.

A head to head comparison of real time PCR and darkground microscopy by Heymans *et al* [85] demonstrated a sensitivity of 87% and a specificity of 93.1% of the real-time PCR assay. This could have implications for those working in general practice who may not have access to darkfield microscopy facilities.

Palmer *et al*[83] reported on the use of PCR targeting the 47kDa lipoprotein gene on samples from ano-genital and oral swabs in the UK. Compared to clinical diagnoses and serology they demonstrated a sensitivity of 94.7% and 80% in primary and secondary syphilis respectively and a specificity of 80% and 98.6%. They concluded that as darkground microscopy was not available in many clinics or that physicians and technicians had become deskilled in performing darkground microscopy, that PCR would be a useful adjunct to syphilis diagnosis. PCR also has the significant advantage of being applicable to oral and peri-anal lesions, which may be commonly seen in MSM.

A group from Switzerland[86] also using an assay targeting the 47kDa published findings on the use of PCR on diverse biological samples. They reported a specificity of 100% when compared to matched controls (95% confidence interval 59-100%). This group also used whole blood samples, and demonstrated that the mean C_T values of blood samples in HIV positive patients was significantly higher than in HIV negative patients (p=0.02) perhaps reflecting a higher concentration of *T. pallidum* in the blood of HIV positive patients. Interestingly it is the first PCR study to test the urine of patients with primary or secondary syphilis, however PCR of the urine was reported to have low sensitivity 29-44%, meaning that testing of this easily obtainable sample may not be clinically relevant.

PCR has been useful in making the diagnosis of syphilis in diagnostically difficult cases as a number of case reports demonstrate. PCR has aided the diagnosis of, amongst others, secondary syphilis presenting as gastric syphilis[67], panuveitis with retinal necrosis[87], osteomyleitis[88], pulmonary syphilis[89] and intersitial orchitis [90].

1.3.3.5 - Molecular typing of *T. pallidum*

Molecular subtyping of *T. pallidum* can be used to identify the diversity and epidemiology of syphilis infection. The use of subtyping can demonstrate the heterogeneity of syphilis infections during an outbreak. Molecular typing utilises a method that is based on the characterisation of 2 repeat genes *arp* (acidic repeat protein) and *tpr* (*T. pallidum* repeat). During the 1990s in

Arizona, the most common subtype identified from 45 samples was 14f[91]. More recently studies from Scotland[92] and Lisbon[93] have demonstrated that strain 14d and 14a were the most common respectively. In the Scottish study, six different subtypes were identified. The majority of patients from whom the 14d subtype was isolated acquired their syphilis in the UK. In Lisbon however, less genetic diversity was seen and the predominant type was 14a, which was not isolated in the Scottish study, suggesting that there is minimal contact between the two communities. Further study on the molecular epidemiology of syphilis in Europe as a whole would be interesting to map regional variations in syphilis strain types. Marra *et al* (in press) analysed 158 samples isolated from patients from Seattle, Baltimore, San Francisco, China, Ireland and Madagascar. 14d was the most common subtype identified and was identified in 5/6 sites. Additionally a change in circulating strain was demonstrated over time in Seattle between 1998 and 2008. A strong trend was also seen with strain type and HIV status suggesting separate sexual networks within the MSM community. Patients with neurosyphilis were most commonly infected with strain 14d. Additional work on the strain types associated with neurosyphilis may have important implications in the future for the investigation and management of patients with syphilis.

1.4 - Management of Syphilis

The management of syphilis has not changed significantly since 1943 when penicillin was first shown to be effective in the treatment of the infection[94]. Despite nearly sixty years of use in the treatment of syphilis infection, no documented resistance to the antibiotic has occurred. This is in contrast to azithromycin. It was hoped that azithromycin could be an alternative to penicillin particularly in cases of incubating syphilis or in regions where parenteral penicillin was not readily available[95-101]. In 2004 a number of treatment failures associated with the use of azithromycin to treat syphilis were reported[102]. Subsequently the single base pair mutation in the *T. pallidum* rRNA gene that confers azithromycin resistance was described[103]. This led to concern regarding the use of azithromycin in the management of syphilis, particularly in the developed world.

As syphilis can be an indicator disease for HIV[104, 105], all patients who are diagnosed with syphilis should also have a HIV test performed. In addition due to the fact that many centres report a high prevalence of untreated syphilis amongst newly diagnosed HIV patients[106, 107], all HIV patients should have syphilis serology preformed at diagnosis and 3-6 monthly in high risk groups (such as MSM) and yearly in all other patients.

The mainstay in the treatment of syphilis is parenterally administered penicillin. While guidelines differ on the duration of therapy[63, 69], the basic principals remain the same (table 1.4)

Syphilis Stage	UK Guidelines[63]	CDC Guidelines[69]
Incubating	1) Benzathine penicillin G 2.4 MU- single dose 2) Doxycycline 100mg b.d. x 14 days 3) Azithromycin 1g stat	1) Benzathine penicillin G 2.4 MU- single dose
Early (Primary/ Secondary/ Early latent)	1) Benzathine penicillin G 2.4 MU- single dose 2) Procaine penicillin 600000 U x 10 days 3) *Doxycycline 100mg b.d. x 14 days	1) Benzathine penicillin G 2.4 MU- single dose 2) Doxycycline 100mg b.d. x 14 days
Late (late latent/ cardiovascular/ gummatous)	1) Benzathine penicillin 2.4 MU weekly x 2 weeks (3 doses) 2) doxycycline 100mg b.d. x 28 days	1) Benzathine penicillin 2.4 MU weekly x 2 weeks (3 doses) 2) doxycycline 100mg b.d. x 28 days
Neurological Syphilis	1) procaine penicillin 1.8-2.4 MU plus probenecid x 17 days 2) benzylpenicillin 18-24 MU daily x 17 days. 3) **doxycycline 200mg b.d x 28 days	1) benzylpenicillin 18-24 MU daily x 10-14 days. 2) procaine penicillin 2.4 MU plus probenecid x 10-14 days***

* other alternatives listed azithromycin, erythromycin, ceftriaxone and amoxicillin plus probenecid.

** other alternatives amoxicillin plus probenecid or ceftriaxone.

*** penicillin desensitisation the preferred alternative in penicillin allergic patients.

Numbers indicate first line, second line treatment etc.

HIV positive patients should be treated as per the guidelines above.

Table 1.4 – Syphilis Treatment Guidelines

In the absence of Irish National guidelines on the management of syphilis, the author in collaboration with the consultants of the GUIDE clinic in St. James's Hospital devised departmental guidelines for the management of syphilis. These guidelines were initially devised in June 2009 and revised in March 2010. This was in response to wide variation in practice, due in part to the differing international guidelines. Guidelines were distributed electronically or in paper copy to all clinical staff, and the treatment algorithm was displayed in all clinic rooms. These departmental guidelines are reproduced in appendix 1.

All patients should be clinically assessed upon the diagnosis of syphilis. Careful history taking regarding symptomatology and sexual history to stage the disease will impact on potential treatment. Obstetric history and blood donation history may also be helpful. Any patients presenting with syphilis of unknown duration or late latent syphilis should have a full neurological and cardiovascular exam. Any abnormalities should be followed with appropriate imaging and lumbar puncture if indicated. Controversy surrounds the role of lumbar puncture in the investigation of patients with asymptomatic syphilis, and will be discussed elsewhere in the text.

1.5 - Recognition of a Problem: Five cases of Secondary Syphilis Presenting with Ocular Symptoms

Ocular manifestations of syphilis are uncommon[44, 108]. We present five cases diagnosed with ocular syphilis four of whom had a delay in their diagnosis. These patients presented over an eight-month period. Four of the patients were MSM. Four patients were human immunodeficiency virus (HIV) negative, one patient had previously tested HIV positive.

The number of reported cases in the western world had been steadily declining [109, 110], however since 2000 most developed countries have reported a significant increase in the number of new syphilis cases being diagnosed, many of these among men who have sex with men who are HIV co-infected. In the UK, there was a 15-fold increase in syphilis incidence between 1998 and 2003[111]. In Ireland, syphilis rates fell to a historic low in 1999 followed by a dramatic increase from 2000-2003[40].

Ocular involvement is an uncommon manifestation of syphilis [44, 108, 112]. It may be overlooked as it can have a broad spectrum of presentation. Posterior uveitis, retinal vascular involvement, placoid maculopathy, retinal detachment, involvement of the cornea, episcleritis and scleritis and interstitial keratitis presenting as open angle glaucoma have all been described[113-116]. It has also been described as an indicator for HIV infection[105]. Delay in the diagnosis can lead to irreversible visual loss due to optic nerve and retinal atrophy[112].

This cluster of cases of ocular syphilis presenting in a short time period prompted a review of all the cases of syphilis diagnosed in the Department of Genitourinary Medicine and Infectious Diseases (GUIDE), to ascertain if there was an increase in the number of cases of syphilis presenting to the department and to study the epidemiology of syphilis in Dublin.

1.5.1 - Case reports

All five patients presented with decreased visual acuity (VA). All the patients were male and four identified MSM as a risk for acquisition of their syphilis infection. Patient characteristics are outlined in table 1.5. Two of the patients described a rash prior to the onset of their visual symptoms, and one patient described a systemic illness consisting of malaise, fatigue, generalised lymphadenopathy and hair loss before he complained of blurred vision. One patient described fatigue alone and the final patient gave a history of inguinal adenopathy that was noticed at the time of onset of his visual symptoms. One patient was known to be HIV positive with a CD4 count of 398 cells/mm^3 and was virally suppressed on an anti-retroviral regimen of truvada, atazanavir and ritonavir at the time of syphilis diagnosis. Three of five patients underwent lumbar puncture; all three had a pleocytosis on analysis of their cerebrospinal fluid.

All of the HIV negative patients had a delay in the diagnosis of their syphilis. The time from onset of ocular symptoms to diagnosis ranged from six weeks to four months. The patient with known HIV had no delay from time of

presentation to diagnosis. All of the patients received parenteral penicillin therapy (either intramuscular procaine penicillin and probenecid or intravenous benzylpenicillin for 14 days). All but one patient had a significant improvement in their visual signs and symptoms following treatment. Of the HIV negative patients, three had repeat HIV testing at three months, all tests were negative.

Age	Risk	Preceding symptoms	Ophthalmic findings	RPR*	RPR 3mths
43	MSM	Malaise, fever, lymphadenopathy & alopecia.	Decreased VA bilaterally, Neuroretinitis & choroiditis	1:256	1:16
45	MSM	Rash & general malaise.	Decreased VA, extensive chorioretinitis & disc oedema	1:512	1:8
43	MSM	Rash.	Decreased VA, Dense vitritis & choroiditis.	1:128	1:8
40	MSM	Fatigue.	Decreased VA, Neuroretinitis & choroiditis	1:128	1:16
50	Heterosexual unprotected intercourse	Inguinal lymphadenopathy.	Decreased VA, anterior uveitis, vitritis & focal retinitis.	1:128	1:4

Table 1.5- Characteristics of patients presenting with ocular syphilis.

* All non-treponemal tests were confirmed with *T. pallidum* particle agglutination (TPPA)

1.5.2 - Discussion

Ocular manifestations of sexually acquired syphilis can occur at any stage of the disease, and are part of the manifestations of neurosyphilis. The presentation of ocular involvement is varied reflecting the fact that syphilis is often termed "the great impostor", however the most common presentation of syphilis in the eye is uveitis. This can occur as early as six weeks after primary infection. When uveitis occurs as part of secondary syphilis it often follows the resolution of other signs and symptoms.

Sexual history may give a clue to the possible aetiology of a patient's uveitis, four of our patients were MSM and all reported episodes of unprotected sexual intercourse in the months preceding the onset of symptoms. These details were not elicited at initial presentation.

Ocular involvement may be more severe in patients with HIV co-infection[17], is frequently bilateral and may more often involve the posterior segment [57, 117]. Syphilis is now more common than cytomegalovirus as a cause of uveitis among patients on highly active anti-retroviral therapy[57]. Syphilis manifestations are related to immune response in HIV positive patients[118]. Patients with lower CD4 counts (<200cells/mm^3) diagnosed with neurosyphilis are less likely to normalise their cerebrospinal VDRL than those with CD4 counts >200cells/mm^3[119].

As ocular syphilis is part of the spectrum of neurosyphilis, it should be treated with parenteral penicillin daily for a period of ten to seventeen days[63, 69]. Treatment is in either the form of intravenous penicillin G or intramuscular procaine penicillin G with probenecid orally. Patients with reported penicillin sensitivity should be desensitized and treated with penicillin. Opinions differ on whether or not all patients with neurosyphilis should have a lumbar puncture prior to initiation of treatment [120]. For those who do undergo lumbar puncture, repeat lumbar puncture after therapy to ensure normalization of CSF findings is useful in monitoring patients. Some guidelines suggest that if CSF findings have not normalized two years post therapy that treatment should be repeated.[63]

The Jarish-Herxheimer (JH) reaction, a systemic reaction following antibiotic therapy can occur in the treatment of any stage of syphilis but is most common in secondary syphilis[44]. It occurs one to two hours after administration of therapy particularly penicillin. There are case reports of significant deterioration in symptoms or serious adverse events, such as retinal detachment, secondary to the JH reaction following therapy[121]. Steroid therapy prior to initiation of antibiotic therapy is recommended in patients with ophthalmic syphilis to prevent the JH reaction[63].

Ocular syphilis can be difficult to diagnose and if left untreated can lead to irreversible visual loss. Recent reports have suggested an increase in the rates of syphilis among older patients[115]. If not considered during the evaluation of patients presenting with ocular symptoms of unclear aetiology

the diagnosis may be missed. Four of our five patients had a delay of up to four months in the diagnosis of ocular syphilis after presenting with ophthalmic symptoms and all of these patients were HIV negative.

Careful history taking regarding risks factors for the acquisition of the infection, history of possible s syphilitic lesions or systemic illness and or rash prior to the onset of visual symptoms may offer vital clues in making the diagnosis of this treatable disease.

1.6 - The objectives of the project

1. To study the epidemiology of syphilis infection in Dublin.
   (a) To study newly diagnosed patients to ascertain if there been a significant demographic or behavioural shift amongst these patients compared to patients diagnosed with syphilis in 2001.

2. To study the use of real time PCR as a diagnostic tool in the assessment of patients presenting with early syphilis, and to assess the value of syphilis IgM as an adjunct to traditional syphilis serology. As a secondary benefit of molecular epidemiological surveillance the prevalence of azithromycin resistance among newly diagnosed syphilis infections will be recorded to ascertain if there been a change since 2004. This latter work will be of significant therapeutic importance given the present widespread use of macrolide antibiotics.

3. To study HIV positive patients previously diagnosed with neurosyphilis and to re-evaluate those previously diagnosed and treated for neurosyphilis and develop institutional evidence-based guidelines for treatment.

4. To study the knowledge of syphilis, among sexual health clinic attendees and the management of syphilis by physicians specialising in infectious diseases and genitourinary medicine.

Chapter 2 –

The Epidemiology of Syphilis in Dublin 2007-2009

2.1 – Introduction

Following the discovery of penicillin, the incidence of syphilis fell dramatically, despite a rise during the 1960s, the rates of syphilis infection never reached pre-antibiotic levels. With the HIV epidemic, came increased awareness of sexually transmitted infections and an increase in safer sex practices and rates fell to historic lows in the late 1990s[40, 109, 110]. However, with the advent of highly active antiretroviral therapy (HAART) in 1996 attitudes changed again[122]. A shift in the demographic of the infection occurred and since the early 2000s, outbreaks in the western world have centred predominantly on men who have sex with men (MSM).

Post HAART, the first syphilis outbreak was reported in Hamburg in 1997[109], similar trends were seen in other European countries in the late 1990s and early 2000s. The largest outbreaks were reported in Paris, London and Dublin[109].

Hopkins *et al*[41] first reported on a large outbreak of syphilis in Dublin in 2001. This followed historically low levels of reported cases of syphilis during the 1990s[40, 123]. The mean number of cases reported per year in the 1990s was 15, five of which were classified as infectious[42] (i.e. primary secondary and early latent cases). In the eighteen months from January 2000 to June 2001, 181 cases of syphilis were reported, of which 121 (66.85%) were early infectious syphilis. In the preceding three years, the numbers of new diagnoses were in single figures and all cases were reported amongst heterosexual patients. In this initial report of the 2000-2003 outbreak, 92% of

patients were men who have sex with men (MSM) and the estimated incidence amongst MSM for the year 2000 was 46-77 per 100,000 compared to an estimated incidence of 1 per 100,000 in heterosexual patients. The mean age of patients in 2000-01 was 35 years and 92% were Irish, 22.5% of patients were HIV positive and of these patients 36% had newly diagnosed HIV[41]. The number of cases of syphilis diagnosed in Dublin remained high in the early 2000s[42], between January 2000 and June 2003, 610 cases were diagnosed; 58% of which were early infectious cases. Of the early infectious cases 17.4% of cases occurred in HIV positive individuals. The number of reported cases peaked in July 2001[40]. The vast majority of cases were diagnosed in Irish patients with only 18.1% of cases in patients born outside Ireland. HIV was concurrently diagnosed in 3.5% of patients with early infectious syphilis cases[40].

Worryingly 53% of patients who tested HIV negative at initial presentation with syphilis during the 2000-2003 outbreak did not undergo follow up HIV testing[42], leading to concern that HIV seroconversion in these patients may have gone unnoticed. HIV negative patients have been shown to be less likely to attend for serological follow up post syphilis treatment[124], meaning the opportunity for repeat HIV testing is lost.

The number of new cases decreased from 2003 on, due to several public health initiatives. The provision of additional clinical resources, employment of personnel for partner notification and contract tracing, design and distribution of educational materials and alerts for medical professionals, targeted

information campaigns among the MSM community and the innovative introduction of onsite testing in venues where MSM were likely to meet sexual partners were all employed[40].

On a European level, the overall number of reported cases of syphilis increased substantially in most Western European countries between 1998 and 2007[125], although a downturn in numbers was recognised from the mid 2000s. Similar outbreaks to the Dublin epidemic were reported in the U.K. [64, 126, 127]. Men accounted for the majority of the increase in cases. In Central and Eastern Europe, the pattern was reversed with a decrease in the number of cases in the same time period with the male to female ratio remaining unchanged. In studies of lesbian, gay, bisexual and transgender (LGBT) patients, many respondents have been found to be unwilling to disclose their sexual orientation to medical practitioners. Only 2-55% of LGBT patients in Eastern Europe were found to feel comfortable revealing sexual orientation to a health care provider[128].

Similar outbreaks amongst MSM have been identified worldwide. In the USA rates reached their lowest level since 1941 (when national reporting began) in 2000[110]. Between 2000 and 2003 rates increased 19%, with the increase solely accounted for by men[110]. In Canada, in British Columbia, which had reported an ongoing epidemic amongst it's heterosexual population in the 1990s, the number of cases amongst MSM rose in the early 2000s and 6% of cases were re-infections[129]. Re-diagnosis of syphilis was associated with being HIV positive, a history of chlamydia or gonorrhoea or being MSM. In

San Francisco, HIV infection was found to be the only factor associated with an increased risk of re-infection[130]. The importance of these patients presenting with re-infection, is that an epidemic may be sustained by a core group of people with repeated infection, maintaining a reservoir of infection within a specific community. Targeting such a group of core transmitters may impact on an epidemic.

The concern that HIV and syphilis can facilitate the transmission of one another[131, 132] adds an extra dimension to any syphilis outbreak. In Western Europe sexually transmitted infections are disproportionately diagnosed among HIV positive MSM[109]. In onsite testing in New York bathhouses, 44.9% of MSM reported having had unprotected anal intercourse in the recall period[133]. In Spain, in MSM between 2005 and 2007, HIV prevalence amongst syphilis cases was 29.8%[106]. In Boston, HIV infected MSM were more likely to have a seroreactive syphilis test than HIV negative men[134]. In addition, HIV positive patients diagnosed gonorrhoea, Chlamydia or syphilis were more likely to be asymptomatic[134]. The related trend of an increase in HIV diagnoses amongst MSM in North America, Western Europe and Australia since the year 2000[135] is especially worrying as no significant difference is seen between HIV positive and negative MSM reporting unprotected anal sex[136].

Many MSM will also have female partners[133], and spread of a syphilis outbreak into a heterosexual population may lead to an increase in cases of congenital syphilis[137-139]. Indeed MSM who do not identify themselves to be

"gay" may be one of the most difficult groups to target, in a pilot programme of HIV testing In bathhouses in New York City, men who were married to a woman were significantly less likely to have ever tested for HIV[133]. Estimating the MSM specific incidence of infections can be difficult, population surveys have found that approximately 2.6% of men report prior sexual contact with another man[140], although some feel this may be an underestimate[141].

Despite the inexpensive treatment available for syphilis, the overall economic cost of the infection should not be underestimated. The estimated direct medical cost of syphilis infection in 2006 in Illinois was $66,333, or a cost per case of $511.49[142]. While HIV has been associated with modulation of syphilis infection[48, 143, 144], response to therapy in HIV positive and negative individuals does not seem to differ significantly[124].

In the control of syphilis, the two main tools available are treating infectious cases and promotion of behavioural change and very little has changed in this approach in the last 60 years[145, 146]. While contact tracing or partner notification is not as cost effective as selective screening[147] it still has an important role in the identification and treatment of syphilis cases[23]. The approach set out in 1937 by the then surgeon general, Dr. Thomas Parran of; case finding, prompt and effective therapy, treatment of sexual partners and screening of selected populations[148] is still very relevant today.

Behavioural interventions have the potential for increasing awareness of sexually transmitted infections and for behaviour modification. Peer mediated

interventions have been shown to work in some risk groups[149]. Individually delivered cognitive behavioural therapy has been shown to significantly reduce transmission risk acts (such as unprotected anal or vaginal sex) in HIV infected MSM[150], unfortunately there was failure to maintain the effect ten months after conclusion of the intervention, highlighting the need for ongoing education of patients at risk of sexually transmitted infections. Interventions such as male circumcision which has been shown to decrease the transmission of HIV[151], have not been shown to have the same effect for *T. pallidum* infection[152].

There have been concerns expressed over the methods of reporting syphilis cases in Ireland. Dual reporting from both laboratories and clinicians led to duplication of case reporting. There is also concern that under-reporting is occurs as has been seen world-wide, in Germany in 1994 it was estimated that 84% of syphilis cases were not reported[153].

In response to a cluster of cases of ocular syphilis (Chapter 1) and given the concerns around syphilis reporting, the number of patients diagnosed with syphilis from 2007 to 2009 was reviewed.

2.2 – Methods

2.2.1 – The study population

All patients attended either the department of Genitourinary Medicine or Infectious Diseases (GUIDE) in St. James's Hospital, Dublin 8 or the Gay Men's Health Service (GMHS) in Baggot Street Hospital, Dublin 4.

2.2.1.1 – The GUIDE Clinic

The GUIDE clinic is the largest provider of HIV and sexual health services in the Republic of Ireland. The clinic has a total of more than 25,000 attendances per year, and provides outpatient clinics for HIV positive patients, sexual health and infectious diseases clinics.

The GUIDE clinic provides ongoing care for HIV positive individuals both as inpatients and outpatients. There are over 1500 HIV positive patients actively engaged in ongoing care, of whom 26.25% are men who have sex with men (MSM). The majority of the HIV positive MSM are less than 50 years of age (figure 2.1), and 71.49% are Irish. All HIV services, including the provision of highly active antiretroviral therapy (HAART) are free of charge.

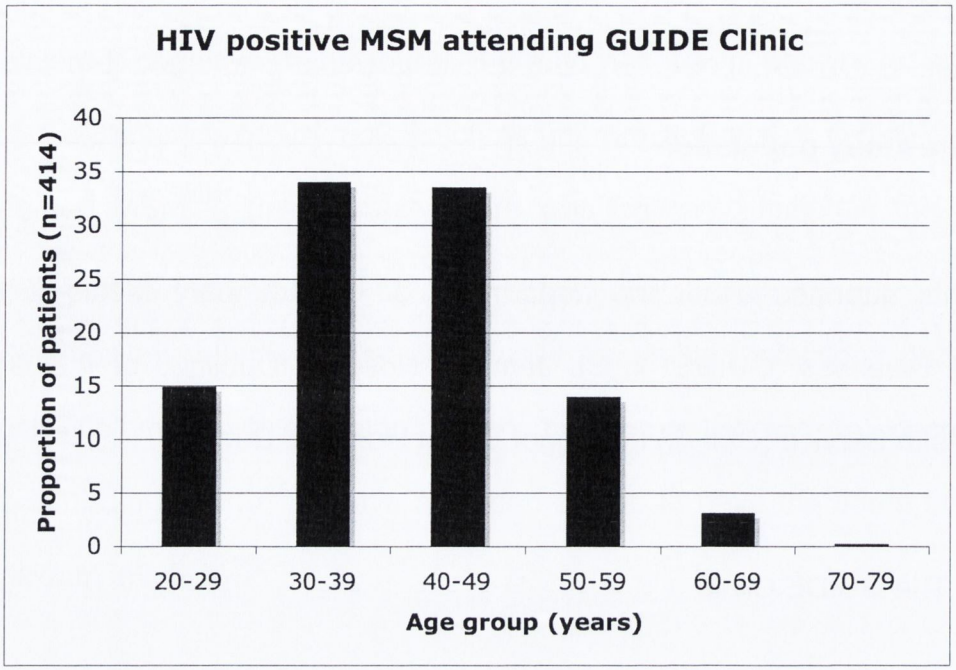


Figure 2.1 – Age Group of HIV positive MSM attending the GUIDE clinic

The GUIDE clinic also provides free sexual health screening and follow up care. There are five sexually transmitted infection (STI) clinics a week. Services can be accessed free of charge and do not require doctor referral. Walk in clinics operate on a first come first served basis, with the exception of symptomatic patients, who are seen on a triaged basis. In 2007 there were 6907 new attendances to the STI clinic and a total attendance of 14704 patients. In 2008 and 2009 there were 6792 and 4027 new attendances respectively and a total attendance of 13901 in 2008 and 17665 in 2009.

2.2.1.2 – The GMHS Clinic

The GMHS clinic is an outreach clinic of the GUIDE department and is a sexual health clinic with the specific aim of providing sexual health care for MSM. It is an evening clinic, which, operates on a first come first served basis, with the exception of symptomatic patients who are triaged. Until October 2009 there was only one full STI clinic per week with a second evening session offering blood testing for HIV, syphilis and hepatitis. Since October 2009, two evening clinics have operated offering full sexual health screening and treatment to attendees.

The GMHS clinic had 655 newly registered patients in 2009, and saw greater than 5000 patients in total. There were 580 and 536 newly registered patients for the years 2008 and 2007 respectively. The proportion of Irish patients did not change year on year, with 63%, 64% and 65% of patients being Irish in 2007, 2008 and 2009 respectively. The age of profile of newly registered patients is demonstrated in table 2.1.

Age (years)	<19	20-24	25-29	30-39	40-49	50-59	>60
2009 (n=655)	3%	21%	30%	30%	11%	3%	2%
2008 (n=580)	5%	29%	34%	20%	9%	2%	1%
2007 (n=536)	4%	24%	31%	25%	10%	5%	1%

Table 2.1 – Age group of newly registered patients in GMHS clinic

2.2.2 – Study Methods

All positive syphilis serology results from the department of Microbiology in St. James's Hospital from January 2007 to December 2009 were identified. Patients whose serology was drawn at the GUIDE sexual health clinic or HIV clinic or at the Gay Men's Health Service (GMHS) Clinic were included. Patients were included if they had a newly positive syphilis serology or in the case of patients with previously diagnosed and treated syphilis infection, had a four-fold rise in RPR titre.

Cases were then correlated with a database of syphilis diagnoses held in the GUIDE department or case notes were reviewed to ensure that only new infections were captured. Data was collected on patient demographics, risk factor, country of origin, HIV status, stage of syphilis diagnosis and treatment received. In the case of HIV infected individuals, results of CD4 count and HIV viral load at the time of syphilis diagnosis was also recorded along with HAART regimen in those patients on therapy.

Statistical analysis was performed using SPSS version 17.0 commercially available software

Crude incidence rates were calculated using population data available form the 2006 Irish Census[154]. For the purposes of statistical analysis, sexually active adults were taken persons over the age of 15 years.

2.3 – Results

Four hundred and thirty nine new diagnoses of syphilis were made in the GUIDE and GMHS clinics in the three-year period from January 2007 to December 2009. These diagnoses were made in 412 patients. 287 (65.38%) cases were diagnosed in the GUIDE clinic and 152 (34.62%) cases in the GMHS clinic. Of the 24 patients who had more than one episode of syphilis in the study period, 21 patients had two episodes of syphilis infection and three patients had 3 episodes. The mean age of patients at diagnosis was 35.7 years (range 17-73 years). 412 (93.8%) cases occurred in men. The greatest numbers of diagnoses were made in 26-35 year olds, and over half of the diagnoses were made in those less than 35 years old (table 2.2 & figure 2.2).

The number of syphilis diagnoses increased year on year from 2007 to 2009. 95/439 (21.6%) occurred in 2007, 136/439 (31%) in 2008 and 208/439 in 2009 (47.4%) (figure 2.3). When the number of cases is broken down by year quarter, again a yearly and quarterly increase can be seen (figure 2.4). The proportional increase was 43% from 2007 to 2008 and an increase of 118.9% from 2007 to 2009. The number of diagnosis made in each age group did not differ significantly year on year (p=0.75).

Age Group (years)	Total Number of Patients	Percent
<= 25	74	16.9
26 - 35	156	35.5
36 - 45	132	30.1
46 - 55	65	14.8
56 - 65	8	1.8
66+	4	.9
Total	439	100.0

Table 2.2 – Age group of patients presenting with syphilis 2007- 2009

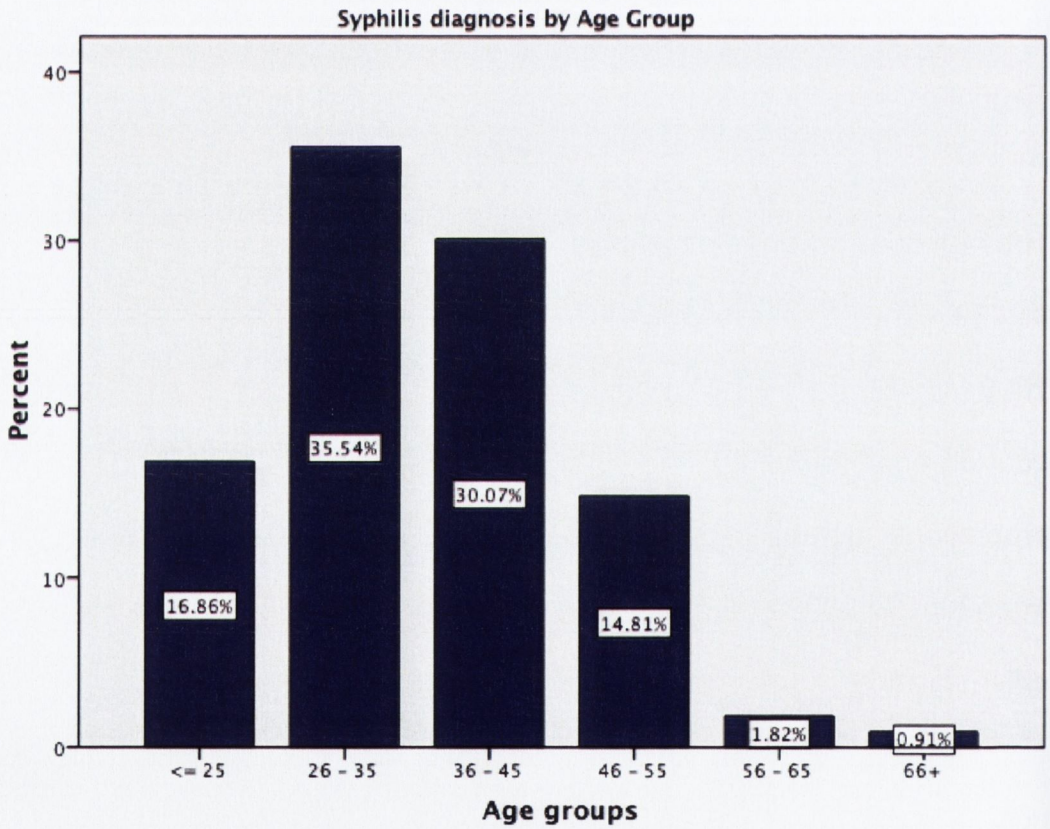


Figure 2.2 – Age groups of patients diagnosed with syphilis 2007-09.

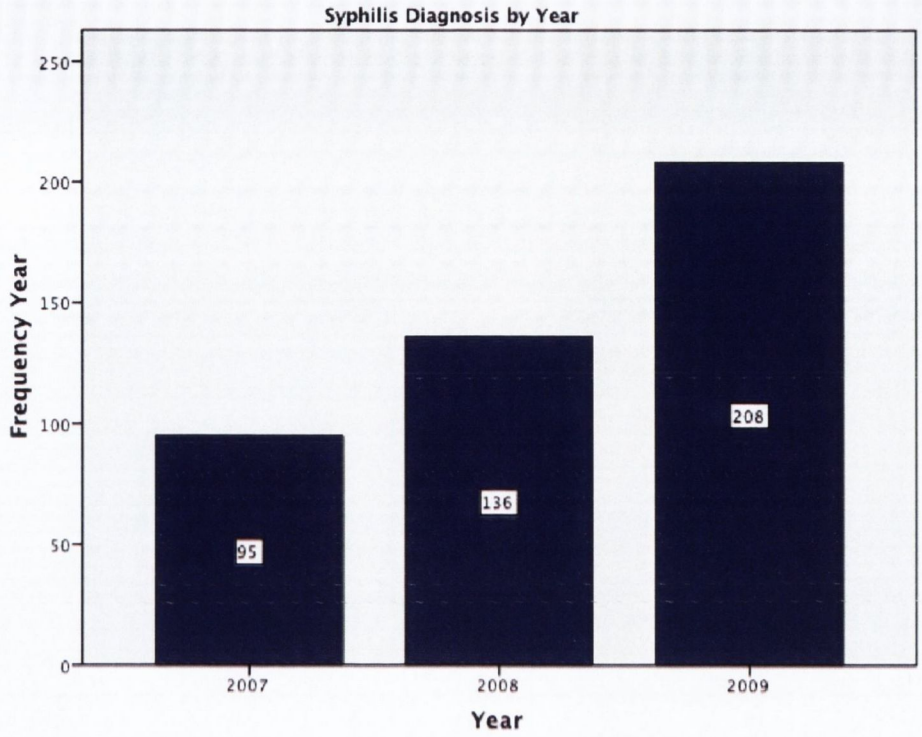


Figure 2.3 – Number of syphilis diagnoses per year

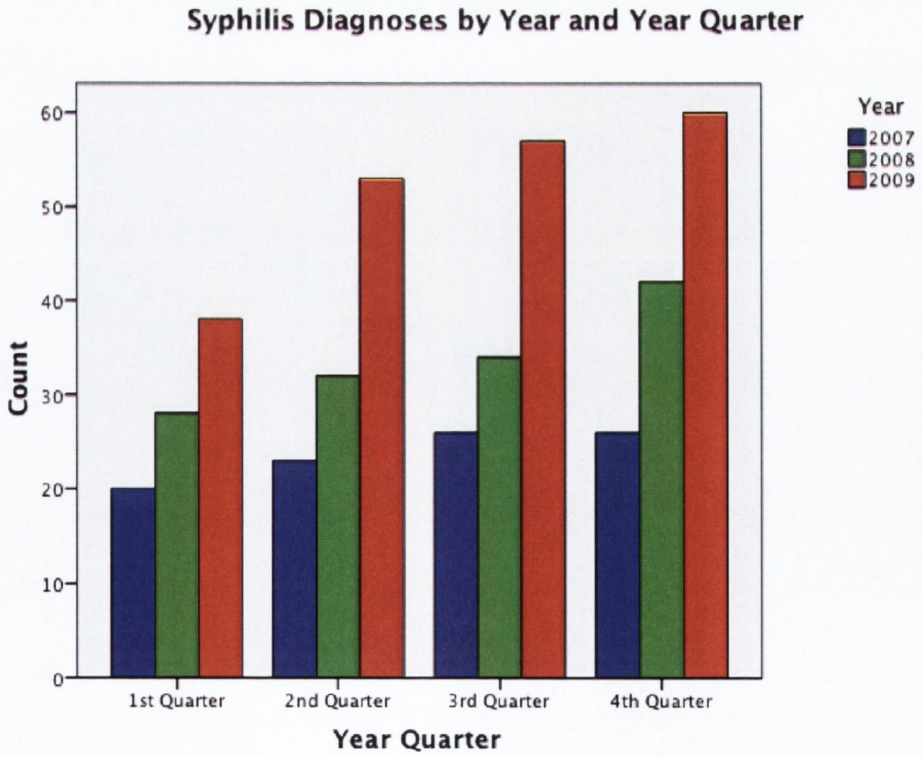


Figure 2.4 – Number of Syphilis diagnoses per year quarter.

Crude incidence rates for the population of Ireland (15 years of age or older)[154] are 6.16 per 100,000 population for 2009. If the proportion of MSM is 2.6% as has been reported by others[140] the crude incidence rate for all of Ireland amongst MSM would be 378.16 per 100,000 population.

Information on the risk of acquisition of syphilis was available in 433/439 (98.6%) of cases. 381 (86.8%) of cases occurred in MSM. The remaining 52 cases in heterosexual patients, 29/52 cases (6.6% of total) were diagnosed in patients from an area of high syphilis prevalence. There was a significant difference in presentation of MSM compared to heterosexual groups ($p<0.001$) (figure 2.5). 66.3% of syphilis diagnoses occurred in Irish patients (figure 2.6), all geographical areas were represented in the remaining patients.

Of the 439 diagnoses of syphilis made, data relating to the stage at diagnosis was available for 418 cases (table 2.3). Early infectious syphilis accounted for the majority of cases. 96/418 (22.96%) were primary cases, 104/418 (24.88%) were secondary cases and 164/418 (39.23%) were early latent cases. The stage at diagnosis differed significantly between the three years ($p=0.013$). In 2009, 24.46% of cases were primary syphilis compared to 22.34% and 21.32% in 2007 and 2008 respectively, while the proportion of secondary cases in 2009 dropped to 20.21% from 27.66% and 29.41% in 2007 and 2008 respectively (figure 2.7). The one case classified as a reactivation was in a patient who despite adequate treatment had a four-fold rise in RPR and denied any sexual contact post penicillin therapy.

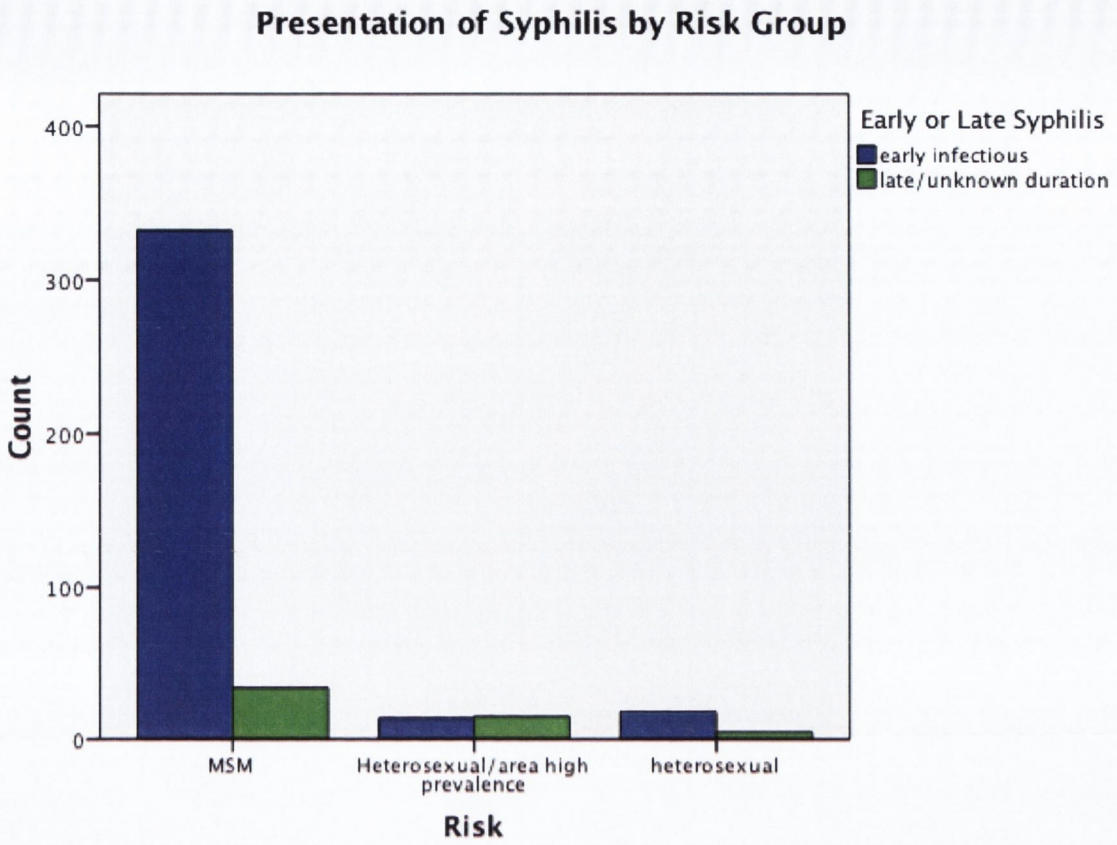


Figure 2.5 – Presentation of syphilis in different risk groups

Stage	Number of Patients	Percent
Primary	96	21.9
Secondary	104	23.7
Early latent	164	37.4
Late latent	53	12.1
Reactivation	1	.2
Sub-Total	**418**	**95.2**
Unavailable	21	4.8
Total	**439**	**100.0**

Table 2.3 – Syphilis Stage at Diagnosis.

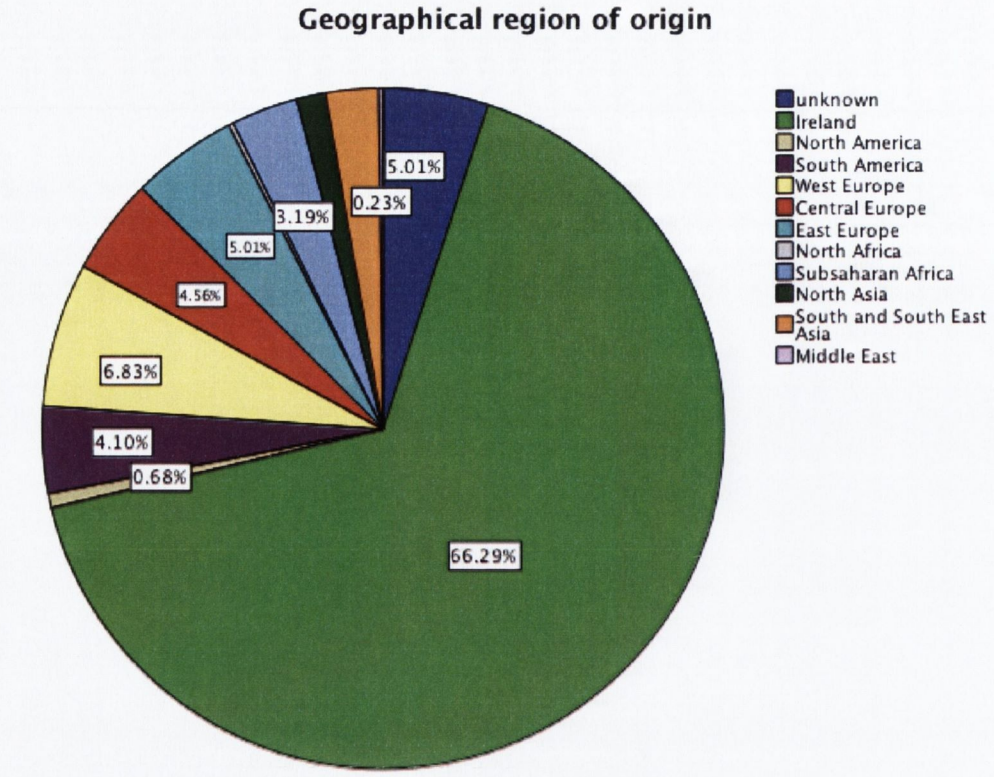


Figure 2.6 – Region of Origin of Patients diagnosed with Syphilis.

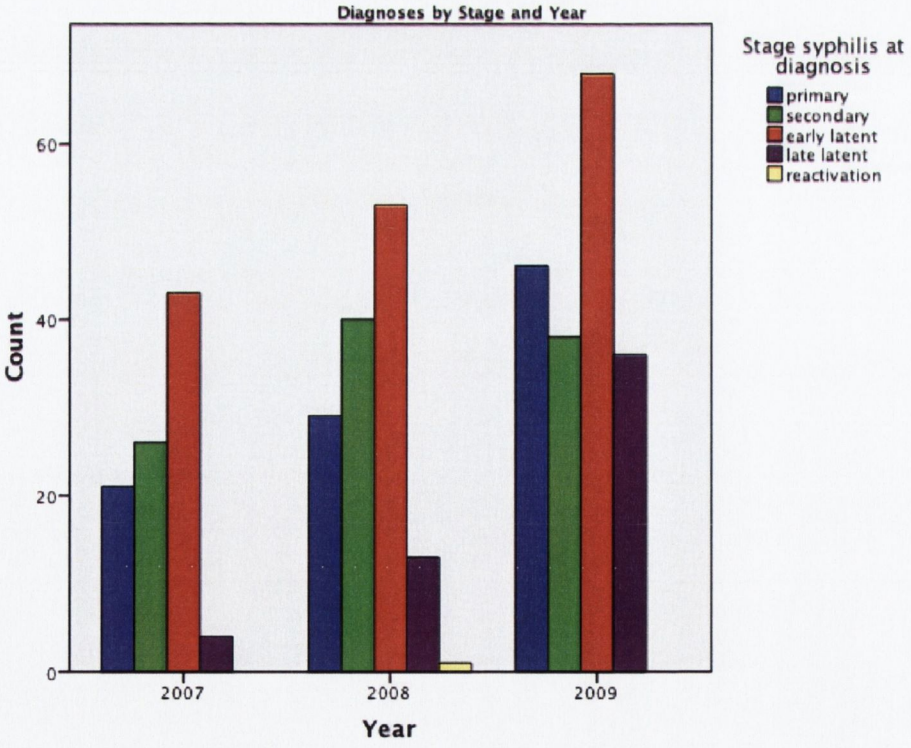


Figure 2.7 – Syphilis stage at diagnosis year by year.

47.8% of cases were of symptomatic syphilis (i.e. either primary or secondary syphilis) 52.2% were of asymptomatic syphilis (i.e. latent syphilis of any duration). The rate of return for HIV testing amongst patients presenting with primary syphilis was low, only 22% of patients had repeat HIV serology. HIV status was unavailable for 18 (4.1%) of cases. Of the remaining cases, 126/421 (28.7%) occurred in HIV positive patients.

2.3.1 - HIV positive patients

Of the HIV positive patients, all but one were MSM. The mean age of HIV positive patients was 38.59 years (95% CI 37-40.6 years, range 20-62 years) this was significantly older than the HIV negative patients, mean 34.1 years (95% CI 32.9-35.2 years, range 17-73 years) two-tailed significance $p<0.001$. 68.3% of diagnoses of syphilis occurred in Irish HIV positive patients.

The mean CD4 count at diagnosis in HIV positive patients was 508 cells/mm^3 (median 525 cells/mm^3, range 13-1283 cells/mm^3). 48 (38.1%) of HIV positive patients were virally suppressed at the time of their syphilis diagnosis. The mean duration since commencement of HAART was 24 months in those patients receiving therapy. No significant alteration in HIV viral load occurred pre and post syphilis diagnosis and treatment.

42/126 (33.3%) episodes of syphilis occurred in patients who received a new diagnosis of HIV at the time of their syphilis diagnosis. The mean age of patients newly diagnosed with HIV was 34.79 years (95% CI 31.7-37.9 years)

significantly lower than that of patients who were aware of their HIV serostatus mean 40.3 years (95% CI 38.6-42.05 years) p=0.002. The mean CD4 count between the two groups did not differ significantly at 509 cells/mm^3 (95% CI 418-601 cells/mm^3) in the new diagnosis group and 510 cells/mm^3 (95% CI 462-558 cells/mm^3) in those previously diagnosed with HIV. The mean HIV viral load in those patients newly diagnosed with HIV was 52253 copies/ml (range 303- 500001 copies/ml).

There was no statistical difference in the presentation of HIV positive and HIV negative patients when patients were divided into groups of symptomatic and asymptomatic presentation (p=0.8).

2.3.2 – Syphilis Re-Infection

68 (15.5%) episodes of syphilis infection were diagnosed in patients who had had previously been diagnosed and treated for syphilis. These patients had a mean age of 42.1 years (95% CI 40.1-44.1, range 25-73 years) significantly higher than those diagnosed with syphilis for the first time (mean 34.6 years, 95% CI 33.6-35.6, range 17-67 years) p<0.001.

The mean time since first diagnosis of syphilis was 48.58 months. This did not differ significantly among HIV positive patients (mean 45.63 months, 95% CI 36.1-55.1 months) and HIV negative patients (mean 53.9 months, 95% CI 27.5-80.3 months).

43/68 (63.2%) cases of re-infection occurred in HIV positive patients, this accounted for 34.1% of cases diagnosed in HIV positive individuals. This differed significantly from the proportion of cases of re-infection diagnosed in HIV negative patients at 0.08% $p<0.0005$. Only one patient who presented with a syphilis re-infection had a new diagnosis of HIV, all the other patients were aware of their HIV diagnosis at the time of syphilis re-infection.

Of the cases of syphilis re-infection, the number of previous infections was; 1 prior episode in 56/68 (82.3%) cases, 2 prior episodes in 9/68 (13.2%) and 3 prior episodes in 3/68 (4.4%) cases.

The IgM response was not seen as frequently in patients presenting with syphilis re-infection, 34/68 (50%) compared to those presenting with a first episode of syphilis, 242/368 (65.8%) Patients presenting with a syphilis re-infection were significantly less likely to demonstrate a positive IgM response $p <0.0001$.

2.4 - Discussion

The rising number of syphilis diagnoses over the three years from 2007 to 2009 is concerning. The vast majority of cases are occurring in MSM and efforts must focus on the MSM community.

The concern with the rising rate of syphilis diagnoses is that an associated increase in new HIV diagnoses will also be seen. There is a suggestion in this data that serosorting is occurring within the HIV positive MSM community in Dublin. The majority of syphilis re-infections were occurring in HIV positive MSM, and it has been shown that while sero-sorting will decrease the transmission of HIV, subsequent increases in other STIs may occur[155]. The message of safer sex must be reiterated to HIV positive patients engaged in ongoing care.

Whatever the reasons for the current increase in syphilis diagnoses, which are most likely multifactorial, it cannot be ignored that there is currently a syphilis problem among MSM in Dublin. In response to this, a multidisciplinary team was set up, in which the author had an active part. This team consisted of medical professionals involved in sexual health and HIV services, as well as those in the Department of Public health, along with representatives of the gay lesbian and transgender community in Dublin. Initiates included the streamlining of syphilis reporting to the Dept. of Public Health, educational campaigns, the provision of additional sexual health screening services in the

GMHS clinic and a pilot of onsite testing, in venues where MSM are likely to meet sexual partners.

Work must continue in an effort to decrease the number of new syphilis diagnoses. Education not only of the MSM community about the value of being tested for syphilis but also of providers who may not recognise the symptoms of syphilis is of the utmost importance. Identification of infectious individuals and prompt and adequate treatment of their syphilis is our most effective tool in combating the current outbreak.

Chapter 3 –

The use of Polymerase Chain Reaction (PCR) & Syphilis IgM in the diagnosis of Syphilis & Azithromycin resistance in *T. pallidum* isolates in Dublin

3.1 – Introduction

As *T. pallidum* is not amenable to laboratory culture, the diagnosis of syphilis relies in the most part on serological testing. Darkground microscopy can be used in patients presenting with primary or secondary lesions that are amenable to sampling, however most diagnoses are made on a positive serological test. Serology is an indirect method of diagnosis. Serological diagnosis is useful for screening at risk patients and monitoring response to therapy, however, it lacks sensitivity in some stages of syphilis and lacks specificity in some populations[61].

The first serological test for syphilis was developed in 1906 by Wassermann[61] which was around the same time that the use of dark ground microscopy was also first described. Wassermann *et al* used antigen from liver tissue from newborns who had died from congenital syphilis[156]. Extracts of tissues, which did not contain the organism, were found to be equally effective. These complement fixation tests made a huge impact on the diagnosis of syphilis, however they were complicated to perform and took 24 hours to complete[61]. Subsequently work using water and sodium chloride extracts of liver led to the first flocculation tests that did not require complement fixation. These however, had the disadvantage of variation in the concentration of antigen present. In the 1940s with the isolation of cardiolipin, standardised serological tests were developed[61].

Traditionally laboratories use an algorithm of screening samples with a non treponemal test such as an RPR, and confirming positive results with a treponemal test such as TPPA[61]. However, when this algorithm was reviewed in New York laboratories concern arose that potential positive samples would be missed[81]. The current algorithm would suggest screening with an automated treponemal test such as an enzyme immunoassay (EIA) and testing reactive samples with non-treponemal tests such as the RPR and VDRL and treponemal tests such as TPPA subsequently[81]. This is the practice in the department of microbiology in St James's Hospital.

It is reported that up to 30% of patients presenting with primary syphilis will have negative syphilis serology[61, 65]. As a result it is recommended that patients presenting with suspected primary syphilis and negative dark ground microscopy should return for repeat dark field microscopy examinations and syphilis serology[63]. Given that low numbers of both microbiology laboratories and GUM clinics have darkground microscopy facilites[66], and the concern that practitioners may become deskilled in obtaining dark ground microscopy samples[64], the reliance on serology for the diagnosis of syphilis has increased. Early diagnosis is important in interrupting the spread of infection, however there is a wide differential diagnosis for genital ulceration[44] and the positive predictive value of clinical examination alone is 78%[157].

With the advent of newer molecular techniques and the complete sequencing of the *T. pallidum* genome in 1998[36], the use of polymerase chain reaction (PCR) as a method of diagnosing syphilis was investigated. Several different

PCR assays for *T. pallidum* targeting varying genetic sites have been described. The two most common assays target either the 47kDa integral membrane lipoprotein gene[83, 158, 159] or the DNA polymerase I gene of *T. pallidum* (*pol*A)[67, 82, 84, 85].

In 2000 Liu *et al*[84] reported on the use of a PCR method targeting the *pol*A gene of *T. pallidum.* The primers were designed to take advantage of the high cysteine content and additional inserts in the DNA polymerase I gene of *T. pallidum*[160]. To determine the specificity of the assay the authors tested it against a panel of organisms. The assay was found to be specific to *T. pallidum*. When the sensitivity of the assay was investigated, it was shown to consistently detect as low as 10 organisms per sample, which was further improved to one organism per sample by labelling the primers with fluorescent dye[84].

Leslie et al[82] developed an assay in 2006 that has subsequently been used by others prior to the detection of azithromycin resistance in *T.pallidum*[161]. Genital, anorectal, mouth and superficial skin swabs along with cerebrospinal fluid (CSF) were analysed. The detection limit of the assay determined by using serial dilutions of a positive control was 35 organisms per swab (1.75 target copies per reaction) at a Ct of 38.4. The authors set an assay cut-off at cycle 38, and samples with a Ct value greater than 38 were deemed negative. When compared to serological testing the assay showed 95% agreement with a sensitivity of 80.39% and specificity of 98.4% for the detection of early syphilis.

More recently a Dutch group looked the clinical value of real-time PCR with regard to primary and secondary syphilis[85]. An assay targeting the *pol*A gene of *T. pallidum* was utilised. Interestingly the authors described a great deal of discrepancy between the real-time PCR and the dark-field microscopy results, 47 cases were positive by both methods whereas there were 53 discrepant results- 7 cases were positive by dark-field microscopy only and 46 were positive by PCR alone. The sensitivity and specificity of the assay are reported as 87% and 93.1% respectively. However, when the authors looked at those samples received from patients with suspected secondary syphilitic lesions (skin scrapings of abraded lesions) the specificity was low at 43%. They conclude that real-time PCR is a fast and reliable test for primary syphilis.

Following infection with *T. pallidum* early and widespread dissemination is thought to occur due to the many organ systems, including the central nervous system (CNS) that the organism can invade[44]. To date much of the knowledge of the pathogenesis of *T. pallidum* has been evolved from animal studies. Following intratesticular injection with treponemes, orchitis develops and resolves after 7 -11 days as host mechanisms, particularly activated macrophages and opsonic antibodies clear the organisms[162]. In rabbit models to investigate the dissemination of treponemes, real-time quantitative PCR was used to detect treponemal DNA in blood following intratesticular injection[163]. As early as 24 hours post injection *T. pallidum* DNA was detected in plasma and whole blood. By day ten post injection, DNA was detectable in whole blood, plasma, serum and peripheral blood mononuclear cells. The

increases coincided clinically with the development of orchitits and serologically with RPR titres. From 10-14 days post inoculation DNA was detected in 40% of brain tissue samples, 50% of kidney samples and nearly all liver and spleen samples. The observation of uneven distribution of bacteria in various blood components, demonstrated that serum samples are less reliable source for detecting circulating treponemes than whole blood or plasma. One Swiss study demonstrated a higher Ct value in the PCR of blood of patients presenting with primary syphilis compared to lesions swabs whereas the Ct value from the blood of patients presenting with secondary syphilis was variable[86].

The diagnosis of neurosyphilis can be especially problematic[120] particularly given that up to on third of patients with neurological involvement will be asymptomatic[44]. Pre-antibiotic era data suggests that patients with asymptomatic neurosyphilis that fails to resolve may be more likely to develop long term neurological sequelae[50]. In the rabbit model 40% of brain tissue samples were *T. pallidum* DNA positive by day ten post intratesticular inoculation. In humans, an early study investigating the value of PCR in investigating neurosyphilis, found 52.6% of CSF samples positive in patients undergoing lumbar puncture for the investigation of neurosyphilis in late latent disease[164]. Subsequent series demonstrated treponemal DNA to be detectable by PCR in the CSF at all stages of disease[165] this mirrored the findings of rabbit inoculation studies in the 1920s, however the presence of *T. pallidum* DNA in the CSF did not induce any consistent laboratory findings.

In a Dutch series while all patients with acute neurosyphilis were found to have syphilis DNA in their CSF prior to treatment while in patients with asymptomatic neurosyphilis DNA was detectable in the CSF of only 2 of 16 patients[166]. In the follow up of patients with acute neurosyphilis, 6/7 patients were found to have persistently detectable *T. pallidum* DNA in their CSF 1-21 months after intravenous penicillin treatment. Again, no correlation was found between CSF VDRL positivity and the presence of *T. pallidum* DNA within the CSF.

Penicillin has been used for years in the treatment of syphilis, it was first reported in 1943 that penicillin cured syphilis[94]. Penicillin G Benzathine was introduced in 1956[167] and has been used in the treatment of syphilis ever since. Despite it's long term and widespread use there has been no reported resistance of *T. pallidum* to penicillin. Parenteral penicillin is the treatment of choice in the treatment of syphilis[63, 69]. Benzathine penicillin at a dose of 2.4 MU intramuscularly weekly from one to three weeks for non-neurological infection and either procaine penicillin 1.2MU-2.4MU intramuscularly, with the addition of probenecid, or Benzylpenicillin intravenously in the case of neurosyphilis is the recommended treatment for syphilis infections.

However, despite the effectiveness of these treatments for syphilis there can be practical issues that arise in administering the therapy. The parenteral route makes the treatment less tolerable for many patients than orally administered medication. When multiple doses of penicillin must be given over days or weeks, the concern arises that patients may not return for further

treatment. In addition in patients reporting a penicillin allergy, therapy can be further complicated by the need for penicillin desensitisation, which while the preferred choice may not always be practicable[99]. Up to 10% of patients, report a penicillin allergy. In a recent survey of U.S. practitioners Dowell *et al*[168] found that while 79% of doctors treating patients with syphilis would recommended penicillin desensitisation, 39% state that it is not always done, citing patient refusal and practical reasons setting up the procedure as the most common problems encountered. Tetracyclines, the alternative therapy for penicillin allergic patients cannot be administered in pregnancy and may not be as effective in treating syphilis.

Starting in the early 2000s several studies looked at the effectiveness of azithromycin in the treatment of syphilis. This semi-synthetic azalide[169] antibiotic (a subclass of the macrolides) has the distinct advantage of being orally administered, it also has a long half life of 68 hours and is used in the treatment of other sexually transmitted infections[170, 171]. In 1990 azithromycin was shown to inhibit protein synthesis in *T. pallidum*[172] and animal models demonstrated it's efficacy in the treatment of syphilis infected rabbits[95]. A small pilot study in 1994 demonstrated a cure in 11 of 13 patients given 500mg of azithromycin daily for ten days[173]. Further studies examining the use of a single dose of 2g of azithromycin or two doses of 2g one week apart followed. In a pilot of study of 74 patients, no significant difference was found in serological response in comparable groups of patients receiving azithromycin therapy or Benzathine penicillin[100]. A similar study with much larger patient numbers performed in Rakai, Uganda, evaluated the use of

azithromycin either alone or in combination with Benzathine penicillin in the treatment of syphilis[97]. The study showed higher cure rates amongst those patients with higher initial RPR titres receiving either azithromycin alone or azithromycin therapy combined with Benzathine penicillin. However the study was a secondary data analysis and non-specifically set up to evaluate azithromycin therapy, in addition all patients with confirmed serological syphilis infections received penicillin.

The first randomised controlled trial, studied a single dose of 2g of azithromycin versus 2.4 MU of Benzathine penicillin in patients in Tanzania[96]. The study found equivalent rates of cure with azithromycin and Benzathine therapy (97.7% and 95% respectively) at nine months post treatment. Additionally using azithromycin in patients with incubating syphilis was found to be cost effective alternative in situations where penicillin therapy was not available[98] and it's use was reported to be effective in certain outbreak situations[174]. These studies lead to the hope that azithromycin would be a plausible alternative to penicillin therapy, particularly in penicillin allergic patients.

In 2004, eight cases of azithromycin treatment failure amongst patients with syphilis were reported in San Francisco[102]. Three of the patients had primary lesions, which failed to resolve following administration of 2g of azithromycin, two of these lesions were penile ulcerations, which remained darkfield positive post azithromycin therapy. The remaining cases were seronegative patients

who received azithromycin following contact with syphilis and subsequently seroconverted.

Azithromycin is a macrolide antibiotic and the target site for macrolides is the large (50S) subunit of the bacterial ribosome[175]. Many cases of macrolide resistance in clinical strains of a variety of pathogens can be linked to the alteration of specific nucleotides in the 23S rRNA within the large ribosomal subunit. Adenosine 2058 is the key nucleotide involved in macrolide interaction on the ribosome. A2058 to G was the first rRNA mutation to be shown to confer erythromycin resistance in organisms and gives the highest level of resistance relative to other mutations. This A2058G mutation was identified in the street 14 strain which is resistant to macrolide antibiotics such as azithromycin[176].

In a multicentre study published in 2004, Lukehart *et al*[103] reported on azithromycin resistance in the United States and Ireland. Samples from Ireland were obtained through the GUIDE department in St. James's Hospital. Of the 114 samples, tested 32 (28%) were found to have the A2058G mutation. The centre with the highest proportion of azithromycin resistant samples was Dublin, where 15/17 samples or 88% were found to have the mutation compared to 22% in San Francisco, 13% in Seattle and 11% in Baltimore. In San Francisco the proportion of samples containing the mutation was also found to have increased form 4% in the period 1999-2002 to 37% in 2003[103]. Interestingly when eighteen historical samples were analysed from various locations from 1912 to 1987 only one, the street 14 strain was found

to have macrolide resistance. The street 14 strain was isolated from a patient whose failure to respond to erythromycin therapy was reported in 1977[169]. The majority of patients who presented with syphilis, which was resistant to azithromycin therapy, were MSM.

Several centres have since evaluated the rate of azithromycin resistance amongst patients presenting with syphilis. In San Francisco between 2000 and 2004, 46 out of a total of 118 contained mutant 23S rRNA genes[177]. The mutation was identified in 1/25 (4%) isolates from 2000-2002 and in 13/32 (41%) from 2003. This prompted the discontinuation of the use of azithromycin for the treatment of primary and secondary syphilis and for incubating syphilis, which had commenced in 1999 in response to the rapid increase in syphilis diagnoses in San Francisco[101, 169, 174]. A similar pattern of emerging resistance was seen in British Columbia, Canada[178] and in Seattle, USA[179]. In the Canadian series, 1/47 (2%) of samples were resistant from 2000- 2003 rising to 4/9 (44%) in 2004. In Seattle, the odds of a syphilis strain having the mutation increased significantly over the years 2001-2005 (p=0.006). However, worldwide resistant rates vary, with no resistant isolates identified in Madagascar (2008) [180], 28.6% in Alberta, Canada (2007-2008)[181] to 100% in Shanghai, China (2007-2008)[182].

Marra *et al*[179], have shown that the mutation is present in greater than two syphilis strains, which refutes the argument of a single resistant strain circulating amongst some high risk populations. In addition, they found that patients who had received azithromycin therapy in the twelve months prior

were twice as likely to have a resistant strain of syphilis, suggesting that antibiotic selection has contributed to the increase in macrolide resistant *T. pallidum.*

3.1.1 – PCR Assay Principle

TaqMan PCR chemistry target detection is based on specific hybridization between a probe and a specific target region within the treponemal genome. The probe is labelled with a fluorescent dye thus enabling the detection of a specific PCR product as it accumulates during PCR cycles. The amount of fluorescence is proportional to the product being produced for a given sample, therefore the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. This use of fluorogenic probes eliminates post-PCR processing and reduced assay labour and material costs. As probes can be labelled with different distinguishable reporter dyes, amplification of two distinct sequences is permitted in one reaction tube. This allows for the development of a separate heterologous amplification reaction that facilitates the inclusion of an internal control in the assay. The spiking of exogenous internal control into patient samples, which is then co-amplified with the target, not only controls the entire process from nucleic acid extraction to detection, but also serves to check for possible PCR inhibition.

Cycle sequencing is a simple method in which successive rounds of denaturation, annealing, and extension in a thermal cycler results in a linear amplification of extension products. The chain of bases has an equal affinity

for both dideoxynucleotide triphosphates (ddNTP's) and deoxynucleotide triphosphates (dNTP's). However, ddNTP's lack the free 3' hydroxyl group necessary for another base to join the chain so it is terminated at that point. By incorporating into the reaction mix the four ddNTP's, each tagged with a different fluorescent dye, the growing chain is simultaneously terminated and labelled with the dye that corresponds with that base. When electrophoresed on a gel or in a capillary, the extension products migrate in size order and the DNA sequencer detects fluorescence from the four different dyes. Each dye emits at a different wavelength when excited by an argon ion laser. All four colours and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection.

3.2 – Materials and Methods

All patients who had a sample sent for darkfield microscopy examination between January 2007 and March 2009 were identified. Data was collected on patient demographics, the specimen site, concurrent and subsequent syphilis serology. Samples sent for syphilis serology were screened with syphilis enzyme immunoassay (EIA). Positive samples had a rapid plasma reagin (RPR), *Treponema pallidum* particle agglutination (TPPA) and immunoglobulin M (IgM) performed.

Patients with a positive syphilis IgM from January 2007 to December 2009 were identified. The stage of infection in all patients with positive syphilis IgM was recorded. The rate of positive syphilis IgM in patients presenting with

syphilis re-infection was also assessed. Syphilis re-infection was defined as a four-fold increase in serum RPR despite adequate syphilis treatment in patients reporting further risk for syphilis acquisition.

3.2.1 – Sample Collection for PCR analysis

Samples (n = 107) were collected from patients (n=92) attending the Genitourinary Medicine and Infectious Diseases (GUIDE) clinic. Whole blood collected in EDTA containers, exudate from suspected primary genital lesions, cerebrospinal fluid (CSF) and tissue samples from patients undergoing biopsy were collected.

Patients with lesions, which clinically resembled primary syphilis, had samples of the exudate sent for PCR testing (n=35). In addition patients who presented with potential incubating syphilis or early infectious syphilis (i.e. clinical primary or secondary disease or those asymptomatic with positive serology who acquired their syphilis within the last two years) had whole blood drawn (n=57). Patients undergoing lumbar puncture for investigation of neurosyphilis also had a sample of CSF collected for analysis (n=12). Any patient who had a skin or other lesion requiring biopsy had a sample collected for PCR analysis where possible (n=3). All samples were stored at -20°C until DNA extraction was performed.

Darkground (dark-field) microscopy was performed as per the GUIDE clinic protocol on any samples suspected of being a primary or secondary syphilitic

lesion. Microscopy was performed on a microscope fitted with a reflecting dark-field condenser using a x 40 objective. All samples were inspected by laboratory staff experienced in darkground microscopy. The remaining sample was placed in in 250 µl Abbott multi-Collect Specimen Collection kit (Multi-collect Buffer; Abbott Molecular, Des Plaines, IL) and stored at -20°C until DNA extraction was performed. Syphilis serology was performed by the use of a screening enzyme immunoassay (EIA) using the ARCHITECT Syphilis TP assay (Abbott Molecular, Des Plaines, IL). Sera found to be EIA positive was then tested with a non-treponemal rapid plasma reagin (RPR) diagnostic test, the Syphscreen RPR Test (Axis-Shield, Dundee, Scotland) and a treponemal *T. pallidum* particle agglutination (TPPA) the SERODIA-TP-PA test (Malvern, PA USA). T. pallidum IgM was also performed on selected samples using the Mercia Syphilis M assay (Microgen Bioproducts, Surry, UK)

The samples were collected and given and a non-identifying study number. Data was collected on patient demographics.

Template DNA from the samples found to be positive for *T. pallidum* DNA were analysed to investigate for azithromycin resistance, determined by the presence of the A2058G mutation. Samples included exudate from genital lesions (n=19), whole blood (n=12), and tissue (n=2) and CSF (n=1) samples.

3.2.2 – Sample storage

All samples were stored at - 20°C until DNA extraction was performed. Genital exudate samples were stored in 250 µl Abbott multi-Collect Specimen Collection kit (Multi-collect Buffer; Abbott Molecular, Des Plaines, IL)

3.2.3 – *T. pallidum* DNA using real-time PCR

3.2.3.1 – Primers and Probes

The primers and probes used in the assay to detect *T. pallidum* were those previously described by Leslie *et al*[82]., (Table 3.1). The test probe is a *T. pallidum*-specific FAM-labelled MGB hydrolysis probe. The target is a 67-bp sequence within the *pol*A gene of *T. pallidum,* sequence (nucleotides 2001 to 2067) (GenBank accession no. TPU57757). *T. pallidum* primers and probe are supplied by Applied Biosystems (Applied Biosystems, USA). The final concentration of both forward and reverse primers is 900 nM and 250 nM for the probe. The primers and probes are stored at - 20°C and denoted TP- F, TP- R and TP- Pr.

The *T. pallidum* PCR assay was designed as a dualplex assay to include the detection of Phocine herpes virus (PHV) as an internal control (IC). PHV DNA was added to each sample to monitor the process from extraction to detection and to detect inhibitors to PCR. The PHV gB gene was selected as the target for the IC assay to generate a 94bp amplicon. PHV primers and probe are supplied by Applied Biosystems. The final concentration of the PHV forward

and reverse primers is 150 nM and 100 nM for the probe. The PHV IC was kindly supplied as a 1000× stock cell culture by Dr. Hubert Niesters, Laboratory for Medical Microbiology, University Hospital, the Netherlands. All primers and probes used were checked for possible sequence homology by sequence comparison using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Name		Sequence	Reference
TP-F	Forward primer	5'-AGG ATC GCC CAT ATG TCC AA-3'	Liu et al[84] J Clin Microbiol 2001;39:1941-6
TP-R	Reverse primer	5'-GTC AGC GTC TCA TCA TTC CAA-3'	Liu et al[84] J Clin Microbiol 2001;39:1941-6
TP-Probe	MGB Probe	5' FAM- ATG CAC CAG CTT CGA-NFQ	Leslie et al[82] J Clin Microbiol 2006;45:93-6.

Table 3.1 – *T. pallidum* primers and probe

FAM = 6-carboxyfluroscein fluorescent dye; NFQ = non fluorescent quencher

3.2.3.2 – *T. pallidum* DNA extraction

DNA was extracted from 250µl of Abbott multi-collect buffer using QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) with no modifications. Briefly, 200µl of sample was added to 200µl of buffer AL and 20µl of proteinase K and mixed by pulse vortexing. 3µl of carrier DNA (poly A) and

10µl of internal control PHV were also added. The quantity of dilution of PHV stock was a pre determined volume (validated for use in clinical diagnostic assay to give Ct value of ~33-36). The lysate was incubated at 56°C for 10 minutes. Following the addition of 230µl of 96% ethanol the mixture was then applied to the mini-column and centrifuged at 14000RPM for 1 min. Two washes of 500µl of AW1 and AW2 wash solutions and were then applied with centrifugation at 8000 RPM for 1 min and 14000RPM for 3 min respectively. DNA was eluted in 60µl of ethanol by centrifugation at 8000 RPM for 1 minute following incubation at room temperature for 5 minutes.

Each batch of samples processed for extraction contained at least one negative control (negative serum only) and a Nicholls strain DNA – (kindly supplied by Sheila Lukehart, Seattle) positive control of unknown DNA copy number. The quantity of Nicholls strain DNA added was pre determined to give Ct value of ~33. The DNA was stored at -80°C until real time PCR was performed, however If the PCR assay was to be performed within 24 hours, the DNA was stored at 4° C.

3.2.4 – PCR Set-Up and Amplification and Detection

The assay was optimised in a dualplex format for the simultaneous detection of *T. pallidum* and PHV IC, where the IC was designed as a primer-limited assay. The final 20µl PCR reaction mixture contained 10 µl of TaqMan™ FAST Universal PCR Mastermix (Applied Biosystems, California, USA), 0.9 µM of *T. pallidum* primers, 0.25µM of *T. pallidum* MGB probe, 0.1µM of PHV primers, 0.15µM of PHV-MGB probe and 7.0µl of extracted DNA. The PCR

reaction components, their volumes used and final concentrations achieved are outlined in Table 3.2. The real-time PCR protocol was performed using an ABI Prism® 7500 Fast sequence detection system (Applied Biosystems, USA). The thermal cycling protocol comprised of 20 secs at 95°C (AmpliTaqGold® enzyme activation), 45 cycles of 95°C for 3 seconds (denaturation) and 60°C for 30 seconds (combined annealing and primer extension). A cycle threshold (Ct) value is generated as the amplified sample crosses the threshold level of detection set during the exponential phase of the PCR reaction. A negative and non-template control was included in each run. Samples were deemed positive with a Ct of 38.

Reagent	**1 Rx (µl)**	**Final Concentration**
TaqMan™ FAST Universal PCR Mastermix TaqMan	10.0	1x
TP-F	0.8	900 nM
TP-R	0.8	900 nM
TP-Probe	0.8	250 nM
PHV–F	0.2	100nM
PHV – R	0.2	100nM
PHV - Pr	0.2	150nM
Sub Total	**13.0**	
DNA extract	7.0	
TOTAL VOLUME	**20.0**	

Table 3.2 – PCR reaction components

3.2.5 – DNA Amplification for the detection of Azithromycin Resistance

In order to determine the azithromycin resistance a selected section of the 23S rRNA gene of the *T. pallidum* genome was amplified. Two different methods were used for varying biological samples. Exudate samples obtained from genital lesions were tested using end point PCR, whereas due to the amplification of an unexpected 500bp band; blood, tissue and CSF samples were analysed with a nested PCR technique.

3.2.5.1 – Primers and Probe

The outer primers used in the assay were those previously described by Lukehart *et al.,*[103] TP23S OF and TP23S OR (table 3.3). The target is a 628-bp sequence within the 23S rRNA gene of *T. pallidum,* sequence (appendix 4).

The inner primers used in the assay were those previously described by Pandori *et al.,*[161] TPAZF and TPAZR (table 3.3). The target is a 201-bp sequence within the 23S rRNA gene of *T. pallidum,* sequence (See appendix 4).

T. pallidum primers and probe are supplied by Sigma (Sigma-Aldrich, Ireland). The final concentration of both the forward and reverse primers is 1000nM.

Name	Primer Function	Target	Sequence	Reference
TP23SOF	Primer-Sense	628 bp 23S rRNA gene	5'-GTA CCG CAA ACC GAC ACA G-3'	Lukehart et al[84] N Engl J Med 2004;351:154-8
TP23SOR	Primer-antisense		5'-TAG GTG GGC GGT TTG ACT-3'	
TPAZF	Primer-Sense	201 bp 23S rRNA gene	5'-GAC TCT GGA CAC TGT CTC G -3'	Pandori et al[84] Antimicrob Agents Chemother 2007;51:3425-30.
TPAZR	Primer-antisense		5'-CAA CAG TGA AAT ACC ACC C-3'	

Table 3.3 – Inner and Outer Primers

3.2.5.2 – Amplification reagents and parameters for end-point PCR

PCR amplification was carried out on the DNA extracts from genital exudate samples using the HotStar Hi Fidelity Polymerase Kit (Qiagen GmbH, Hilden, Germany). All PCR reactions were run on either the GeneAmp 9700 PCR System thermal cycler or the Veriti Thermal Cycler (Applied Biosystems, Foster City, California). The PCR reaction components, their volumes used and final concentrations achieved are outlined in table 3.4. In addition to extracted DNA from clinical samples, Nicholls strain DNA, street 14 strain DNA

and three controls samples known to be azithromycin resistant were also included (samples from S. Lukehart, Seattle). Also included was a non-template control and a pre-extracted negative control were with each run. The PCR cycling parameters were as follows; Initial activation step: 5.00 min at 95 °C, followed by 35 cycles of denaturation 15 seconds at 94 °C, annealing 1 min at 55 °C and extension 1 min at 72 °C. Final extension step: 10 min at 72 °C. Samples were stored at 4°C until further analysis.

Reagent	**1 Rx (µL)**	**Final Concentration**
5x HotStar HiFi PCR Buffer	10	1x
Forward primer TPAZF	5.0 (10µM)	1000nM
Reverse primer TPAZR	5.0 (10µM)	1000nM
HotStar HiFiPCR Taq	1.0	2.5 Units
Nuclease Free Water	24	
DNA extract	5	
Total Volume	**50**	

Table 3.4 – End point PCR components

3.2.5.3 – Nested PCR

Blood, CSF and tissue samples were amplified using a nested technique. The initial amplification used outer primers TP23S-OF and TP23S-OR as described by Lukehart *et al*[103]. The target of these primers is a 628 bp sequence of the 23S rRNA gene of *T. pallidum.*

PCR amplification was carried out on the DNA extracts from blood, CSF and tissue samples using the Taq Core Kit. All PCR reactions were run on either the GeneAmp 9700 PCR System thermal cycler (Applied Biosystems, Foster City, California) or the Veriti Thermal Cycler (Applied Biosystems, Foster City, California). The PCR reaction components, their volumes used and final concentrations achieved are outlined in Table 3.5. Also included was a non-template control and a pre-extracted negative control were with each run. The PCR cycling parameters were as follows; Initial activation step: 3 min at 94 ºC, followed by 35 cycles of denaturation 60 seconds at 94 ºC, annealing 1 min at 55 ºC and extension 1 min at 72 ºC. Final extension step: 10 min at 72 ºC. Samples were stored at 4ºC until further analysis.

Components	1 reaction (µL)	Final Concentration
10x TaqCore PCR Buffer	5	1x
dNTP	1	200 µM of each dNTP
Primer Forward- A*	2.5	500 nM
B	2.5	
Primer Reverse- A*	2.5	500 nM
B	2.5	
Taq DNA Polymerase	0.25	2.5
Nuclease Free Water	36.75	-
DNA extract	2	-
Total Volume	**50**	

Table 3.5 – Nested PCR components

*Primers A= outer primers, B= inner primers

3.2.5.4 - DNA detection - Agarose Gel Electrophoresis

Following amplification by either method, Amplified PCR products were electrophoresed on a 1.5% agarose gel (Sigma) at 90V for 110 minutes. Gels were prepared using 0.5M Tris Borate EDTA (TBE) buffer (Sigma), 0.5µg ethidium bromide ml^{-1} and cast in a 7X15cm casting tray. The Gel Pilot 100bp Plus Ladder (Qiagen GmbH, Hilden, Germany) was used as a molecular weight marker on each gel. PCR products were visualized using a UV transilluminator and a photographic record was taken. The expected

amplicon sizes for the first and second round PCR amplification were a 628 bp and 201bp amplicon respectively.

3.2.6 – DNA Sequencing

3.2.6.1 – PCR Product Purification

If single band was visible from patient samples, the PCR product was purified using the Qiaquick® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Purified DNA fragments were eluted in a final volume of 30µl room temperature NFW.

3.2.6.2 – Estimation of DNA Concentration

DNA concentration was estimated using a ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington USA). Purified eluate (2µl) was loaded onto the instrument and using a path length of 0.2mm, the 260/280 absorbance ratios and the DNA concentration (ng/µl) were calculated by the software.

3.2.6.3 – DNA Dilution

All PCR products were diluted to a 20ng/100bp/10µl concentration for DNA sequencing using NFW water

3.2.6.4 – DNA Sequencing Procedure

DNA sequencing was carried out using the BigDye terminator V3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK), according to manufacturer's instructions with reagent volume modifications. The manufacturer recommends using 4µl BigDye terminator per 20µl reaction. A prior in-house validation performed demonstrated that Big Dye terminator could be diluted prior to use without lessening the strength of the fluorescent signal emitted. Therefore, BigDye terminator was diluted 1:8 prior to use to reduce the cost of the reaction. Modified reaction component volumes are listed in table 10.4 Amplification using Round 2 primers was performed in a 96-well microtitre plate (Applied Biosystems, Warrington, UK) in the GeneAmp 9700 PCR System thermal cycler under the following conditions: 96°C for 1min (denaturation), followed by 25 cycles of 96°C for 10sec (denaturation), 51°C for 10sec (primer annealing), 60°C for 4min (elongation) and a final elongation step of 10min and held at 4°C. Products were sequenced in forward and reverse directions using the round two primer pairs. The primers used and summaries of their details are listed in Table 3.6.

Reagent	Per one reaction
BDT v3.1	0.5µl
5x Sequencing reaction buffer	5.5µl
H_2O	10µl

Table 3.6 – BigDye terminator components

3.2.6.5 – Ethanol/Sodium Acetate Precipitation

Ethanol/Sodium Acetate was used to completely remove excess terminators from the reaction. After PCR amplification was completed, 2µl of 3M sodium acetate and 50µl of 96% molecular grade ethanol (Analab) was added to each well of the microtitre plate. Reaction components were mixed by pipetting up and down four times. Plates were sealed with an adhesive strip (Applied Biosystems, Warrington,UK) and incubated for 15 minutes at room temperature. Incubated samples were centrifuged at 1400 x g for 45 minutes in a Beckman Allegra 6A centrifuge (Beckman Coulter, Fullerton, CA, USA). Immediately following centrifugation, the adhesive seal was removed; the plate was inverted onto tissue paper and spun upside-down up at 185 x g to empty the wells. A 70µl volume of 70% ethanol (Analab) was added to each well; plates were sealed and spun again at 1400 x g for 15minutes as described previously. The plate was removed from the centrifuge and inverted as described previously and spun upside-down again at 185 x g for one minute to thoroughly remove all ethanol. All minute traces of ethanol

were finally removed by heating the plate to 95°C for one minute in the GeneAmp 9700 System thermal cycler. Pellets were resuspended in 20µl of the injection buffer HiDi Formamide (Applied Biosystems, Warrington, UK). Automated DNA sequencing was then performed on all samples using the ABm 3130 Analyzer (Applied Biosystems, Warrington, UK)

3.2.7 – Analysis of DNA Sequences

3.2.7.1 – Identification of A2058G mutation

Sequence analysis was carried out using DNASTAR® Lasergene (DNASTAR, Madison, WI). A consensus sequence was assembled and edited using SeqMan sequence analysis software. All nucleotide were aligned using MegAlign and the A2058G sequence mutation was identified.

3.3 – Results

3.3.1 – Syphilis IgM

68 samples were sent for darkfield examination (figure 3.1). 4 samples were incorrectly labelled or were unsuitable for analysis. Of the remaining 64 samples, the mean age of patients was 36.4 years (range 19-63 years). 58 (90.6%) were collected from men. 35 samples were penile samples, 2 perianal, 2 vaginal and 2 vulval. The remaining samples were from unspecified ano-genital sites.

15 (22.6%) of samples were positive by darkfield microscopy. The mean age of patients with positive darkfield samples was 36.6 years (range 22-58 years). 100% were male. 13/15 (86.7%) patients had positive syphilis serology (figure 3.1). All of these patients had a positive immunoglobulin M (IgM). Results of the patients' RPR tests are presented in figure 3.2.

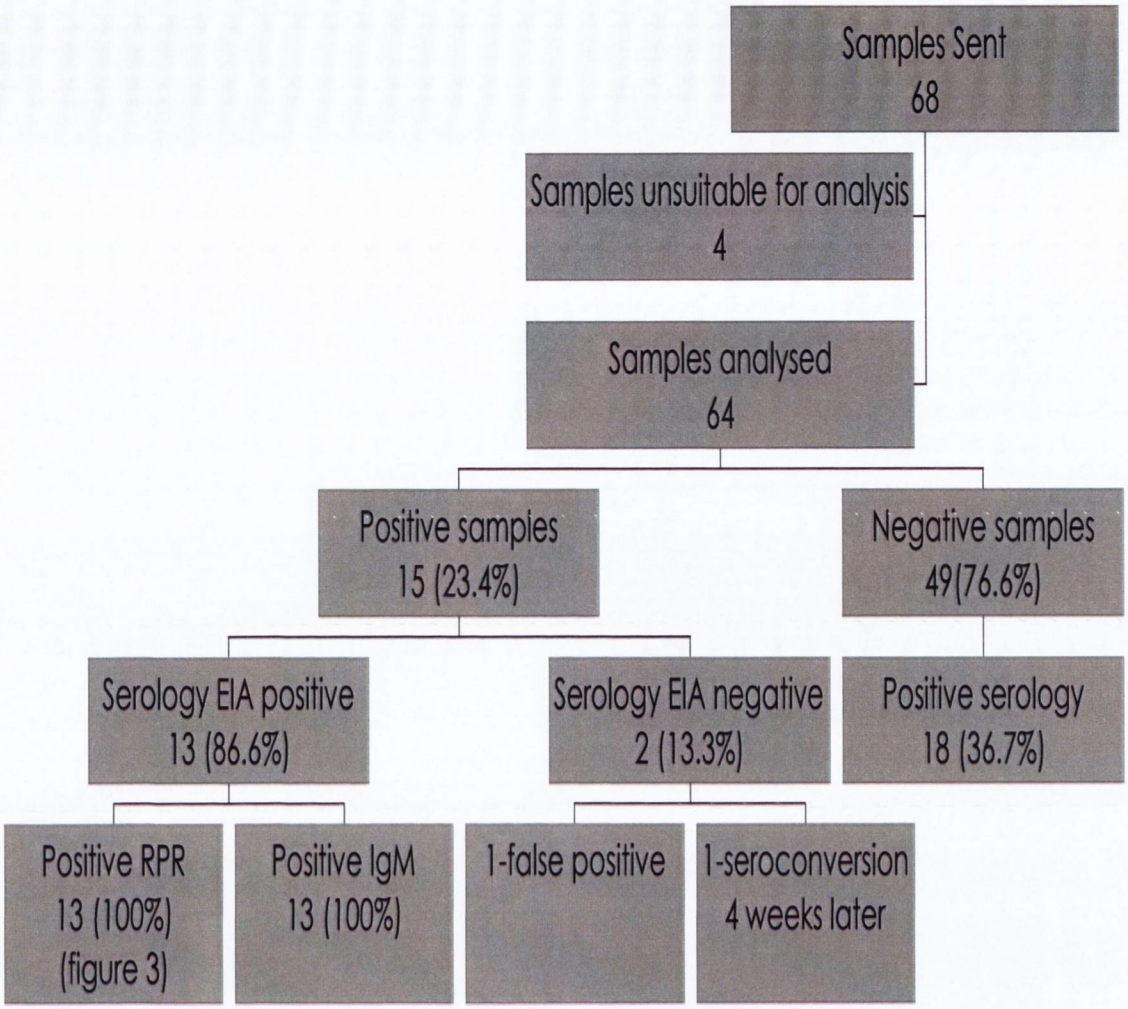


Figure 3.1 – Darkground Samples analysed

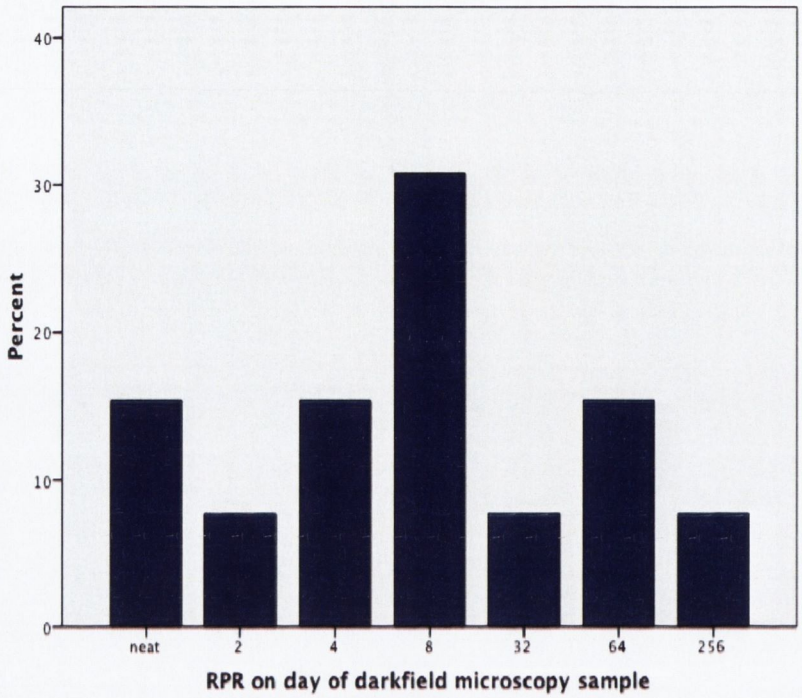


Figure 3.2 – Results of RPR test in patients with positive Darkground Microscopy

		Re-infection		Total
		Yes	No	
IgM	Positive	34	242	276
	Negative	30	76	106
Total		**64**	**318**	**382**

Table 3.7 - IgM response in syphilis re-infection

69.2% of patients had an RPR of 1:8 or lower. 2 /15 (13.4%) patients had negative syphilis serology, 1 patient had positive serology 4 weeks later. The other patient remained seronegative; real time PCR analysis of the darkfield specimen was negative confirming a false positive result. Excluding the false positive result, 92.8% of patients had positive serology the day of their darkfield microscopy. All of whom had a positive syphilis IgM. Compared to darkfield microscopy EIA and IgM both had a sensitivity of 85.7%.

Of the 49 patients who had negative darkground microscopy, 18/49 (36.7%) had a positive EIA on the day their genital specimen was obtained. One patient had no serology drawn. Of the 18 patients with a positive EIA, 16/18 (88.9%) had a positive RPR, all 18 had a positive TPPA and 14/18 (77.8%) had a positive IgM. Of the 30 patients who had a negative EIA on the day that darkground microscopy was done, 8/30 (26.7%) had repeat serology performed, all tests were negative.

Between January 2007 and December 2009, 278 patients had a positive syphilis IgM. 265/278 (95.3%) of patients were male and the mean age of patients was 34.3 years (range 17-67 years). 90.6% of patients were MSM.

80/278 patients (28.8%) were diagnosed with primary syphilis, 87/278 (31.3%) were diagnosed with secondary syphilis and 103/278 (37.1%) were diagnosed with early latent disease. Only 3/278 (1.1%) were diagnosed with late latent or syphilis of unknown duration. Patients were more likely to have a positive syphilis IgM if they were diagnosed with early infectious syphilis, compared to

those diagnosed with late latent or syphilis of unknown duration (chi square $p<0.00003$).

In the case of syphilis re-infection, 64 patients were diagnosed with syphilis re-infection. Of those patients, 34/64 (53.1%) had a positive IgM response compared to 242/318 (76.1%) patients who presented with syphilis for the first time. Patients presenting with syphilis re-infection were significantly less likely to demonstrate an IgM response than those presenting with their first episode of syphilis, chi square $p=0.0004$ (table 3.7).

3.3.2 - PCR

A total of 107 samples from 92 patients were collected (table 3.8). The mean age of the patients was 37.8 years, with an age range of 19 to 64 years. The majority of the patients 88/92 (95.7%) were male. The largest risk for acquisition of syphilis was men who have sex with men (MSM) with 78.3% of patients belonging to this category, however data on risk for acquisition was unavailable for 10/92 patients (10.9%). When these patients were excluded the proportion of MSM patients rose to 87.8%.

Of the 108 samples collected 57 were whole blood samples, 35 were genital samples from lesions which were clinically suspected to be primary syphilis, 12 were CSF and three were tissue samples (table 3.8).

Sample Type	Number
Whole blood (EDTA)	57
Exudate from genital ulcer (Suspected primary syphilis)	35
Cerebrospinal fluid (CSF)	12
Tissue	3
Total	**107**

Table 3.8 – Samples Received for PCR analysis

All of the genital lesions were collected from patients with clinically suspected primary syphilitic lesions. The blood samples were all collected from patients with early latent syphilis or patients presenting with a clinical syndrome suggestive of secondary syphilis. The three tissue samples were collected from two patients with a widespread rash, who underwent skin biopsy and one patient who underwent vitreal biopsy after presentation with decreased visual acuity and posterior uveitis whose syphilis serology was subsequently positive.

103 samples were analysed using the TaqMan Real-time PCR technique. A total of 54/57 blood samples, 35/35 genital exudate samples, 12/12 CSF samples and 3/3 tissue samples were analysed. The three blood samples not analysed were received on patients who also had a genital exudate submitted for PCR testing. Of the 103 samples analysed 36/103 were PCR positive

(34.95%) (figure 3.3). These 36 samples were obtained from 30 patients. Of the blood samples analysed 12/54 (22.22%) were positive. Of the genital exudate samples, 21/35 (60%) were positive. Two of the three tissue samples (66.66%) were positive and one CSF sample (8.3%) was positive.

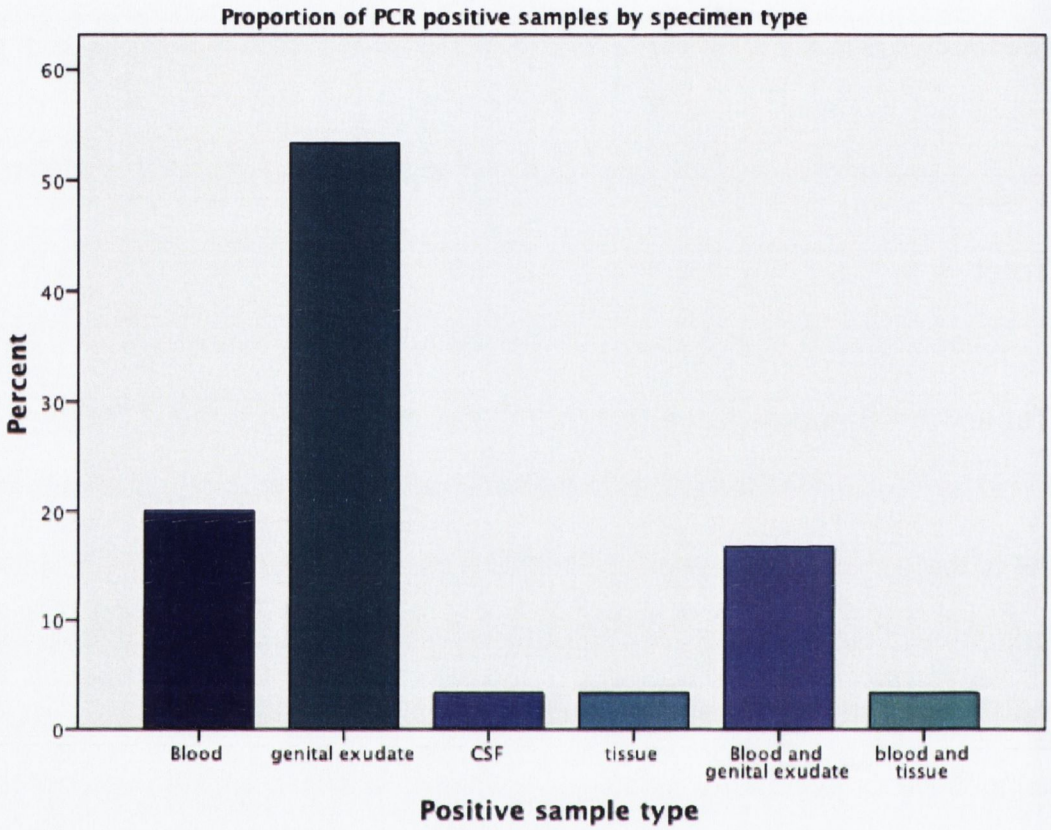


Figure 3.3 – Proportion of samples positive by PCR

The mean Ct of the blood samples analysed was 35.76, (95% confidence interval for the mean 34.1-37.41) this value was significantly higher than the mean Ct value for the genital exudate samples at 28.44 (95% CI 26.26-30.62) $p<0.0001$ (figure 3.4). The one positive CSF sample had a Ct of 34 and the two tissue samples both had a Ct value of 35.

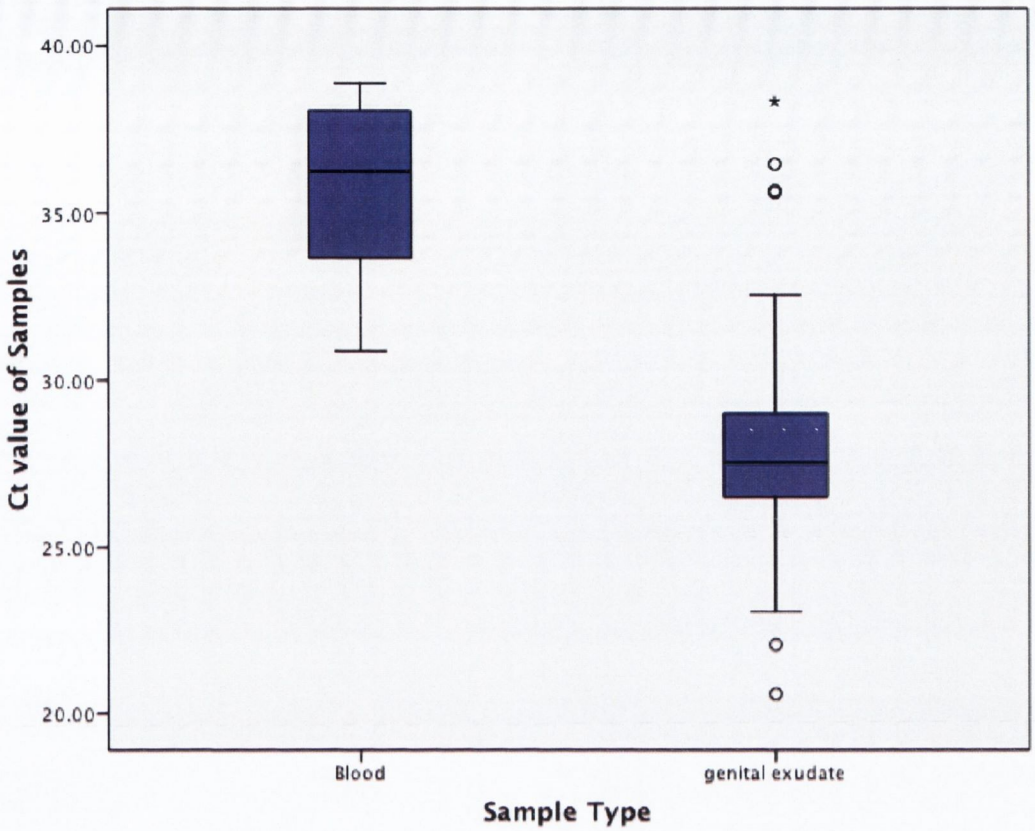


Figure 3.4 – Mean Ct value of blood and genital exudate samples

Patient number	Ct genital exudate	Ct blood	Ct tissue
1	23.06	38.87	
2	35.56	34.5	
4	27.4	33.0	
5	27.3	37.1	
31	27.1	34.25	
7		30.87	34.7

Table 3.9 – Ct values in patients with samples from different sites

Five patients had both a positive genital exudate samples and a positive blood sample by PCR. All but one patient had a higher Ct value on their genital exudate sample. One patient had a positive sample from both blood and tissue, the Ct of the tissue sample being higher (table 3.9).

Of the 21 genital exudate samples, all 21 patients had a dark ground microscopy sample performed on the same day. There were two discrepant results; two samples were darkground microscopy negative and PCR positive. Of the two samples with a negative dark ground microscopy one had relatively high Ct value on PCR of 38, while the other had a Ct value of 32.54. Three patients had a dark ground microscopy, which was positive, however their PCR was negative. Compared to darkground microscopy the sensitivity and specificity were 86.3% and 75% respectively (table 3.10). The kappa value of 0.614 was found for agreement between the tests.

Of the 92 patients, 88/92 (95.65%) had syphilis serology drawn on the same day that the sample for PCR was obtained. Of the 88 patients who had serology drawn 73/88 (83%) had positive syphilis serology (table 3.11 & 3.12).

		PCR result		Total
		Positive	Negative	
Darkground result	Positive	19	3	22
	Negative	3	9	12
Total		22	12	34

Table 3.10 – Darkground Microscopy and PCR results

		Serology			Total
		Positive	Negative	Not available	
PCR result	Positive	29	0	1	30
	Negative	44	15	3	62
Total		73	15	4	92

Table 3.11 – Syphilis Serology and PCR Results

		Serology		Total
		Positive	Not available	
Positive sample type	Blood	6	0	6
	Genital exudate	16	0	16
	CSF	1	0	1
	Tissue	0	1	1
	Blood and genital exudate	5	0	5
	Blood and tissue	1	0	1
Total		29	1	30

Table 3.12 – Positive Serology & Positive PCR- Specimen Type

Of the thirty patients with positive PCR results, 29/30 (96.67%) had positive syphilis serology, results were unavailable for one patient. Of the patients with negative PCR results, 44/62 (70.97%) had positive serology. This most likely reflects the fact that patients were recruited with early latent syphilis prior to treatment, or with a clinical syndrome suggestive of secondary syphilis.

Of the patients who presented as having contact with syphilis, secondary or early latent syphilis, a total of 9/57 (15.79%) had positive PCR results. Six patients had positive blood results, one patient had positive CSF, one a positive tissue sample (vitreous biopsy) and the final patient was PCR positive in both blood and tissue (skin biopsy). 42/57 (73.68%) of the same group of patients had positive serology (table 3.13). Eight of the patients with positive PCR had positive serology and serological results were unavailable for the final patient.

		Serology			Total
		Positive	Negative	Not available	
PCR result	Positive	8	0	1	9
	Negative	42	6	0	48
Total		50	6	1	57

Table 3.13 – Serology and PCR results in patients with Early latent/secondary syphilis

3.3.3 – Azithromycin Resistance

Thirty samples were found to be positive by Real-time PCR. A total of 12 of blood samples, 19 genital exudates, 1 CSF and 2 tissue samples were analysed. All types of biological sample were initially analysed using end point PCR. This was found to work well for genital exudates samples, from which the target 201 bp sequence successfully amplified.

However, in the case of blood samples the first test samples were found to contain a 500 bp sequence when the amplicon was electrophoresed. When this amplicon was sequenced, it was found to contain homo-sapiens DNA, from chromosome 2, when entered into the GeneBank. As a result, further samples of blood, CSF and tissue were analysed using the nested technique described. Following amplification with the nested technique a 201 bp sequence was successfully amplified.

Of the thirty samples analysed, 29 samples successfully sequenced. The remaining sample failed to amplify any DNA product. Of the 29 samples that successfully sequenced, 27/29 (93.1%) were found to contain the A2058G mutation. Figure 3.5 shows the region of the A → G mutation in that confers azithromycin resistance in red. The sensitive strain Nicholls is shown in yellow. Control 1, 2 and 3 are samples from S. Lukehart Seattle known to have the mutation. Samples 4, 41, 89 and 26 are samples obtained from patients attending the GUIDE clinic. Samples 4 and 41 have the mutation, while samples, 89 and 26 are azithromycin sensitive.

Figure 3.5 – A2058G mutation

Interestingly the two patients who had isolates, which did not contain the A2058G mutation both described the risks for acquisition of their syphilis to be outside Ireland.

3.4 – Discussion

This series demonstrates a high degree of correlation between positive darkfield microscopy examination and syphilis serology. While the disadvantage with serology compared to darkground microscopy is that the result is not available while the patient is still in clinic, a large proportion of patients presenting with primary syphilis will have positive serology results.

It is also interesting to note that nearly 37% of patients with negative dark ground microscopy results had positive syphilis serology, allowing for the diagnosis and treatment of syphilis cases that may other wise have been missed. In addition although the rate of repeat testing in those with negative results was low (27%), all repeat tests were negative, suggesting a low false negative rate. This may give solace to physicians who do not have readily available darkfield microscopy facilities and who rely wholly or in part on syphilis serology in the diagnosis of primary syphilis.

The use of PCR as a diagnostic tool is attractive. Given the poor availability of darkground microscopy and the increasing numbers of syphilis diagnoses, there is certainly a need for an easily accessible, reliable test for the diagnosis of syphilis. When compared to darkground microscopy the sensitivity and specificity of PCR were found to be 86.3% and 75% respectively with a kappa value of agreement of 0.614.

The utility of PCR is primarily amongst patients presenting with lesions suspicious for primary syphilis. In this series, only 12 blood samples were positive out of 54 analysed. In all of those patients, syphilis serology was positive. In 5 of 12 patients, there was also a positive genital swab sample. This mirrors the rabbit model, which demonstrated that dissemination of treponemes occurs early after inoculation. This spirochetaemia must be transient as the majority of patients presenting with secondary or early latent syphilis had negative PCR results from blood (48/57, 84.21%). In addition, only one CSF sample was positive, which would indicate that the use of PCR may not be a helpful addition in the diagnosis of neurosyphilis.

In 2004 when syphilis treatment failures following azithromycin therapy were reported[102] and the A2058G mutation associated with azithromycin resistance was described[176], Dublin had the highest rate of Azithromycin resistance[103]. It appears little has changed; 27/29 (93.1%) of isolates are azithromycin resistant. In the two patients with sensitive isolates, the risk for acquisition of their syphilis was outside Ireland, suggesting that perhaps the situation may well be worse than 2004.

The data from Africa[180] suggests that there may not be overlapping of sexual networks between the developed and developing world. In addition, there is less likely to be selective pressure in Sub-Saharan Africa contributing to azithromycin resistance. Some have suggested that azithromycin could be used in developing nations as an alternative to penicillin[183]. This approach may be risky given that selective pressure plays a role in the development of

azithromycin resistance[179]. If large-scale treatment of syphilis with azithromycin in Africa were to occur, we may see changing patterns of macrolide resistance amongst *T. pallidum* in that continent in years to come.

Any plans to establish Irish guidelines for the management and treatment of syphilis must take into consideration the high level of macrolide resistance in *T. pallidum* isolates in Dublin. Little has changed since the original study in 2004. If azithromycin is to be used in pregnant patients reporting penicillin allergy, it must be done only with extreme caution and with very close follow up of both mother and baby. Azithromycin cannot be recommended for use in the treatment of syphilis in Dublin and should not be used in the epidemiological treatment of syphilis contacts.

Chapter 4 –

Considerations in the Management

& Prevention of Syphilis Infection

4.1 – The Role of Lumbar puncture in the Management of Syphilis in HIV positive patients

The role of lumbar puncture in the management of patients with syphilis is controversial, particularly in the investigation of asymptomatic patients[120, 184-186]. Patients with asymptomatic neurosyphilis (i.e. those with no symptoms but with abnormal CSF analysis) are more likely to progress to long term neurological sequelae if left untreated[44, 50].

Syphilis and HIV are particularly suited to being acquired together, both are sexually transmitted and risk factors for acquisition are the same. Syphilis is known to facilitate the transmission of HIV[132], both by disrupting the normal epithelial barrier, and by large amounts of HIV being present within a chancre[187]. In addition, syphilis has been reported to have the immunological effects on HIV infection[143, 144, 188-190] and HIV is known to modulate both the manifestations of syphilis and the serological response to therapy[47, 48, 186, 191-193].

Neurosyphilis i.e. syphilitic infection of the central nervous system (CNS) can present at any time after infection. It can be classified into early and late disease. Early in the course of syphilis, most forms of neurosyphilis will involve the cerebrospinal fluid (CSF), meninges and vasculature, while later disease that occurs in tertiary syphilis tends to affect the brain parenchyma and spinal cord. In the pre-antibiotic era 25 to 33% of patients with syphilis had neurological involvement[50], a third had asymptomatic neurosyphilis, a

third tabes dorsalis, 10% had paresis, 10% meningovascular disease and the remaining patients had a variety of other manifestations including symptomatic meningitis and cranial nerve abnormalities.

Post neuro-invasion by treponemes, spontaneous resolution may occur in some cases without an inflammatory response. Asymptomatic neurosyphilis where persistent meningitis occurs in the absence of symptoms is a consequence of failure to clear organisms from the CSF. These patients are more likely to develop long-term neurological sequelae than those with normal CSF[50]. Symptomatic meningitis occurs more frequently in the first year after infection. Signs are similar to other forms of meningitis, however visual acuity may be impaired if there is eye involvement and cranial neuropathies can occur. Meningovascular syphilis may manifest as thrombosis, ischaemia and infarction. Late neurosyphilis manifested as tabes dorsalis and paresis is now uncommon. Although case reports show that sporadic cases do still occur[51].

Analysis of the CSF in patients, be they HIV infected or not, is an easy decision if patients present with symptoms suggestive of neurological syphilis. Current guidelines would recommend lumbar puncture in these patients, and repeat CSF analysis to assess adequate response to treatment[63, 69]. Controversy arises with regard to asymptomatic patients, in particular HIV co-infected patients[120, 184, 185]. Standard therapy for early infectious syphilis (e.g. 2.4 MU Benzathine penicillin intramuscularly in a single dose) does not reach treponemicidal concentrations in the CSF. As a result intravenous

benzylpenicillin or intramuscular procaine penicillin[194] with probenecid are the recommended regimens[63, 69].

In the 1980s and 1990s there were reports of aggressive and dramatic forms of secondary syphilis in HIV positive patients that did not respond to standard therapy. In addition, there were reports of relapse of neurosyphilis despite administration of standard therapy for CSF involvement of syphilis[77, 195]. This lead to the concern that perhaps standard therapy was not adequate amongst HIV positive patients presenting with neurosyphilis. There have been reports of asymptomatic HIV positive patients relapsing with neurosyphilis despite adequate treatment for early syphilis[196, 197].

The mechanism of clearance of organisms from the CSF is probably similar to the immune response peripherally, where opsonized organisms are cleared by activated macrophages. A study in non-human primates demonstrated increased CD4 cells and gamma interferon responses in the CSF as the bacteria were cleared form the CNS consistent with a "Th-1-type" cellular immune response[52]. While it is still unclear whether human host response or organism characteristics determine the risk of developing symptoms following neuro-invasion, a rabbit model has suggested that there are particularly neuroinvasive strains of *T. pallidum*[53]. More recently the same group presented data on the strain type in 66 patients with syphilis between 2001 and 2007[54]. Three molecular methods were used to assign strain type and five strains were identified in the 66 patients. The strain type was seen to change significantly over time. Strain type was also significantly associated

with neurosyphilis; none of the patients with neurosyphilis had stain type 11, while 54% of those infected with strain type 10 had neurosyphilis. Molepo *et al*[55] looked at molecular typing in patients with neurosyphilis and found type 14 a to be the most common (53.8% of 13 patients).

With an increasing proportion of patients co-infected with syphilis and HIV (chapter 2), the management of HIV positive patients diagnosed with syphilis will become increasingly more relevant. HIV positive patients are also reported to have more severe manifestations of primary and secondary syphilis[47, 48]. There is concern that standard neurosyphilis therapy may not be effective in HIV positive patients. Gordon *et al*[197] reported in 1994, on clinical and laboratory follow up of 11 HIV positive patients with neurosyphilis, 4/11 (36%) had CSF abnormalities 6 months post follow therapy. Similarly Malone *et al*[198] demonstrated a high rate of serological failure of HIV patients infected with syphilis, particularly those with a reactive CSF VDRL at initial presentation while Rolfs *et al*[192] reported that HIV positive patients were significantly more likely to have defined serological failure 6 months post therapy compared to HIV negative patients. Not all reports were consistent when it came to HIV positive and negative patients, in other series no differences in response to standard therapy were reported in HIV infected versus HIV un-infected patients [193]. These studies are all pre HAART era.

Animal studies of intrathecal administration of *T. pallidum* in Macaques have demonstrated the importance of CD4 mediated cytokines in the clearance of the organism from the CSF[52]. This indication that CD4 positive T cells have a

central role in the immune control of syphilis infection may be clue to the altered manifestations in HIV positive patients.

More recently, post HAART, studies have sought to readdress the question of serological response to syphilis therapy in HIV infected patients. Marra *et al*[119] studied 59 patients, 78% of whom were HIV co-infected with neurosyphilis. HIV infected patients were less likely to normalise CSF VDRL, and this finding was more pronounced in those patients with low CD4 counts. Interestingly treatment with non-neurological dose penicillin prior to lumbar puncture also affected CSF normalisation, a possible explanation put forth by the authors being the down regulation of immune response following Benzathine therapy that slows the immune response following neurosyphilis appropriate therapy.

Manavi *et al* reported similar responses to therapy among HIV positive and negative patients enrolled in an observational study between 2003 and 2005[191]. Serological failure in HIV positive patients has also been shown to be associated with lower CD4 counts[143], and the use of HAART to be associated with less serological failures[199].

In the identification of patients at risk for neurosyphilis it has been shown that HIV positive patients with a CD4 count of less than 350 cells/mm^3 and an RPR greater/equal to 1:32 are 18.6 times more likely to have neurosyphilis than patients without these features[118]. In HIV negative individuals neurosyphilis is more common in those with an RPR greater than or equal to 1:32. Application of these criteria has been shown to improve the ability to

identify asymptomatic neurosyphilis[200], compared to recommended guidelines[69]. The use of serum RPR as a predictor of CSF normalisation is a potential for future management of patients with neurosyphilis, however it's accuracy is lower among patients co-infected with HIV and not receiving HAART[201]. In Investigating patients with late syphilis, a negative serum VDRL has been shown to predict normal CSF analysis, irrespective of HIV status[202].

Similar to syphilis, HIV enters the central nervous system early in the course of infection, and has shown to be present at all stages of disease[203, 204]. Patients with lymphocytic meningitis were shown to have high CSF HIV viral loads by Morris *et al*, and the HIV viral load was shown to correlate with the number of infiltrating lymphocytes[205]. However, no correlation with serum HIV viral load was demonstrated. As syphilis infection has been shown to increase the serum HIV viral load in HIV co-infected patients[144, 189, 190], it would not be difficult to imagine that there may also be an impact on CSF HIV viral load.

More recently, higher CSF HIV viral loads have been demonstrated in patients with neurosyphilis versus those with systemic syphilis alone or no syphilis co-infection[206]. CSF HIV viral loads have been shown to be suppressed on HAART[207 208], however this may be dependent on the antiretroviral regimen chosen[209]. Strain differences have been found in HIV isolated from the blood and CNS[210 211] in the same individuals, indicating that compartmental evolution of HIV may differ.

High HIV CSF viral loads have been associated with poor neurocognitive performance[212], what is yet unknown is whether neurosyphilis has any long term neurological sequelae, by increasing CSF HIV viral load, or the persistence of treponemes in the CSF even following treatment[166].

4.1.1 – Methods

4.2.1.1 – Patients with prior syphilis infection

HIV positive patients who had been diagnosed with and treated for syphilis prior to 2007 were identified. All of these patients had received standard dose therapy at the time of diagnosis, according to syphilis stage. Patients were asked to consent to lumbar puncture and clinical neurological examination. Demographic data, CD4 count and HIV viral load were recorded.

4.2.1.2 – Patients with new diagnosis of syphilis

HIV positive patients newly diagnosed with syphilis were prospectively recruited. A new diagnosis of syphilis was defined as patients with seroreactive syphilis test with no prior history of syphilis treatment or a documented previously negative syphilis serology or a four-fold rise in serum RPR in patients who had previously treated syphilis infection.

These patients also underwent lumbar puncture based on the following criteria.

1. Symptoms suggestive of syphilitic involvement of the CNS (as determined by the practitioner performing the initial assessment)
2. An RPR greater than or equal to 1:32
3. A CD4 count less than 350 cells/mm^3.

Data was collected on patient demographics, CD4 count and HIV viral load. All CSF was analysed for white cell count, protein, and RPR. In the case of patients newly diagnosed with syphilis CSF was also sent to the National Virus Reference Laboratory, Belfield, Dublin 4 for measurement of HIV viral load.

In the case of CSF white (WCC) and red cell counts (RCC) the average of the three counts over samples 1, 2 and 3 was used for further statistical analysis. In the case where the white or red cell count was recorded as less than one cell per high power field (hpf), the value was taken as zero. In the case of HIV viral load results, a value less than 50 copies/ml was taken as 49 copies/ml for statistical analysis.

All data was stored and analysed using SPSS 17.0 commercially available software.

4.1.2 – Results

4.1.2.1 – Patients with Prior Syphilis Infection.

Patient records for 141 HIV positive patients who had syphilis infection prior to 2007 were available. 30 patients were no longer attending the GUIDE clinic, 4 patients were known to have died and a further 17 had transferred care to another centre. Of the remaining 90 patients, 41/90 (45.5%) consented to lumbar puncture, and CSF was successfully obtained in 36/41 (87.8%).

One patient complained of headache, photophobia and neck stiffness and was diagnosed with meningitis due to syphilis re-infection and was excluded from further analysis.

The mean age of patients was 41 years (range 23-69 years) (figure 4.1). 26/35 (74.3%) of patients were MSM, the remaining patients were from a region of high syphilis prevalence. 31/35 (88.6%) were men.

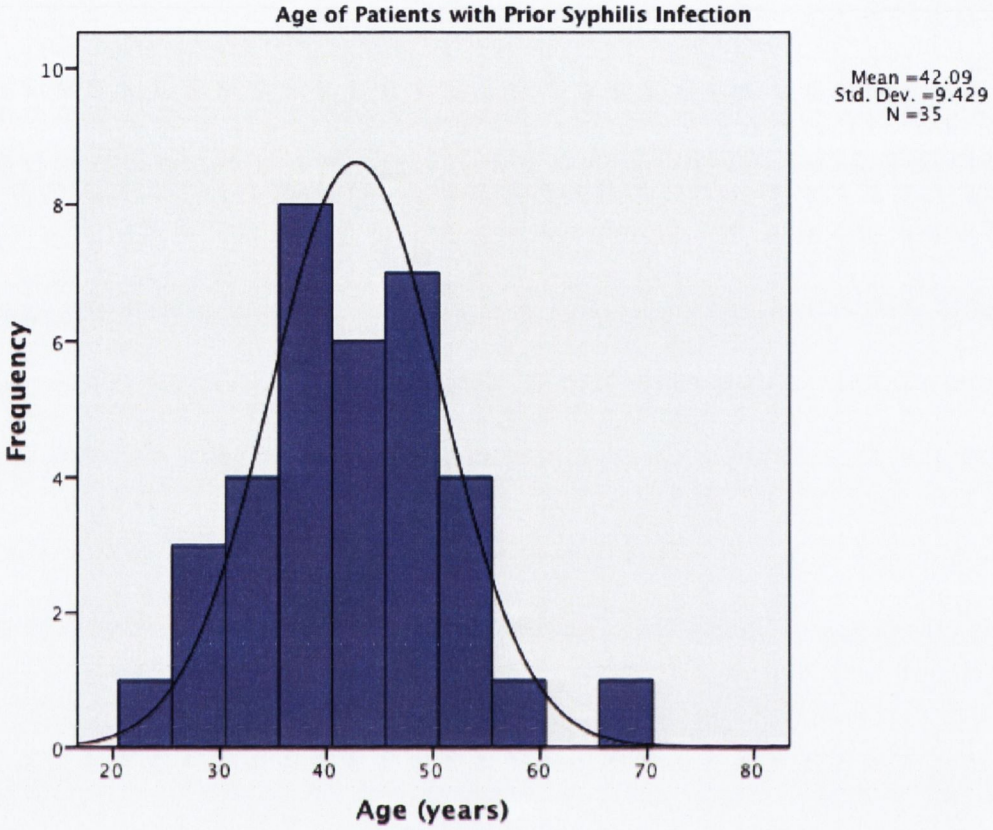


Figure 4.1 – Age of Patients previously diagnosed with syphilis

4.1.2.1.1 – Clinical Examination

All patients had a normal neurological exam, except one patient who had suffered a serious head injury in the interim and had long term neurological sequelae directly related to the head trauma. Two patients complained of decreased hearing, they were 59 and 40 years old respectively. Formal audiology examination is pending.

4.1.2.1.2 – CSF Analysis

The results of patients CSF analysis is displayed in table 4.1. The mean white cell count of all patients was 1.41 cells/hpf, (range 0-10.7 cells, mean 0.7 cells/hpf). Only one patient had a mean white cell count of greater than 10, at 10.7 cells/hpf. A measurement of CSF protein was available for 33 patients, the mean CSF protein was 36.45 mg/dL (range of 16-68 mg/dL). Ten patients had a CSF protein level above the lab specified upper limit of normal, 40 mg/dL.

		CSF protein	CSF glucose	Mean CSF WCC/hpf
N	Valid	33	33	35
	Missing	2	2	0
Mean		36.45	3.570	1.414
Median		33.00	3.400	.700
Mode		33	3.4	.0
Minimum		16	2.7	.0
Maximum		68	5.5	10.7

Table 4.1 – CSF results in patients with previously treated syphilis

4.1.2.2– Patients newly diagnosed with Syphilis

Thirty-seven patients newly diagnosed with syphilis underwent lumbar puncture. The mean age of patient was 35 years (range 22-56 years) (figure 4.2). This was significantly lower than those patients with a prior diagnosis of syphilis (p=0.0001). 35/37 (94.6%) of patients were male. 20/37 (54%) received a new diagnosis of HIV concurrent with their syphilis diagnosis.

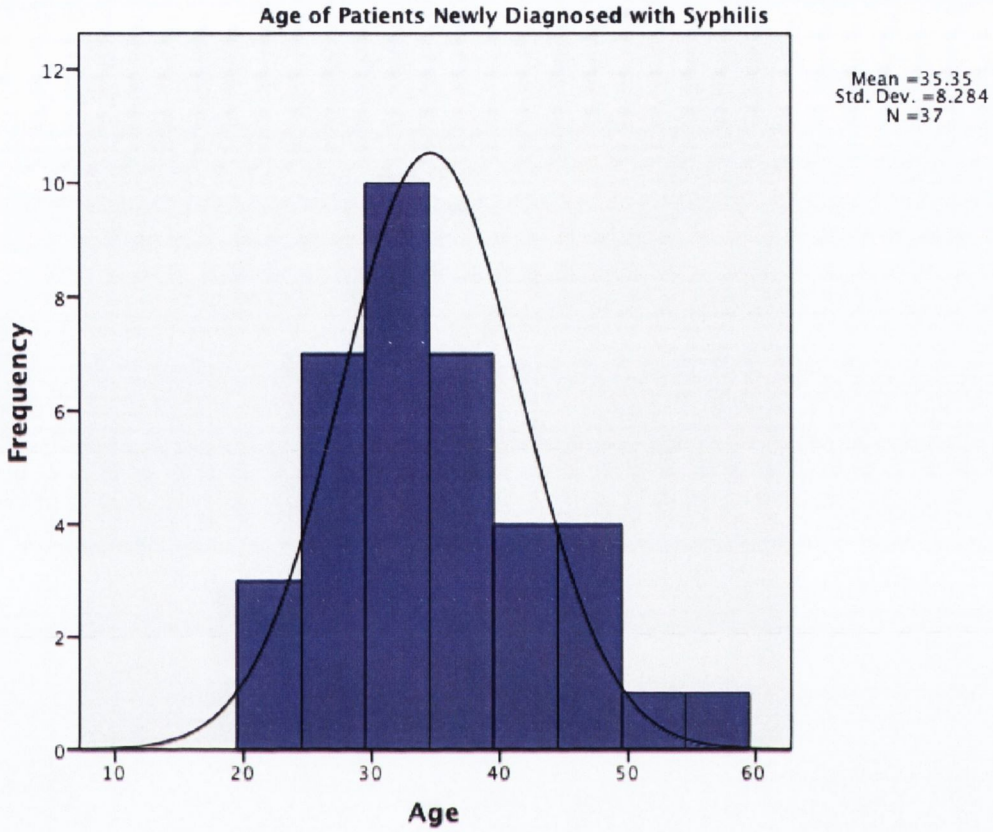


Figure 4.2 – Age of patients newly diagnosed with syphilis

4.1.2.2.1 – CSF Analysis

The results of the patients CSF analysis is presented in table 4.2. The mean CSF WCC was 7.6 cells/hpf (range 0-56 cells/hpf). Results of CSF protein analysis were available for 36/37 patients. The mean CSF protein count was 42.61mg/dL (range 15-43). 16 patients had a CSF protein higher than the laboratory defined upper range of normal of 40.

CSF RPR was performed on 34/37 (91.9%) of samples. The results of CSF RPR results are presented in table 4.3. The majority 31/35 (83.8%) of patients had a negative CSF RPR.

		Mean CSF WCC	**CSF protein**	**CSF glucose**
N	Valid	37	36	36
	Missing	0	1	1
Mean		7.6432	42.61	3.275
Median		2.7000	38.50	3.250
Mode		.00	43	3.5
Minimum		.00	15	2.7
Maximum		56.00	110	3.9

Table 4.2 – CSF results in patients newly diagnosed with syphilis

	Frequency	Percent
Negative	31	83.8
Neat	3	8.1
Not done	3	8.1
Total	37	100.0

Table 4.3 – CSF RPR results

Seven patients had a mean WCC of greater than 10 cells/hpf. In these patients the mean CSF protein was 50.3 mg/dL (range 33-81 mg/dL). 5/7 (71.4%) had a CSF protein above the laboratory cut off of 40 mg/dL. There was no statistical difference between a normal CSF protein level (i.e. <40 mg/dL) and a high protein level (i.e. >40 mg/dL) in patients with a WCC less than or greater than 10/hpf (p=0.15, kappa value 0.195). 1/7 (14.3%) had a positive RPR.

Based on laboratory values, 9/37 (21.6%) had a diagnosis of neurosyphilis (table 4.4). 1 patient had a positive RPR alone, one patient had a positive RPR and a protein >40 mg/dL, 2 patients and a mean WCC >10/hpf, 4 patients had a mean WCC >10/hpf and a protein level >40 mg/dL, and 1 patient had a positive CSF RPR, WCC >10/hpf and protein >40 mg/dL.

			CSF RPR		Total
			Negative	Neat	
Mean WCC <10/hpf	CSF protein >40 mg/dL	Protein <40		1	1
		Protein >40		1	1
	Total			2	2
Mean WCC >10	CSF protein >40 mg/dL	Protein <40	2	0	2
		Protein >40	4	1	5
	Total		6	1	7

Table 4.4 – Neurosyphilis Diagnoses

4.2.2.2.3 – CSF HIV viral load

HIV viral load (VL) was performed on 27/37 (72.9%) samples, in the remaining samples there was insufficient CSF remaining to perform an analysis of CSF HIV viral load. 25/27 (92.6%) of samples had a detectable CSF HIV VL. The mean detectable CSF HIV VL was 10415.6 copies/ml (range 5-193000 copies/ml). The mean CSF HIV viral load in patients with a diagnosis of neurosyphilis based on laboratory parameters was 41125 copies/ml (95% CI -64451.08-146701.8 copies/ml) compared to those without neurosyphilis was 2493.72 (95% CI 540.88-4446.67 copies/ml) p=0.03 (two tailed significance). Serum HIV VL was available for 32/37 (86.5%) patients. Patients who had a detectable serum HIV VL were more likely to have a

detectable CSF VL (p=0.04). 6 patients had a HIV viral load of less than 50 copies/ml in serum, however had a detectable HIV CSF VL. 3 patients had HIV VL CSF values of less than 50 copies/ml, while 3 patients had CSF HIV VL values of greater than 50 copies/ml (table 4.5).

CSF HIV VL (Copies/ml)	Number of Patients
5	1
18	1
22	1
62	1
399	1
14500	1
Total	**6**

Table 4.5 – CSF HIV VL in Patients with serum HIV VL <50 copies/ml

4.1.2.2.3 – Serum RPR & CD4 count.

29/37 (78.4%) of patients had a serum RPR of greater than or equal to 1:32. There was no statistical difference in the frequency of diagnosis of neurosyphilis among patients with an RPR of greater than or equal to 1:32 versus those with an RPR less than 1:32 (chi square p=0.32).

The mean CD4 count of patients who underwent lumbar puncture was 521 cells/mm^3 (range 13-964). There was no statistical difference in the mean CD4 count among patients who were diagnosed with neurosyphilis (mean 526 cell/mm^3, 95% CI 303.2-749.91 cells/mm^3) versus those who were not (mean 520 cells/mm^3, 95% CI 433.25-607.46 cells/mm^3) p=0.95. Similarly no statistical difference was found in the CD4 percentage.

4.2 – Survey of Infectious Diseases and Genitourinary Medicine Physicians on the Management of Syphilis in Ireland.

Controversies exist in both the assessment and treatment of patients with syphilis[120]. Much of the discrepancy in opinion arises from the lack of large clinical trials, leading to portions of the guidelines being based on expert opinion. International guidelines differ[63, 69]. Recently Dowell et al [168] surveyed US based infectious diseases practitioners on their practices pertaining to the investigation and assessment of patients diagnosed with syphilis. Not surprisingly, a wide variation in practice was found. 1007 infectious diseases consultants who reported treating adults with infectious diseases were surveyed by means of an online survey distributed to members of the Emerging Infections Network[213]. There was an overall response rate of 46%. The majority of respondents (86%) had seen between 1 and 20 patients with syphilis in the last year, and 87% had been consulted on a patient with syphilis in the same time-period.

Darkfield microscopy was unavailable to 81% and as a result 56% of respondents said that in deciding to treat primary syphilis that they would send an RPR and treat presumptively. Similarly, most (63%) had been consulted on a penicillin allergic patient with syphilis and 79% recommended desensitisation. Of those who said that desensitisation was not always done, the reasons given were patient refusal or difficulty arranging same. On the issue of lumbar puncture in HIV positive patients without neurological or

ophthalmic signs or symptoms, 65% responded that they would perform a lumbar puncture if the patient had a CD4 count of 150 cells/mm^3, whereas 44% if the CD4 was 550 cells/mm^3. Physicians who had treated more than 5 patients in the preceding year were significantly less likely to perform a lumbar puncture p=0.007.

4.2.1 – Methods

A modified version of the survey used by Dowell *et al*[168] was used (obtained with thanks, from Dr Deborah Dowell, Centers for Disease Control and Prevention) to survey Irish Infectious Diseases and Genitourinary Medicine consultants and trainees (appendix 2). There are currently 9 consultants in Infectious Diseases (ID) and 3 in Genitourinary Medicine (GUM) working in teaching hospitals in the Republic of Ireland and 22 Specialist Registrars (SpR) training in these specialties. Practitioners were contacted via email and requested to complete an attached word document survey. This survey could then be emailed back as a word attachment or printed off and posted back. Reminder emails were sent to encourage practitioners to complete the survey. The complete survey is contained in appendix 2.

4.2.2 – Survey Results

25 of 34 (73.5%) physicians surveyed responded. 40% were consultants and 60% specialist registrars. Similar to the US survey the majority (76%) of respondents had treated between 1 and 20 patients with syphilis in the last year. (figure 4.3)

92% of respondents stated they had treated more than 50 patients with HIV in the last year and 64% treated between 1 and 20 patients with gonorrhoea infection. 72% had been consulted on a patient with syphilis in the past year. 68% of respondents said that darkfield microscopy was available to them and that they used it, and the same percentage said that they would treat presumptively and send an RPR in the case of primary syphilis.

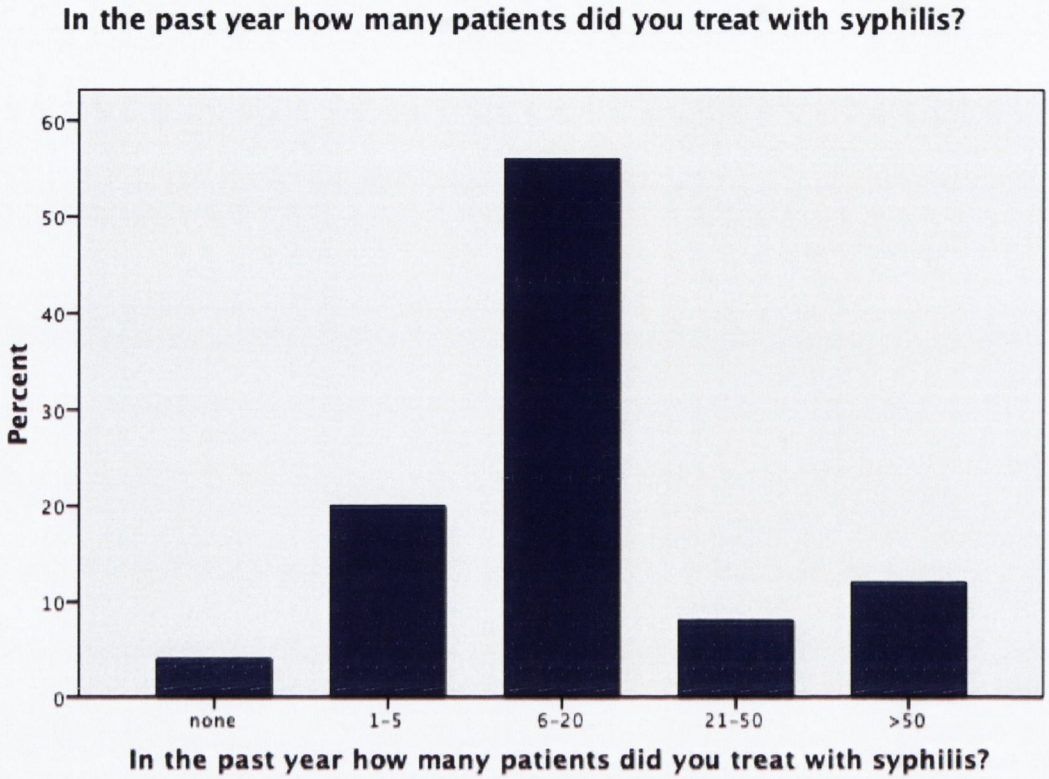


Figure 4.3 – Number of patients treated with syphilis in the preceding year

52% treat secondary syphilis in a HIV positive patient with three doses of Benzathine penicillin, compared to 40% who give only one dose. This did not vary significantly between consultants and SpRs (chi-square p=0.9). Unlike the US survey treatment of a patient with secondary syphilis did not significantly differ between practitioners who had seen less than five patients with syphilis in the last year and those who had seen more than five p=0.2.

Irish respondents again differed to their US counterparts when asked what was the lowest RPR they were willing to follow (i.e. retest periodically without re-treatment or lumbar puncture) post treatment in patients reporting no re-exposure. 40% of respondents were willing to follow a titre greater than 1:4 in HIV negative patients versus 48% in HIV positive patients $p<0.001$, a trend reserved from the American findings.

When it came to the issue of lumbar puncture to investigate asymptomatic HIV positive patients with syphilis, 72% would perform a lumbar puncture in a patient with an RPR of 1:32 with a CD4 count of 150 cells/μL while 44% would if the patient's CD was 550 cells/μL (p=0.21).

68% responded that they had treated a penicillin allergic patient with syphilis. 66.7% recommended penicillin desensitization, however only 28% stated that desensitization was always done and practical reasons were the most frequent response for why desensitization was not always done.

9 respondents did not answer the questions on alternative therapy in penicillin allergic patients, of those who did answer, 12/16 (75%) would use tetracycline therapy.

4.3 – Delayed diagnosis of Secondary Syphilis.

Given that identification of patients with syphilis is paramount in controlling the numbers of new cases of infection, it is vital that symptomatic patients with syphilis infection are recognised and treated promptly[23]. Previous studies have demonstrated that attendance at non-specialised sexual health clinics is independently associated with missed and delayed diagnosis of syphilis[214]. In Australia when the records of MSM who presented with syphilis were reviewed, of 34 men who had been referred by a general practitioner, 30/34 (88%) referral letters contained no evidence that a diagnosis of syphilis was considered[215]. This occurred more frequently in secondary syphilis. This may not be surprising given syphilis' reputation as "the great imitator". In addition the knowledge of sexually transmitted diseases among health care providers has been demonstrated to be low in certain settings[216].

The Internet has allowed a wealth of knowledge, including medical knowledge become available the population as a whole. More recently the provision of home testing from internet sites for certain sexually transmitted infections has become available[217].

Anecdotally it was felt that many patients presenting with secondary syphilis had been seen by a medical practitioner prior to attendance at the GUIDE clinic and that the correct diagnosis was often missed. In addition, it was felt that many patients were using the Internet to self diagnose prior to attendance at the clinic.

4.3.1 – Methods

Patients who presented to the GUIDE clinic with secondary syphilis from January 2007 to December 2009 were identified. Patients were then contacted via telephone by two medical students enrolled in Trinity College Dublin. Telephone contact was performed under the supervision of the author and any patient concerns that arose were addressed by return phone call. Surveys were conducted over a two week period, and patients were surveyed (appendix 3) about the time to presentation post onset of symptoms, attendance at other health care professionals, and the use of the internet in an attempt to self diagnose. Four attempts were made to contact patients via telephone. Messages were not left on answering services, so as not to breach patient confidentiality. Data was collected, stored and analysed in MS excel.

4.3.2 – Results of Telephone Survey

70 patients were identified who had presented with secondary syphilis. There were no telephone contact details available for ten patients. Of the remaining 60 patients, 38 (63.33%) were contactable and 37 participated in the telephone survey. All patients were male and the mean age was 39.54 years (range 24-58 years). 18 (48.65%) were HIV co-infected.

31/37 (83.75%) recalled their symptoms prior to diagnosis. The mean duration of symptoms prior to diagnosis was 52.73 days (range 7- 168 days). The most commonly reported symptoms were rash (61%), genital lesion (39%), general malaise (52%), headache (29%) and fever (29%). Other reported symptoms were alopecia, lymphadenopathy, general pain and hearing or visual loss.

23/37 (62.16%) patients recalled having seen a medical practitioner prior to attendance at the GUIDE clinic. Of these patients, 16/23 (69.57%) reported having attended a general practitioner (GP) while the other patients reported having attended a physician other than a GP. The majority of these patients (12/23, 52.17%) attended only one physician prior to attendance at the GUIDE clinic. 6/23 (26.09%) saw two practitioners, 2/23 (8.7%) attended three, and one patient (4.35%) saw four medical personnel prior to attendance at the sexual health clinic. The remaining patients could not recall the number of practitioners attended.

Of the patients who attended another practitioner, 9/23 (39.13%) reported having been given an alternative diagnosis to syphilis, and 3/23 (13.04%) patients responded that no diagnosis was given. The remaining patients were given the diagnosis of syphilis. Common alternate diagnoses were "fungal infection", "'flu like illness", general rash/hives or unspecified sexually transmitted disease. 8/23 (34.78%) of patients were given treatment, predominantly antibiotics or topical cream. No patient received targeted syphilis therapy.

7/37 (18.92%) of patients reported having delayed their own attendance at the GUIDE clinic, the main reasons for which were underestimating symptoms or time constraints.

12/37 (13.51%) reported having used the Internet in an attempt to self-diagnose. 7 of these 12 patients (58.33%) arrived at the correct diagnosis. An additional 5/37 (13.51%) reported having discussed their symptoms with family or friends who assisted in diagnosing syphilis prior to attendance at the clinic.

Only 4/37 (10.81%) of patients used the Internet to get information about the GUIDE clinic prior to attendance. All of these patients reported that the website was easy to use.

4.4 – Knowledge of Syphilis among Sexually Transmitted Infection Clinic Attendees.

In fight against the spread of syphilis, identification of infectious cases is paramount. As many patients may be asymptomatic[44], the identification of patients infected with *T. pallidum* is in the large part reliant on the selective screening of at risk populations[147]. Education about the transmission and consequences of sexually transmitted diseases will increase self-presentation for testing for syphilis and is a cornerstone of any public health initiative. Targeting such education campaigns to at risk groups or those cohorts of patients with poor knowledge should increase the effectiveness of such public health campaigns.

In England in 2005, it was found that 20% of male respondents had not heard of syphilis[218], while a common perception among MSM was that syphilis is a rare disease[219]. In the United States, 65% of adolescents correctly identified syphilis as a sexually transmitted infection[220]. Ethnicity has also been shown to have influence over knowledge of STIs in some studies. In Sydney, Asian students were found to have poorer knowledge than their Australian counterparts[221].

In Ireland a survey conducted during the 2009 lesbian, gay, bisexual and transgender (LGBT) Pride concert, found that 70% of respondents were aware that syphilis infection could be asymptomatic[222], however the

knowledge of syphilis infection and transmission was poorer than in a similar survey conducted in 2001.

4.4.1 – Methods

An anonymous questionnaire, devised by the author was distributed to all attendees of consecutive sexual health clinics at the GUIDE clinic in St James's Hospital over the two-week period from 1st to 12th March 2010. The questionnaires were distributed by two students of the Department of Medicine, Trinity College Dublin supervised by the author. Data collection and statistical analysis was performed using MS excel and SPSS 17.0 commercially available software.

The questionnaire comprised questions related to patient demographics and knowledge of syphilis characteristics and modes of transmission (appendix 3).

4.4.2 – Results of Questionnaire

In the two week period 272 completed questionnaires were collected, the refusal rate was less than 10%. The majority of patients (50.4%) were between the ages 19 to 27, reflective of the age demographic of the Irish population [154] (figure 4.4).

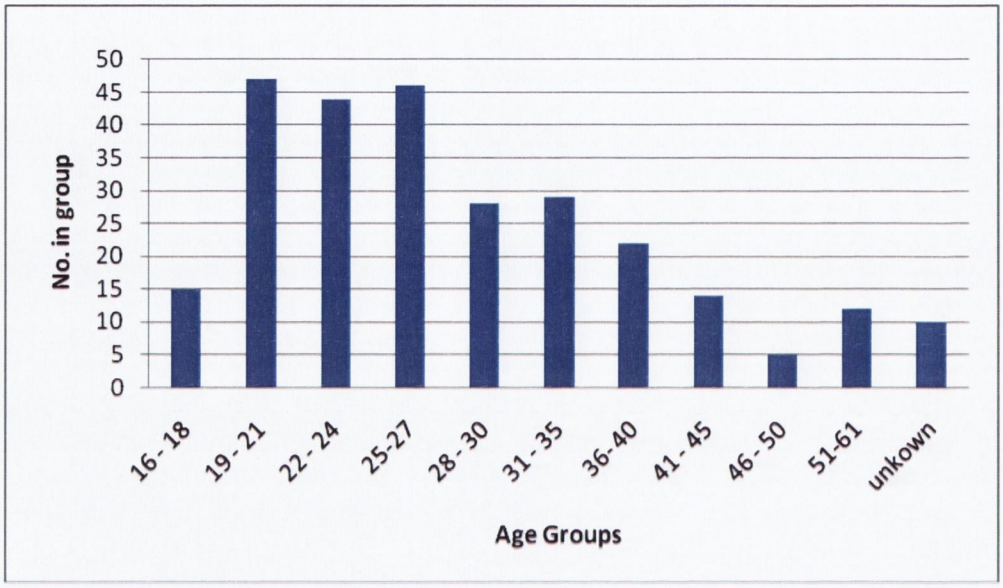


Figure 4.4 – Age Group of STI clinic attendees surveyed

	Number	Percentage
First Attendance	130	47.8
More than once	138	50.7
Unknown	4	1.5
Total	**272**	**100.0**

Table 4.6 – number of first time Attendees at STI clinic

The majority of respondents 232/272 (85.3%), identified themselves as heterosexual, 36 participants identified as homosexual/bisexual, 31 (11.4% of total respondents were MSM. 81.8% of respondents were Irish. Attendees were equally split between first time and repeat attendees (table 4.6).

83.1% of respondents reported that they had previously heard of syphilis. Of the patients who had previously heard of syphilis, the source of their syphilis knowledge was categorised into six categories; word of mouth, health services, media, education STI awareness and other. After the miscellaneous category of "other" media was the most common response. Of those who hadn't heard of syphilis (n=34), 28/34 (82.35%) were Irish, the mean age was 28.7 years. Worryingly, in 13/28 (46.43%) of patients, it was not their first attendance at the GUIDE clinic, and 5/13 (38.46%) reported having attended more than once previously.

The number of correct responses to each individual question on syphilis is represented in table 4.7. The percentage of respondents answering each question correctly varied from 12% who knew that syphilis could be contracted by kissing an infected individual to 96% who correctly responded that syphilis could not be acquired by being in the same room as an infected individual. Due to validity concerns, question 14 was omitted from the analysis.

	Number (%) of respondents with correct answer
STD	232 (85%)
Curable	154 (57%)
Kissing	33 (12%)
Pregnant can pass on	101 (37%)
Gay men only	209 (77%)
Lifelong disease	97 (36%)
Old fashioned disease	199 (73%)
Testing regularly is good	227 (83%)
Unprotected oral sex	160 (59%)
Condom	225 (83%)
Touching infected	227 (83%)
Same room	261 (96%)
Blood transfusion	150 (55%)
Sharing needles	195 (72%)

Table 4.7 – Responses to questions on Syphilis

	General score (p value)	Transmission score (p value)
Gender	0.084	0.121
Nationality	0.616	**0.010**
Orientation (Hetero vs. Homo/Bisexual)	0.342	0.084
MSM vs. Heterosexual men	**0.037**	**0.019**
First attendance vs. repeat	0.533	0.518
Times attended (0, 1, 2+)	0.618	0.776

Table 4.8 – Relationship between patients' characteristics and syphilis knowledge

To analyse the responses on aggregate, the questions were divided into general and transmission knowledge of syphilis. Questions 9-10, 13, 15-17 were classified as general and questions 11, 12, 18 and 19 as transmission knowledge. Each individual was given a general and transmission score calculated on the number of correctly answered questions (figure 4.5). A significant correlation was found between the general and transmission score ($p<0.001$).

The relationship of syphilis knowledge to gender, age, nationality, and attendance is displayed in table 4.8. MSM were found to have better general and transmission knowledge of syphilis than their heterosexual counterparts ($p=0.037$, 0.019 respectively).

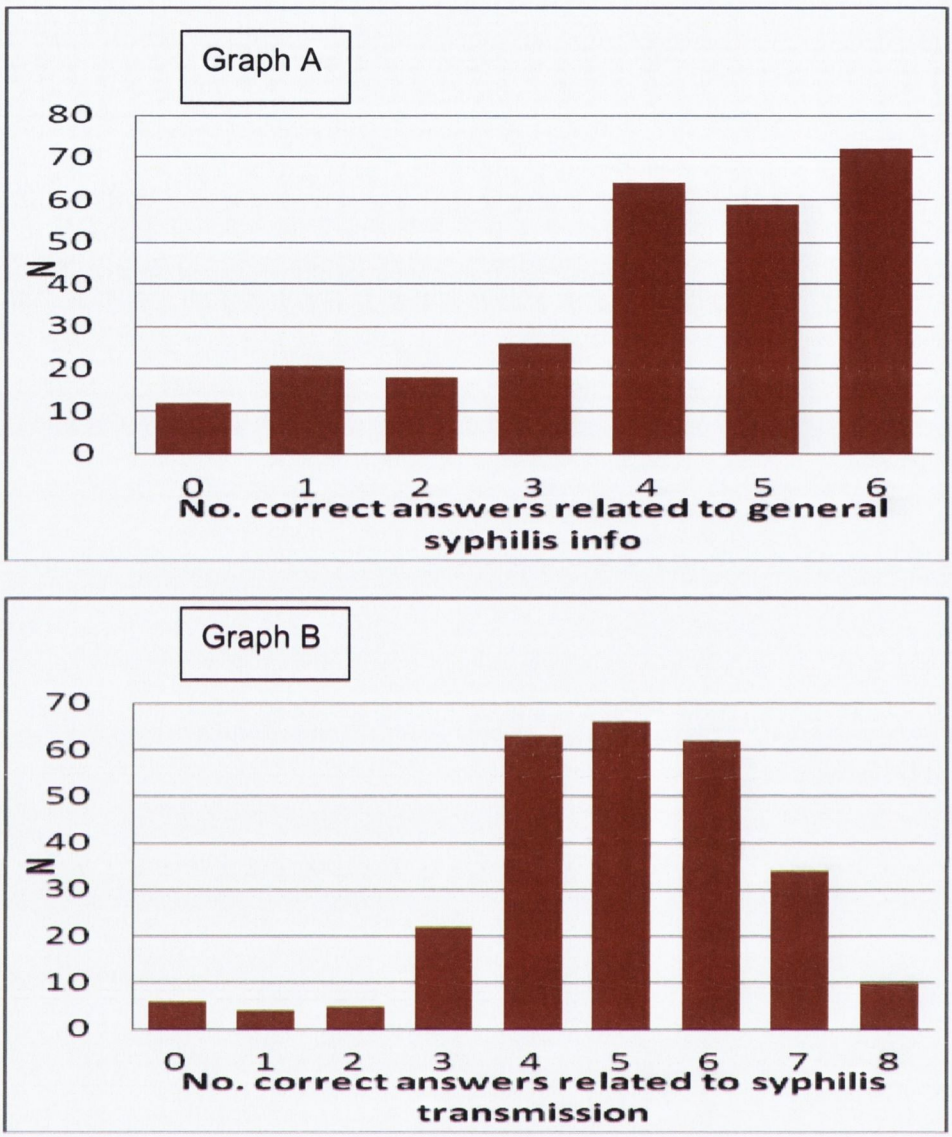


Figure 4.5 – Number of correctly answered questions on syphilis knowledge and transmission

(Graph A= general knowledge, graph B= transmission knowledge)

4.5 – Discussion

The role of lumbar puncture in the investigation of patients co-infected with syphilis and HIV is not yet clearly defined. In this series there is a high rate of diagnosis of neurosyphilis (22%), however given that symptomatic patients were included the rate of asymptomatic neurosyphilis is likely lower. The lack of criteria for definitive diagnosis of neurosyphilis is problematic. If a positive CSF RPR alone was taken as indicative of neurosyphilis, then only 3 patients would have been given the diagnosis. The issue arises that although a CSF RPR is sensitive, it has poor specificity. As a result, surrogate markers such as CSF pleocytosis, and CSF protein are used to make the diagnosis. Given that HIV in particular can case abnormalities of both of these variables, the waters become significantly more muddied.

The finding that none of the patients diagnosed prior to 2007 had abnormal CSF findings is reassuring. All of the patients had received standard dose, stage appropriate therapy at the time of their syphilis diagnosis. This finding strengthens the recommendation of treating HIV positive patients with standard syphilis therapy, as one would in a HIV negative patient. Future work focusing on whether or not HIV positive patients with previously diagnosed and treated syphilis have higher CSF HIV VL than matched controls who have never had syphilis may contribute to the pool of knowledge on the long term prognostic implications of syphilis, and in particular neurosyphilis infection.

The survey of specialists in the disciplines of infectious diseases and genitourinary medicine highlights the variation in practice that occurs in the management of syphilis in Ireland. The response rate of the survey was excellent, likely reflecting the fact that the ID/GUM community in Ireland is small, and reminder emails were sent out to remind physicians to complete the survey. It is unusual that 68% of respondents stated that darkfield microscopy was available to them and that they used it, given that St. James's and the Mater Misericordiæ Microbiology departments are the only centres performing the test. However the largest proportion of ID/GUM practitioners work in these two hospitals and as the place of work of respondents was not recorded, it is possible that the majority of participants work in these centres.

An unusual finding was that practitioners were more likely to follow a higher RPR in treated HIV positive patients vs. HIV negative patients without re-exposure. An opposite trend to that seen in the US study. The fact that no difference was seen in treatment practice amongst those who had seen smaller numbers of syphilis patients compared to those seeing larger numbers likely represents the smaller sample size of the Irish survey. Both this survey and the larger American survey demonstrate the need for large-scale clinical studies into the investigation and management of syphilis particularly in the HIV population.

The retrospective review of syphilis patients who reported to GUIDE has revealed that a significant proportion of patients have had their diagnosis of secondary syphilis delayed prior to attending the GUIDE clinic which

demonstrates the need for greater provider knowledge of syphilis symptoms and treatments.

32% of patients reported having used the Internet to self diagnose. While patients' education is paramount to interrupting the transmission of infection, the concern would arise over the quality of some websites and the information they provide. However just over half of these patients reported having arrived at the correct diagnosis. Efforts should be made within the HSE and sexual health services to ensure that their websites, containing quality information be the top hits in search engine results for certain sexually transmitted infection related keywords.

Although the survey of STI clinic attendees was conducted over a short period of time, several definite trends were observed. The fact that MSM are more likely to have a better knowledge of syphilis than their heterosexual counterparts is indicative of previous and ongoing efforts to educate the MSM community regarding the risks of syphilis. The syphilis awareness, knowledge and action (SAKA) survey[222] conducted during the Dublin LGBT Pride concert in June 2009, similarly showed an young age profile (46% of respondents were under 30 years old). However, the study found that compared to a similar survey conducted in 2001 that MSM had a significantly poorer awareness of the increase in syphilis cases in 2009. Similar to our survey, the knowledge that kissing can spread the infection was low (only 36% of respondents). Knowledge was lowest in men who did not identify themselves as gay, lived outside a city or who had never tested for syphilis.

These two surveys highlight the need for good quality information to be disseminated amongst MSM to encourage them to test for syphilis. While both surveys have sample bias, the fact that individuals presenting to either the GUIDE clinic or attending Dublin Pride have poor knowledge of syphilis, and in particular some of the modes of transmission of syphilis is worrying. Targeting those people who have not attended for testing or who do not identify themselves as gay may be very problematic.

The media was cited as the most commonly used source of information. Using mass media communications and not just gay publications may increase awareness amongst men who do not identify as gay. Ongoing education of younger MSM and continued efforts to encourage presentation for screening must be emphasised. In addition, education of primary health care providers may also increase the threshold of such practitioners to send syphilis serology on any patient in contact with their service.

Education is paramount to decreasing the ongoing spread of syphilis. As highlighted this education must not only target those at risk such MSM, but also health care providers who may come into contact with patients presenting with signs and symptoms of syphilis. Development of national guidelines for the treatment of syphilis would contribute to the standardisation of syphilis treatment amongst physicians specialising in Infectious diseases and Genitourinary Medicine and may also contribute to increased knowledge amongst general practitioners and other physicians who may encounter patients with syphilis, by providing clarity on the management of the infection.

Chapter 5 – Discussion

The rising number of syphilis diagnoses over the three years from 2007 to 2009 is concerning. The fact that there was a 118% increase n the number of cases of syphilis diagnosed between 2007 and 2009 is particularly worrying. The vast majority of cases are occurring in MSM. Intravenous drug users were not identifies as a risk group in this population in contrast to other cohorts[104]. Efforts need to be primarily centred on the MSM community.

The concern with the rising rate of syphilis diagnoses is that an associated increase in new HIV diagnoses will also be seen. In 2008 15% of newly diagnosed HIV patients in the GUIDE clinic in St James's Hospital also had untreated syphilis[107]. Engaging in safer-sex practices is known to decrease the transmission of HIV, and data is suggestive that condoms also decrease syphilis rates[223], although definitively studying the impact of condom use on syphilis rates can be difficult[224]. The use of condoms for anal sex alone may not be protective for syphilis however as it is known that syphilis can be transmitted through oral sex and kissing[44]. HIV negative men are more likely to choose sero-concordant partners and use oral sex as a method of HIV risk reduction[136]. Such sero-sorting has been used to explain increases in STIs among HIV positive MSM[155]. There is a suggestion in this data that serosorting is occurring within the HIV positive MSM community in Dublin. The majority of syphilis re-infections were occurring in HIV positive MSM, and it has been shown that while sero-sorting will decrease the transmission of HIV, subsequent increases in other STIs may occur[155].

The idea of fatigue among MSM to the "safe-sex" message may well be playing a role in the increased number of sexually transmitted infections. Recent outbreaks of hepatitis C among HIV positive MSM[225] engaging in high risk sexual behaviour strengthens this argument. The 2009 Swiss Statement that HIV positive individuals on ART who do not have an STI cannot transmit HIV[226] may exacerbate the situation further. While cognitive behavioural therapy has been shown to decrease the number of transmission risk sex acts among MSM[150], it is interesting to note that the control group in the same study also reported a decrease in risk behaviour. This underpins the need to fully incorporate discussion on sexually activity with all patients attending sexual health services including HIV positive patients engaged in ongoing care, and to incorporate discussion on sexual health and safer sex practices at any point of clinical contact.

At the 2010 Conference on Retroviruses and Opportunistic Infections (CROI), Marra *et al* presented data on syphilis strain typing[227]. Their work supports that hypothesis that certain strains of syphilis are more neuroinvasive that others[53]. Perhaps then, certain strains are also more infectious. In patients who deny any further sexual contact, strain typing has the potential to distinguish new infections from reactivation of a previous infection due to failed therapy. Further epidemiological and phylogenetic research is indicated to better understand this phenomenon. Strain studies along with epidemiology mapping of patients presenting with syphilis in Dublin, and perhaps Europe as a whole should be undertaken.

This study demonstrates a high degree of correlation between positive darkfield microscopy examination and syphilis serology. This is in contrast to previous reports that syphilis serology may take several weeks to months to become positive after appearance of a primary chancre[61]. This data demonstrated that 92.8% of patients with a positive darkground microscopy examination had positive syphilis serology, much higher than the 66% reported by Wheeler *et al*[65]. When compared to dark ground microscopy the sensitivity of EIA is reported to be 57%[65]. However based on our results the sensitivity of EIA compared to dark field microscopy is 85.7%. While the disadvantage with serology compared to darkground microscopy is that the result is not available while the patient is still in clinic, this series demonstrates that a large proportion of patients presenting with primary syphilis will have positive serology results.

The finding that syphilis IgM is more often positive in early infectious syphilis is not surprising, as an IgM response is seen in acute infection. However the loss of IgM response in cases where patients are re-infected with syphilis should be highlighted as some practitioners may rely on the IgM result in asymptomatic patients to determine if the patient has early infectious syphilis. The loss of IgM response may be related to re-infection with the same strain of syphilis, thus the patients immune system does not recognise the infection as "new". Further molecular work on the identification of syphilis strain types circulating in Dublin may contribute further to the knowledge of syphilis IgM response.

Given that *T. pallidum* cannot be cultured and that the manifestations of syphilis are multiple and varied, the use of PCR as a diagnostic tool is attractive. Serology is an indirect method of diagnosis[61], and darkground microscopy requires not only particular clinical manifestations to be present but also experienced clinical staff and expert laboratory personnel. In reality, serology is the mainstay of diagnosis, and in many clinical settings, such as general practice the only option available to physicians. Unfortunately darkground microscopy is not available in all genitourinary clinics, in a 2006 audit, Amin *et al* reported that darkfield microscopy was performed in 1% of microbiology laboratories and 34% of genitourinary clinics in England and Wales[66]. In Ireland, there are currently two laboratories performing darkground microscopy, St. James's Hospital and The Mater Misericordiæ Hospital, which are both in Dublin (Dr. Brendan Crowley- personal communication). Given the poor availability of darkground microscopy and the increasing numbers of syphilis diagnoses, there is certainly a need for an easily accessible, reliable test for the diagnosis of syphilis.

The use of the PCR assay utilised in this series has a documented sensitivity and specificity of 80.39% and a specificity of 98.4% when compared with serology[82]. However, the initial study did not compare the use of darkground microscopy to the PCR assay in those patients presenting with suspected primary syphilis. When compared to darkground microscopy the sensitivity and specificity were found to be 86.3% and 75% respectively with a kappa value of agreement of 0.614. These results are similar for those recently

published by Heymans *et al*[85] who used a similar assay which also targeted the *pol*A gene of *T. pallidum*.

Given that relatively few centres currently perform darkground microscopy and that the PCR assay is quick to perform, PCR may be a useful tool in the investigation of syphilis. Given the high level of agreement between darkground microscopy and the sensitivity and specificity of the PCR assay it would not be unreasonable to offer the test for practitioners who do not have access to darkground microscopy and are involved in the care of patients presenting with syphilis.

There are currently no Irish national guidelines for the treatment of syphilis and practitioners have to rely on those published internationally such as the those issued by the British Association for Sexual Health and HIV (BASSH) and the United Sates Centers for Disease Control (CDC)[63, 69]. The BASSH guidelines list azithromycin as third line treatment in incubating syphilis and as an alternate regimen in the treatment of early and late syphilis (excluding neurosyphilis at any stage) and in the treatment of syphilis in pregnancy. The CDC guidelines acknowledge azithromycin as a potential alternative, but recommend close follow up of any patient not receiving penicillin therapy.

In 2004 when syphilis treatment failures following azithromycin therapy were reported[102] and the A2058G mutation associated with azithromycin resistance was described[176], Dublin had the highest rate of Azithromycin resistance[103]. At that time, 15/17 (88%) samples obtained from the GUIDE clinic were found to

have the mutant 23s rRNA gene that confers resistance to *T. pallidum*. In our series, nearly six years on from the original study, little has changed 27/29 (93.1%) of isolated are azithromycin resistant. In the two patients with sensitive isolates, the risk for acquisition of their syphilis was outside Ireland, suggesting that perhaps the situation may well be worse than 2004. Azithromyicn cannot be recommended for the treatment of syphilis in Ireland.

The role of lumbar puncture in the investigation of patients co-infected with syphilis and HIV is not yet clearly defined. In this series there is a high rate of diagnosis of neurosyphilis (22%), however given that symptomatic patients were included the rate of asymptomatic neurosyphilis is likely lower. The current practice of performing lumbar punctures in symptomatic patients diagnosed with syphilis should continue. The new version of the CDC guidelines which are yet to be published, are likely to include the criteria of a CD4 less than 350 cell/mm^3 and an RPR of greater than/ equal to 1:32 (C. Marra- personal communication) for performing lumbar puncture in asymptomatic patients. The decision to perform a lumbar puncture in any individual patient should be made with care, and in those who do not undergo LP careful monitoring and follow-up should be undertaken.

There was no correlation between the diagnosis of neurosyphilis and either serum RPR or CD4 count. This is in contrast to previous studies[118, 119, 201]. This is likely due to several factors. This series was not specifically designed to address the issue of asymptomatic neurosyphilis; in addition the inclusion of symptomatic patients does not allow the true rate of asymptomatic

neurosyphilis to be determined. Finally the small number of patients studied effects the ability to determine definite trends.

The observation that three patients with undetectable serum HIV viral loads had a detectable CSF HIV VL is interesting. Given the recent data published by de Almeida *et al*[206], demonstrating higher CSF HIV VL in patients with neurosyphilis compared to patients without neurosyphilis and those seronegative for syphilis infection, it could be that syphilis infection up-regulates the viral replication of HIV within the CSF. This may have important implications for HIV evolution within the CSF and serum, as different strains have been demonstrated in both in the same individuals[211]. In addition, as it has been reported that CSF HIV VL has neurological prognostic implications[212], this up-regulation may have long-term consequences for patients diagnosed with neurosyphilis.

The survey of specialists in the disciplines of infectious diseases and genitourinary medicine highlights the variation in practice that occurs in the management of syphilis in Ireland. This mirrors the similar variation seen amongst American practitioners. The fact that the large number of practitioners who recommend penicillin desensitisation do not always see the procedure done is disappointing as this would be best practice in the treatment of a penicillin allergic patient with neurosyphills[63, 69].

Both this survey and the larger American survey demonstrate the need for large-scale clinical studies into the investigation and management of syphilis

particularly in the HIV population. The wide variation in clinical practice is due to the absence of evidence-based guidelines on the assessment and treatment of syphilis. In addition the development of Irish national guidelines might contribute in some way to the standardisation of syphilis therapy in Ireland.

Similar to the situation among the patients presenting with ocular symptoms, a thorough history including sexual history may have given vital clues to arriving at the correct diagnosis. The public health implications for the prompt diagnosis and treatment of syphilis have already been discussed. The additional psychological benefit to patients of making a quick diagnosis and administering effective therapy cannot be overlooked. Yet another benefit is the potential for cost saving by negating the performance of inappropriate or unnecessary tests.

Future efforts should focus on continued medical education of practitioners who may come into contact with patients at risk for syphilis. The form which this education could take is varied and could be modified to individual groups of practitioners[228]. This should include GPs and GP trainees, and medical specialists such as ophthalmologists, neurologists and dermatologists. In addition practitioners who are involved in the admission of general medical patients should be made aware of the increased numbers of syphilis diagnoses and the common manifestations of the disease. Care should also be taken to ensure that medical students are given a broad education in sexually transmitted infections and their presentations.

Appendices

Appendix 1- Syphilis Treatment Guidelines GUIDE Department, St. James's Hospital.

1- Diagnosis

Efforts must be made to stage the infection- full history including history of sexual contacts, blood transfusions/donation, obstetric history- history of miscarriage, history of previous treatment or syphilis tests or history other treponemal infections may be helpful.

Examination for signs of primary/ secondary disease or sequelae of late disease.

All patients should be offered full STI screening and HIV testing.

Offer vaccination e.g. hepatitis A/B where appropriate.

All STI clinic attendees should have syphilis serology performed & all HIV positive MSM every 3-6 months.

Primary

- Darkground microscopy should be performed if primary chancre present - darkground is less reliable on non-penile samples and should NOT be performed on oral lesions. (See clinic rooms for guidelines on performing darkground)
- Syphilis serology should be sent on all patients

Secondary

- Darkground microscopy may be performed on rash if pustular.
- Serology should be sent on all patients.

Early Latent

- Asymptomatic, acquired infection in last 2 years
- Serology should be sent

Neurological Syphilis

Lumbar puncture should be performed on

- All patients with neurological signs/ symptoms
- All patients with ophthalmic signs/ symptoms
- HIV positive patients- with late disease and all those with CD4 <350 and RPR >1:32
- Treatment failure- (12 months after completion of treatment)

  1) Failure to achieve a fourfold decline in RPR despite therapy or

  2) A fourfold increase in RPR where re-infection has been excluded

Late Latent

- Asymptomatic Infection acquired > 2years ago
- Serology should be sent on all patients
- Thorough neurological & cardiovascular exam
- CXR

Late Symptomatic

- Neurological imaging if focal neurology on exam
- Lumbar puncture if signs/symptoms of neurological/ophthalmic disease
- Echo if cardiovascular signs/ significant difference in BP both arms/ abnormal CXR
- Cardiovascular disease may progress despite adequate therapy

2- Treatment

Penicillin is first line treatment.

Penicillin desensitisation should be considered in patients with beta lactam allergy

Patients should be warned re Jarisch-Herxheimer reaction

Presumptive Treatment

Patients who have been in contact with syphilis in last 90 days

Serological testing may be negative

(See section 3 for recommendations on follow up serology)

Benzathine penicillin 2.4 MU IM x 1

Or doxycycline 100mg bd x 14/7

Primary Syphilis/ Secondary Syphilis/ Early latent disease

Benzathine penicillin 2.4 MU IM x 1

Or Procaine 600,000 U OD x 10/7

Or doxycycline 100mg bd x 14/7

Neurosyphilis in Early disease (secondary syphilis)

Procaine penicillin 2.4 MU IM x 14/7 **and** probenecid 500mg QDS PO x 14/7

Or Benzylpenicillin 18-24 MU in 3-4 divided doses per day IV x 14/7

Consider desensitization if penicillin allergy

Consider prednisilone 40-60mg od x 3/7 in ocular/neurological disease commenced 24hours prior to therapy

Alternatives

Doxycycline 200mg BD PO x 28/7

Or Amoxicillin 2g TDS PO + probenecid 500mg QDS PO x 28/7

Or Ceftriaxone 2g IM/IV x 14/7

Late Disease- Late latent or cardiovascular or gummatous disease

Benzathine penicillin 2.4 MU IM x 3

Or procaine penicillin 600,000 U IM OD x 17/7 (probenecid only required in neurological disease)

Or doxycycline 100mg BD x 28/7

Neurosyphilis in Late disease

Procaine penicillin 2.4 MU IM x 17/7 **and** probenecid 500mg QDS PO x 17/7

Or Benzylpenicillin 18-24 MU in 3-4 divided doses per day IV x 17/7

Alternatives

Doxycycline 200mg BD PO x 28/7

Or Amoxicillin 2g TDS PO + probenecid 500mg QDS PO x 28/7

Or Ceftriaxone 2g IM/IV x 14/7

Pregnancy- Early disease

1st & 2nd trimester - Benzathine penicillin 2.4 MU IM x 1

3rd trimester - Benzathine penicillin 2.4 MU IM x 2

Or Procaine 600,000 U x 10/7

Alternatives (baby must be evaluated and treated at birth)

Amoxicillin 500mg QDS + probenecid x14/7

Ceftriaxone 500mg OD x 10/7

Penicillin allergy-

erythromycin 500mg PO QDS x14/7 or azithromycin 500mg PO OD x 10/7.

Baby must be evaluated and treated at birth if mother is not treated with penicillin. Mother must be evaluated and re-treated post partum if erythromycin used.

Pregnancy- late disease- As per non-pregnant patients

3- Follow Up

Initial serology negative

3 months after exposure in cases of high risk exposure & repeat HIV testing

6 weeks and at 3 months in those with darkfield negative ulcerative lesions (where another dx has not been confirmed) and those who may have been a contact of a patient with syphilis

Follow up post therapy

Early-

1,3,6 and 12 months and then 6 monthly until RPR negative or serofast

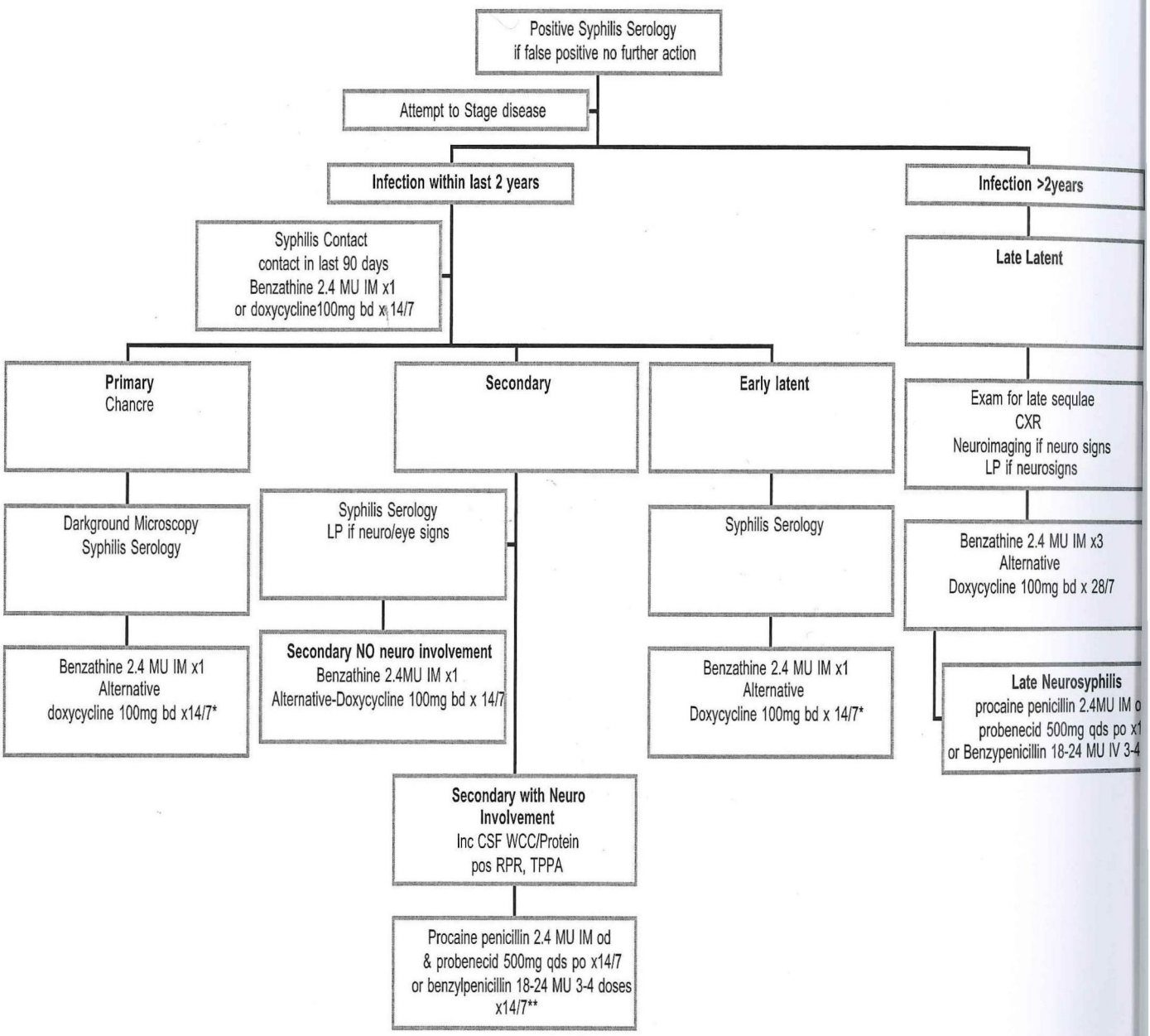
Late-

3 monthly until serofast

Latent infection with initial negative RPR – repeat at 1 month post completion of treatment and no need to do further serology if RPR remains negative

HIV positive MSM patients 3-6 monthly

SYPHILIS TREATMENT



*Penicillin is the treatment of choice
**Consider penicillin desensitisation in the case of penicillin allergy- alternatives Doxycycline 200mg BD PO x 28/7 Or Amoxicillin 2g TDS PO + probenecid 500mg QDS PO x 28/7 or Ceftriaxone 2g IM/IV x 14/7
***Consider penicillin desensitisation in case of penicillin allergy- Alternatives Doxycycline 200mg BD PO x 28/7 Or Amoxicillin 2g TDS PO + probenecid 500mg QDS PO x 28/7 or Ceftriaxone 2g IM/IV x 14/7

Pregnancy- Early disease
1st & 2nd trimester - Benzathine penicillin 2.4 MU IM x 1
3rd trimester - Benzathine penicillin 2.4 MU IM x 2
Or Procaine 600,000 U x 10/7
Alternatives (baby must be evaluated and treated at birth) Amoxicillin 500mg QDS + probenecid x14/7 Or Ceftriaxone 500mg OD x 10/7 if penicillin allergy- erythromycin 500mg po QDS & **evaluation and treatment** of baby at birth.
Pregnancy- late disease As per non-pregnant patients

Appendix 2- Survey of syphilis management in Ireland.

Please mark relevant answers with an x

1. **Are you**
   a. A consultant ☐
   b. An SpR ☐
   c. Registrar ☐

2. **In the past year approx how many patients did you treat with**

   Gonorrhoea
   a. None ☐
   b. 1-5 ☐
   c. 6-20 ☐
   d. 21-50 ☐
   e. >50 ☐

   Syphilis
   a. None ☐
   b. 1-5 ☐
   c. 6-20 ☐
   d. 21-50 ☐
   e. >50 ☐

   HIV
   a. None ☐
   b. 1-5 ☐
   c. 6-20 ☐
   d. 21-50 ☐
   e. >50 ☐

3. **In the past year have you been consulted on patients with syphilis?**

   a. Yes ☐
   b. No ☐

4. **Is darkfield microscopy readily available to you to diagnose primary syphilis?**
   a. No ☐
   b. Yes, I use it ☐
   c. Yes, but I do not use it ☐
   d. Uncertain ☐

5. **In deciding to treat for primary syphilis do you:**
   a. Send RPR and treat while awaiting RPR results ☐
   b. Send RPR and repeat if negative before deciding whether to treat ☐
   c. Send RPR and treat only if RPR is positive ☐
   d. Treat presumptively without sending RPR ☐
   e. Other ☐

If other please indicate current practice.....

6. **For a HIV infected patient with secondary syphilis, how do you treat?**
   a. Benzathine penicillin 2.4 MU x1 ☐
   b. Benzathine penicillin 2.4 MU x3 ☐
   c. Other ☐

If other please indicate current practice.....

7. **For patients with an <u>unchanged</u> non-treponemal titre 12 months after treatment for early latent syphilis and reporting no risks for re-exposure, what is the highest titre you would be willing to follow without LP or re-treatment?**

   I. In a HIV positive patient
   a. Don't know ☐
   b. Negative ☐
   c. Neat (1:1) ☐
   d. 1:2 ☐
   e. 1:4 ☐
   f. 1:8 ☐
   g. 1:16 ☐
   h. 1:32 ☐
   i. 1:64 ☐
   j. >1:64 ☐

   II. In a HIV negative patient
   a. Don't know ☐
   b. Negative ☐
   c. Neat (1:1) ☐
   d. 1:2 ☐
   e. 1:4 ☐
   f. 1:8 ☐
   g. 1:16 ☐
   h. 1:32 ☐
   i. 1:64 ☐
   j. >1:64 ☐

8. **A 34 year old HIV infected man has a secondary syphilis RPR 1:32 and no neurological or ophthalmic symptoms or sign. Do you recommend an LP**

   I. **If his CD4 count is 150**
      a. Yes ☐
      b. No ☐
      c. Uncertain ☐

   II. **If his CD4 count is 550**
      a. Yes ☐
      b. No ☐
      c. Uncertain ☐

9. **Have you ever treated a penicillin allergic patient with neuro-syphilis?**
      a. Yes ☐
      b. No ☐

10. **Have you ever recommended penicillin desensitization for a patient like this?**
      a. Yes ☐
      b. No ☐

11. **If yes has it always been done?**
      a. Always ☐
      b. Sometimes ☐
      c. Never ☐

12. **If not always, why not?**
      a. Patient refusal ☐
      b. Complexity/ difficulty in arranging monitoring/ impractical ☐
      c. Too time consuming/ expense ☐
      d. Too serious an allergy ☐

13. **If not always, how have these patients been treated**
      a. Ceftriaxone ☐
      b. Doxycycline/tetracycline ☐
      c. Azithromycin/macrolide ☐
      d. Other ☐

      ii. If other please indicate current practice.....

Appendix 3- Delayed Diagnosis of Syphilis Questionnaire

Introduce yourself explain that you are a Medical student working in the GUIDE department of St. James's Hospital. Ask if the patient would mind sparing a few minutes to answer some brief questions. If it is not a good time ask would they mind if you contacted them at a more convenient time.

1. Do you remember how long you had symptoms before your diagnosis?
2. What were your symptoms?
3. Did you attend any other doctors/ alternative practitioners prior to attending SJH?
    a. If yes who? How many?
    b. Were you given a different diagnosis?
    c. What was this diagnosis?
    d. Did you receive any treatment prior to diagnosis in SJH?
4. Did you delay your own attendance at SJH?
    a. If yes why?
5. Did you try to diagnose yourself with the use of the Internet?
    a. Did you arrive at the correct diagnosis?
6. How did you hear of SJH clinic?
    a. Did you use the Internet to get information about the clinic prior to attending?
    b. Was this GUIDE website or another?
    c. If the GUIDE website was used did you find it helpful?
7. Any other comments?

Thank the patient for their time and effort in participation of the survey.

Appendix 4- Knowledge of Syphilis Questionnaire

Please indicate your answer by ticking (√) the following box

Section 1

Q1) what age are you in years? .....

Q2) Are you..

- ☐ Male [1]
- ☐ Female [2]

Q3) what nationality are you? .....

Q4) Sexual Orientation..

- ☐ Heterosexual [1]
- ☐ Homosexual [2]
- ☐ Bisexual [3]

Q5) Is this your first attendance at a sexual health clinic?

- ☐ Yes[1]
- ☐ No[2]
- ☐ Not sure[3]

Q6) If no how many times have you attended previously?

.....

Q7) Have you heard of syphilis before today?

☐ Yes[1]

☐ No[2]

☐ Not sure[3]

Q8) If yes where did you hear about it

PLEASE ANSWER THE FOLLOWING QUESTIONS AS TRUE, FALSE or NOT SURE

Q9) Syphilis is a sexually transmitted disease

☐ True [1]

☐ False[2]

☐ Not sure[3]

Q10) Syphilis is curable

☐ True [1]

☐ False [2]

☐ Not sure [3]

Q11) You can get syphilis from kissing an infected person

☐ True [1]

☐ False [2]

☐ Not sure [3]

Q12) pregnant women infected with syphilis can pass it on to their unborn baby

- ☐ True [1]
- ☐ False [2]
- ☐ Not sure [3]

Q13) Only gay men get syphilis

- ☐ True [1]
- ☐ False [2]
- ☐ Not sure [3]

Q14) Syphilis is a virus

- ☐ True [1]
- ☐ False [2]
- ☐ Not sure [3]

Q15) syphilis is a life long disease

- ☐ True [1]
- ☐ False [2]
- ☐ Not sure [3]

Q16) syphilis is an old fashioned disease and is no longer common in Ireland

- ☐ True [1]
- ☐ False [2]
- ☐ Not sure [3]

Q17) testing regularly for syphilis is a good idea

- ☐ true [1]
- ☐ false [2]
- ☐ Not sure [3]

Q18) syphilis can be caught by having unprotected oral sex (i.e. oral sex without using a condom)

- ☐ true [1]
- ☐ false [2]
- ☐ Not sure [3]

Q19) How do you think you can get syphilis? (please tick all of the boxes you think apply)

- ☐ Sex with a condom
- ☐ kissing an infected person
- ☐ touching an infected person
- ☐ being in the same room as an infected person
- ☐ from a blood transfusion
- ☐ sharing needles with an infected person

THIS WILL HELP US TO HELP YOU

THANK YOU

Please check that you answered all the questions that apply to you.

Appendix 2. Primers used in the detection of azithromycin resistance in *T. pallidum* by PCR

gi|6626258:231950-234850 *Treponema pallidum* subsp. *pallidum* str. Nichols

23S

Inner Forward Primer Pandori/Lukehart 2007 Pandori 201 bp Primer Name: TPAZF Tm= 49.5

Inner Reverse Primer Pandori/Lukehart 2007 Pandori Primer Name: TPAZR Tm= 49.7

Sensor Probe Lukehart 2007 Pandori/Lukehart

Anchor Probe Lukehart 2007 Pandori/Lukehart

Outer Forward Primer Lukehart 2004 628 bp Primer Name: TP23SOF Tm= 56.2

Outer Reverse Primer Lukehart 2004 Primer Name: TP23SOR Tm= 56.2

A G mutation at position 2058

GCGAATAGTGGTTTACGGTGGATGTCTTGGAGTTGTCAGGCGATGAAGGTCGTGATAAG
CTGCGAAAAGCCTCGGGGAGGAGCACATGTCCTGTGATCCGGGGATGACCGAATGGGG
TAACCCGACAGGGTAAAGCCTTGTCATTGCCTTCCTGAATGAATAGGGAGGGTAAGGCG
AAACTGGGTGAACTGAACCATCTAAGTAACTTGGGAAAAGAAATCAAGAGAGATTCCGAA
AGTAGTGGCGAGCGAAATTGGAGGAGCCTAAACCTGTGTCTAACAGGGGTTGTAGGGC
CGCGCGGGCTTGCGTTCGGTGGGTGAAATAATCCGGCCTATAGCAGAAAGGTTTTGGG
AAAGCCTGACAGAGAGGGTGAAATCCCCGTATGCGGAATGGGGCGGACCTGCTGGTGC
GGTACCTGAGTACGGCGGGACACGAGGAATCCTGTCGGAATCTGGGTCGACCACGATC
TAAGGCTAAATACTCGACAACTACCGATAGTGGACAAGTACCGTGAGGGAAAGATGAAA
AGAACCCCGGTGAGGGGAGTGAAATAGAACCTGAAACCGTAAACCAACAAGATGTTACA
GCCTGTGAGGGTGGTAGCGTGCCTTTTGTAGAATGAGCCTGCGAGTTACGGTGTGCAG
CAAGGTTAAGGGATAGGAGTCCTGGAGCCGGAGGGAAACCGAGTCTTAAAAGGGCGTG
GTGAGTTGTACGTCGTAGACCCGAAGCCAGGGTGATCTAGTTATGAGCAGGTTGAAACA
GGGGTGAAGCCTTGTGGAGGACCGAACTATAATCTGTTAAAAAAGGTATGGATGACTTG
TGACTAGGAGTGAAAGGCTAAACAAACCTGGAGATAGCTGGTTCTCCCCGAAATGCCTT
TAGGGACAGCCTTATACAAAACTGTCGGAGGTAAAGCACTGGATGGGCTAGGGGGTTTC
ATCGCCTACCAAACCCAATCAAACTCTGAATGCCGGCAGTCAACGTGTGGGAGTGAGAC
TGCGTGCGACAAGGTTCGTAGTCGAGAGGGAAACAGCCCAGACCGTCAGCTAAGGTCC
CGAAATACCGCTTGAGTGTGAAATGAAGTGTGGGTACCTGGACAGCCAGGAGGTTGGCT
TAGAAGCAGCCATTCCTTGAAAGAGTGCGTAATAGCTCACTGGTCGAGTACGCATGCGC

AGATAATGTATCGGGGCTAAGCGGTATACCGAAGCTACGGGTCTTGCATTTTTGGTGCA
AGGCGGTAGGGGAGCATTCCATGTACTGATGAAGGAATATCCGGGAGGAGTTCTGGAG
GGGATGGAAGAGAGAATGCAGGTATAAGTACACGAAAAGGAGGGTGAGATTCCTTCCC
GCCGAAAACCTAAGGTTTCCTGGGTGAAGGTCATCTGCTCAGGGTAAGTCGGCCCCTAA
GGCGAGGACGAGGGTCGTAGTCGATGGGAATCCGGTTTATATTCCGGAACCTCTTGCAA
TTTCGATGGCAGGACGCGTGAGGTGAAGCCCGGCCAAAGATTGGTAGTTTTGGTCTAAG
TATCCGAGCCGTTTTAAGAGCGATAGGCAAATCCGTCGTTCGAGGTAAGGTGCGAGTGC
GACTGGAGCGATGAGCGAAGGGAAGCAGGTGTAGTCATGGCGACGGGAAATACTGTCT
AAGGTTAGGTTGCAAGAGACCGTACCGCAAACCGACACAGGTAGGTAGGATGAGTAATC
TAAGGCGCTCGAGAGAACTCGCGTCAAGGAACTCGGCAAAATACACACGTAACCTCGG
GAGAAGTGTGACCCTTGCCTTTGGTGAGGGTGGCAGAAAGCAGGTCCAGGCGACTGTT
TATCAAAAACATAGCCATCTGCAAATCAGTAATGAGACGTATAGGTGGTGACACCTGCCC
GGTGCTGGAAGGTTAAGAGGAGAGGTTCGTGGTAACACAACGCTTTGAATTGAAGCCCC
AGTAAACGGCGGCCGTAACTATAACGGTCCTAAGGTAGCGAAATTCCTTGTCGGGTAAG
TTCCGACCCGCACGAATGGTGTAACGACTCTGGACACTGTCTCGACGCGAGACTCGGT
GAAATTTATGTACCGGTAAAGAAGCCGGTTACCCATAGTTAGACGG2058AAAGACCCCG
TGAACCTTCACCGTAGCTTACTATTGGAACTTGGTTTACCATGTGTAGTATAGGTGGGAG
ACAGAGAAGCTTGGCCGTCAGGTTAGGCGGAGTCAACAGTGAAATACCACCCTTGGTAC
GTCAGGTTTCTAACCTTTGGCCGTGGATCCGGCAAAGGGACCGTGGTAGGTGGGCGGT
TTGACTGGGGCGGTCGCCTCCTAAAAGGTAACGGAGGTGCGCGAAGGTCTCCTCACAC
CGGTTGGAAATCGGTGCGCGAGTGTAAAGGCACAAGGAGGCTTAACTGCGAGACCGAC
AGGTCGAGCAGATACGAAAGTAGGTCTTAGTGATCTGGCGGTAGCGTGTGGAAGCGCC
GTCACTTAACGGATAAAAGGTACTCCGGGGATAACAGGCTGATTTTCCCCAAGAGTTCA
CATCGACGGGAAAGTTTGGCACCTCGATGTCGGCTCATCGCATCCTGGGGCTGAAGCA
GGTCCCAAGGGTTTGGCTGTTCGCCAATTAAAGCGGTACGTGAGCTGGGTTCAGAACGT
CGCGAGACAGTTCGGTCCCTATCTGCTATGGGCGTTGGATATGTGAGAGGAGCTGCTTT
TAGTACGAGAGGACCGAAGTGGACGAACCTCTGGTGTACCAGTTATCCTGCCAAGGGTA
CGTGCTGGGTAGCTATGTTCGGAAGGGATAACCGCTGAAGGCATCTAAGTGGGAAGCC
CGCCTCAAGATTACATATCCCTGAAGGTTGACCTTCCTGAAGACTCCTTGCACACTACAA
GGTCGATAGGCTGGAGGTCTACGTACCGT

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