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Molecular epidemiology of syphilis in Scotland

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ABSTRACT

Objective: To examine the molecular epidemiology of syphilis in Scotland.

Methods: Ulcer specimens were collected from 85 patients with infectious syphilis. Typing of *Treponema pallidum* was performed using a method that examines variation in two loci; the number of 60-bp repeats within the *arp* gene and sequence variation in the *tpr* genes.

Results: Patients were predominately white men who have sex with men. Treponemal DNA was detected in 75 specimens and a total of six subtypes were identified from 58 typeable specimens (77%). The most common subtypes were 14*d* (44/58, 76%), followed by 14*e* (7/58, 12%), 14*j* (3/58, 5%), 14*b* (2/58, 3%), 14*p* and 14*k* (1/58, 2%).

Conclusions: This study shows that subtype 14*d* is the predominate subtype circulating in Scotland and there is a surprising level of genetic diversity within the Scottish men who have sex with men community.

INTRODUCTION

Although syphilis is a relatively rare disease in the United Kingdom (UK) diagnoses of infectious syphilis have increased since 1997; rising from 301 cases to 3749 cases in 2007.[1, 2] This has been largely due to outbreaks, frequently amongst men who have sex with men (MSM), in a number of cities.[1] In Scotland, diagnoses have increased annually since the outbreak started in 2000/2001.[3] There were a total of 235 and 248 cases in 2006 and 2007 respectively, with the majority of cases in MSM (81%).[4]

If left untreated, syphilis can lead to devastating diseases including cardiovascular syphilis, benign gummatous syphilis and neurosyphilis.[5] The vertical transmission of *Treponema pallidum* to the unborn child may result in the distressing condition of congenital syphilis. In addition, syphilis infection increases the risk of both transmission and acquisition of HIV[6] and co-infection with HIV is a frequent occurrence. The severe consequences of syphilis highlight the importance of implementing effective measures to control its dissemination.

The availability of a molecular typing system allows strain variation to be identified in the absence of an *in vitro* cultivation system for *T. pallidum*. The typing system for *T. pallidum*[7] is based on analysis of the *arp* (acidic repeat protein) gene and subfamily II (*tpr E, G and J*) of the *tpr* (*T. pallidum* repeat) genes. Investigation into the molecular epidemiology of syphilis combines molecular typing with demographic or behavioural data and enables the identification, characterisation and geographical distribution of molecular subtypes, which in turn could inform public health intervention programmes.

This is the first study in the UK, and the second one in Europe, to examine the molecular epidemiology of syphilis and presents evidence of multiple subtypes circulating in this predominantly MSM population diagnosed with infectious syphilis in Scotland. This study was performed as part of the European Surveillance of Sexually Transmitted Infections project (www.essti.org).

METHODS

Clinical specimens

Between August 2006 and December 2007 genital, anal or oral ulcer swabs were collected from patients with suspected infectious syphilis who were attending genitourinary medicine (GUM) clinics in Scotland. The specimens were sent to the Scottish Bacterial Sexually Transmitted Infections Reference Laboratory (SBSTIRL), Royal Infirmary of Edinburgh, for molecular testing. Extracted DNA for samples that were positive for *T. pallidum* PCR were then assigned a study number and clinic number and patient identifiers removed to allow blinded molecular typing at the Sexually Transmitted Bacterial Reference Laboratory (STBRL), Health Protection Agency, London. The DNA samples were transported from SBSTIRL to STBRL overnight on dry ice.

Epidemiological patient data

Epidemiological data were collected at the clinic visit following an interview by health care staff using a proforma designed as part of the National Enhanced Surveillance of Infectious Syphilis in Scotland (NESISS) programme.

DNA extraction and detection of *T. pallidum* DNA by real-time PCR

DNA was extracted by centrifuging 1ml of M4RT media (Remel, Lenexa, USA) or AL lysis buffer (Qiagen, Crawley, UK) at 13000rpm for 1 minute and 800µl of the supernatant was removed. The remaining 200µl and any pelleted cellular material was then processed using the QIAamp blood and body fluids spin protocol (Qiagen) according to manufacturer's instructions and the DNA eluted into 100µl of sterile water. Treponemal DNA was detected by a real-

time PCR (RT-PCR) assay that targets the DNA polymerase A gene (*polA*) of *T. pallidum*. [8]. The presence of treponemal DNA was re-confirmed on receipt at STBRL by the same *T. pallidum polA* RT-PCR. [8]

Molecular typing of *T. pallidum*

Molecular subtyping was performed based on a previously described method [7;9] that examines variation in two loci; the number of 60-bp repeats in the acidic repeat protein (*arp*) and sequence variation in the *T. pallidum* repeat (*tpr*) genes. The final molecular subtype is defined based on the two loci (e.g. 14d = 14 repeats in the *arp* gene and *tpr* RFLP (Restriction fragment length polymorphism) pattern “d”). PCR of the *arp* gene was performed in 50µl reactions containing 1.67U of Expand High Fidelity PCR System *Taq* Polymerase (Roche Diagnostics, Mannheim, Germany), 2.0mM MgCl₂ (Roche Diagnostics), 2mM (each) of dTTP, dCTP, dATP and dGTP (Invitrogen, Paisley, UK), 0.2 µM of primers ARP-F (5'-CAAGTCAGGACGGACTGTCCCTTGC-3') and ARP-R (5'-GGTATCACCTGGGGATGCGCACG-3') and 10µl of DNA template. Thermocycling parameters were as follows: 1 cycle at 94°C for 5 minutes, 45 cycles at 94°C for 50 seconds, 62°C for 1 minute and 68°C for 2.5 minutes and a final extension step of 68°C for 15 minutes. All thermocycling was performed on the MJ Research PTC-225 thermal cycler (Waltham, USA). The *arp* PCR products were electrophoresed in a DNA7500 LapChip on an Agilent Bioanalyser (Agilent Technologies, Wokingham, UK) and the number of *arp* repeats were determined by comparing the size of the amplicons to the Nichols strain of *T. pallidum* which has 14 repeats. [7]

A nested PCR was used to amplify the *tpr* genes. The first round of the nested PCR was performed in 100µl reactions which contained 2.5U of Expand High Fidelity PCR System *Taq* Polymerase with 1.5mM MgCl₂ (Roche Diagnostics), 2mM (each) of dTTP, dCTP, dATP and dGTP (Invitrogen) and 0.2µM primers B1-F (5'- ACTGGCTCTGCCACACTTGA -3') and A2-R (5'- CTACCAGGAGAGGGTGACGC -3') and 6µl of DNA template. Thermocycling parameters were as follows: 1 cycle at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 60°C for 2 minutes and 68°C for 2.5 minutes, and a final extension step of 68°C for 15 minutes. The second round of the *tpr* nested PCR was performed in 100µl reactions as described for the first round, except that 0.2 µM of a second set of primers, IP6-F (5'- CAGGTTTTGCCGTTAAGC -3') and IP7-R (5'- AATCAAGGGAGAATACCGTC -3'), were used. Thermocycling parameters were as follows: 1 cycle at 94°C for 5 minutes, 40 cycles of 94°C for 1 minute, 59°C for 1 minute and 68°C for 2 minutes, and a final extension step of 68°C for 15 minutes.

The *tpr* nested amplicon (16.2 µl) was digested at 37°C for 1h in a 20 µl reaction containing 4x BSA (New England BioLabs, Hitchin, UK) and 10U of the restriction enzyme *Mse* I (New England BioLabs) . Restriction fragments were electrophoresed using the Agilent Bioanalyser (Agilent Technologies). The RFLP patterns were compared to the *T. pallidum* Nichols strain and sized using the ladder and profiles determined by the original publication[7] and additional profiles kindly supplied by the lead author (Allan Pillay, personal communication).

The number of 60-bp *arp* repeats and *trp* RFLP pattern were combined to create the molecular subtype as previously described.[7] The *arp* and the *trp* assays were repeated on all specimens that initially gave a negative result.

Statistical analysis

The Student's t-test was used to determine any significant difference between the mean *poIA* PCR Ct values for fully typeable specimens and non-typeable specimens. Fisher's Exact test was used to assess the significance of differences of demographic and epidemiological data for persons with common subtypes and the remaining study population.

RESULTS

There were 88 treponemal DNA positive ulcer specimens (excluding duplicates) submitted to SBSTIRL and from these a total of 87 specimens from 85 individuals were available for molecular typing during the seventeen-month study period. Twenty specimens were collected in 2006 and 67 were collected in 2007. Other than sex and age, there was no epidemiological information available for 10 patients. The most common epidemiological and demographic characteristics of the patient population are described in Table 1. To summarise, the patient population was predominantly British white homosexual males with a median and mean age of 32 and 35 years respectively (range 16-72).

The presence of treponemal DNA was re-confirmed using the *poIA* PCR in 75 of the 87 specimens. Of these 75 specimens, 58 were positive by both the *tpr* and *arp* assays to produce a full subtype in 77% (58/75) of specimens. Of the 58 fully typeable specimens, a total of six subtypes were identified. The most common subtype was 14*d* (44/58, 76%), followed by 14*e* (7/58, 12%); 14*j* (3/58, 5%); 14*b* (2/58, 3%); 14*p* and 14*k* (each 1/58, 2%).

Six specimens were positive by the *tpr* assay only and in total there were seven different *tpr* RFLP patterns (*b*, *d*, *e*, *j*, *k*, *l* and *p*). A schematic representation of the *tpr* RFLP patterns identified in this study is shown in Figure 1. Three specimens gave a positive *arp* result of 14 repeats, but no *tpr* profile could be determined. One of these specimens was a duplicate patient sample; the other sample gave a positive 'e' *tpr* pattern only. Fourteen repeats were identified in all but one of the positive *arp* results. Insufficient amplicon was generated to determine accurately the size of the remaining fragment, but

the larger size indicated that it contained more than 14 repeats. This specimen had an 'e' *tpr* pattern. There were seven specimens that were negative for both the *arp* and the *tpr* assays. Using the Student's t-test, there was a significant difference ($p < 0.0001$) between the mean *polA* PCR Ct values from specimens that were typeable (overall lower Ct values) and non-typeable (overall higher Ct values).

Subtype 14*d* appeared throughout the whole 17-month collection period, whereas subtype 14*e* was collected over a ten month period (August 2006 to May 2007), 14*j* over a 13 month period (October 2006 to September 2007) and two specimens with the 14*b* subtype were identified in April 2007 and November 2007.

Table 1 compares demographic and epidemiological data for persons with 14*d* and 14*e* subtypes to the remaining study population. Using Fisher's Exact test there was no significant difference between any epidemiological or demographic factor of those patients with subtypes 14*d* or 14*e* and the remaining study population (for all comparisons $p \geq 0.2$).

Place of acquisition was known for 73 patients (86%). Most patients (79%) recorded acquiring infection in an area or city in Scotland, or elsewhere in the UK (7%) in cities where outbreaks of syphilis have been described.[2] Patients also described acquiring syphilis from contacts outside the UK (7%) or quoted multiple possible locations (7%), all of which included an area of Scotland. The most common subtype, 14*d*, was distributed throughout Scotland and other locations throughout England (Table 2). There were six places of possible acquisition declared outside of the UK, but a full subtype was only retrievable from one specimen; Pakistan (14*j*). The other specimens

from individuals that declared possible places of acquisition did not reveal a full subtype; South Africa (*tpr 1*), Canary Islands (*arp 14*), Australia, Thailand and Spain (no *arp* or *tpr* result).

Table 1. Demographic and epidemiological characteristics of study population

	All patients (n=75*)	14d subtype (n=37*)	14e subtype (n=7*)
Sexuality & Gender			
Male MSM	56	29	4
Male heterosexual	14	5	2
Female heterosexual	2	1	
Country of birth			
UK	65	32	6
Ireland	1		1
Norway	1		
Australia	1		
Race			
White British	63	31	6
White Irish	1		1
White other	1		
Chinese	1		
Pakistani	1		
Age†			
Range	16 - 72	16 - 68	22 – 43
Mean	35	36	32
Median	32	33.5	32
Stage			
Primary	61	30	6
Secondary	12	6	1
Specimen			
Anal	12	9	
Genital	58	27	6
Oral	12	6	1
Scrotal	1		
HIV status			
Positive	10	5	
Negative	50	24	5

Table 1 continued

	All patients (n=75 [†])	14 <i>d</i> subtype (n=37 [†])	14 <i>e</i> subtype (n=7 [*])
Oral sex only			
No	33	13	5
Yes	28	16	1
Total contacts in 3 months prior to syphilis diagnosis			
Range	1 - 100	1 - 32	1 - 3
Mean	3	3.5	1.6
Median	3	2	1
Clinic location			
Aberdeen	6	4	
Airdrie	5	2	
Cumbernauld	1		1
Dundee	1	1	
East Kilbride	2		1
Edinburgh	15	8	1
Glasgow	42	22	4
Kirkcaldy	2		
Perth	1		
* No. of patients with available epidemiological data			
† Age data available from all 85 patients; 44 with subtype 14 <i>d</i> and 7 with subtype 14 <i>e</i>			

Table 2. Distribution of *T. pallidum* molecular subtypes (n = 85)

Subtype	No. of specimens	Place acquired (In Scotland, unless otherwise stated)
14d	44	Glasgow (16) , Edinburgh (6), Aberdeen (4), East Kilbride (1), East Lothian (1) Fife (1), Lanarkshire (2), Livingston (1), Alloa or London, England (1), Glasgow or London (1), Glasgow or Manchester, England (1) Blackpool, England (1), Manchester (1), Unknown (7)
14e	7	Glasgow (2), Edinburgh (1), East Kilbride (1), Lanarkshire (1), Stirling (1), Manchester (1)
14j	3	Glasgow (2), Pakistan (1)
14b	2	Glasgow (1), Edinburgh (1)
14p	1	Unknown (1)
14k	1	East Kilbride (1)
e*	2	Edinburgh (2)
e†	1	Dublin, Ireland (1)
j*	1	Aberdeen (1)
k*	1	Blackpool (1)
l*	1	South Africa (1)
p*	1	Unknown (1)
14‡	2§	Glasgow (1), Edinburgh or Canary Islands (1)
No results¶	18	Glasgow (8), Aberdeen (1), Lanarkshire (1), Livingston (1), Glasgow or Thailand (1), Manchester (1), Spain (1), Australia (1), Unknown (3)

* No *arp* result available
† Specimen contained more than 14 60-bp *arp* repeats
‡ No *tpr* result available
§ Three *arp* only results in total, but one was a duplicate sample. The patient's other sample gave a *tpr* 'e' pattern only and the place of acquisition was Edinburgh
¶ No *arp* or *tpr* result available or no treponemal DNA amplified

DISCUSSION

This is the first UK study to examine the molecular epidemiology of syphilis and it demonstrated variation in the subtypes circulating in Scotland and the rest of the UK. Of the 347 cases of infectious syphilis identified throughout Scotland over the study period, 88 (25%) were diagnosed by serology as well as amplification of *T. pallidum* DNA in ulcer specimens, and so were available for this study. Fifty eight of these (66%) were fully typed (17% of all syphilis cases). Six full subtypes (14*b*, 14*d*, 14*e*, 14*k*, 14*j* and 14*p*) were identified in this Scottish study. Subtype 14*d* accounted for 76% of the fully typeable specimens and was the dominant subtype circulating in the Scottish syphilis outbreak during 2006 and 2007. In the only other European typing study performed in Lisbon, Portugal,[10] a lower genetic diversity was observed; three full subtypes from 42 specimens (14*a*, 50%; 14*d*, 45%; 14*f*, 5%). The most prevalent subtype (14*a*) was not found in Scotland. Subtype 14*d* was predominant in South Africa and Madagascar and also demonstrated in the United States.[7;9;11]

The much smaller numbers of the other non-14*d* subtypes may indicate smaller and more localised transmission networks and/or outbreaks which are most probably related to the importation of syphilis strains into the Scottish MSM community. However, it is currently not possible to determine the presence of such networks using the available epidemiological information. There appears to be a higher level of strain diversity where syphilis is endemic, such as in South Africa, Madagascar and southeastern United States.[7;9;12] Subtypes 14*j*, 14*k* and 14*p* have not been described in the six published studies to date.[7;9-13] Further work to investigate the distribution

of syphilis subtypes throughout England and Wales, and any correlation with Scotland would be useful.

Subtype 14*d* predominated for the duration of this study, although a more discriminatory typing method may be required before it can be concluded that the strains characterised as subtype 14*d* are indistinguishable from each other. The predominance of a single subtype may be a reflection of the low prevalence (3.3 per 100, 000) of syphilis in Scotland, with a limited number of subtypes circulating in a relatively small core group. However the possibility that subtype 14*d* represents a more transmissible or even more virulent strain cannot be excluded. Without typing data from when the outbreak started in 2000/2001,[4] it is not possible to say whether 14*d* is the original outbreak strain in Scotland. Further prospective typing studies would determine if subtype 14*d* continues to predominate and perpetuate sustained transmission and endemic spread of syphilis throughout Scotland. In spite of this subtype predominance, this study has demonstrated that there is variation in the molecular epidemiology of syphilis in Scotland. The identification of six different subtypes and one additional different *tpr* profile (“*l*”) is surprising given the low prevalence of syphilis in Scotland. It is interesting that there is such diversity when there was little evidence of overseas travel and most patients declared that the most likely place of acquisition was within Scotland or elsewhere within the UK. This may suggest that syphilis is becoming endemic within the Scottish MSM community; this is supported by the stabilising epidemiology which shows a similar number of cases from 2005 to 2007.[4]

The major demographic of the study population was white British homosexual males, which also matches the published surveillance data,[4] so although only 17% of strains from cases of infectious syphilis in 2006 and 2007 were typed, the data set is most probably representative. There was no evidence to suggest that subtype 14*d* was associated with any particular patient population characteristics or locations, however this does not exclude the presence of other subtypes that are related to particular epidemiological, demographic or risk factors. It was not possible to establish this during this study due to the low numbers of the other subtypes. Where the data are available, the number of patients co-infected with HIV is high, 13% (10/75), and this highlights the need to limit transmission of syphilis with great exigency. Greater understanding of the disease molecular epidemiology may allow a more focused intervention strategy with the aim of limiting syphilis and HIV infection.

The presence of treponemal DNA was not reconfirmed in all specimens (14%). This loss of sensitivity may be due to DNA degradation as a result of prolonged storage or repeat freeze-thawing. The overall sensitivity of this typing method may increase if fresh samples were used. Of the 75 specimens available for typing, only 77% were fully typeable. DNA quality and/or poor assay sensitivity, as indicated by the significant difference of the means of the *poIA* PCR Ct values from the typeable and non-typeable specimens, may explain why seven specimens were negative with this typing assay. The sensitivity of the *arp* gene PCR in particular was low, and in one case the observed weak amplification prevented accurate assessment of band size. The three instances where an *arp* result was obtained but no *tpr* profile was

determined could be due to primer-template mismatch or to insufficient DNA quality to permit amplification of this larger (2186bp) product. Insufficient specimen volume was available for further investigation. Previous syphilis typing studies also have demonstrated the poor sensitivity of this typing method, ranging from 87% to as low as 49%, when applied to specimens which have been confirmed to contain treponemal DNA.[9-13]

This report is timely following the first European study that was performed in Portugal.[10] Introduction of routine typing using this method would allow the creation of a database of syphilis subtypes to monitor the prevalence and distribution of subtypes throughout Europe. However, it is clear that further work is required to establish a more sensitive, robust and more discriminatory typing strategy. Continued molecular typing of syphilis, would allow: (i) the identification of new subtypes; (ii) the detection of outbreak strains; (iii) identification of associations between particular subtypes, their virulence and disease outcome and, (iv) would also determine whether endemic strain levels are reduced by elimination strategies.

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Competing interests: None

CONTRIBUTORS

MC was the project microbiologist, performed the laboratory work, analysed the data and is the lead author for the paper.

SC contributed to the design of the study and assisted in the laboratory work and data analysis.

HP and LW provided the specimens and epidemiological information and contributed to the design of the study.

CI contributed to the design of the study, supervised the project and was the project lead.

All authors contributed to the write up and reviewed the final draft of the manuscript.

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KEY MESSAGES

This is the first study to describe the molecular epidemiology of circulating syphilis strains in Scotland and in the United Kingdom.

The majority of syphilis cases are diagnosed in white men who have sex with men (MSM).

Subtype 14d predominates and the identification of six different subtypes reveals a surprising level of genetic diversity given the low prevalence of syphilis in Scotland.

Epidemiological and molecular typing data has shown that syphilis is becoming endemic within the Scottish MSM community.

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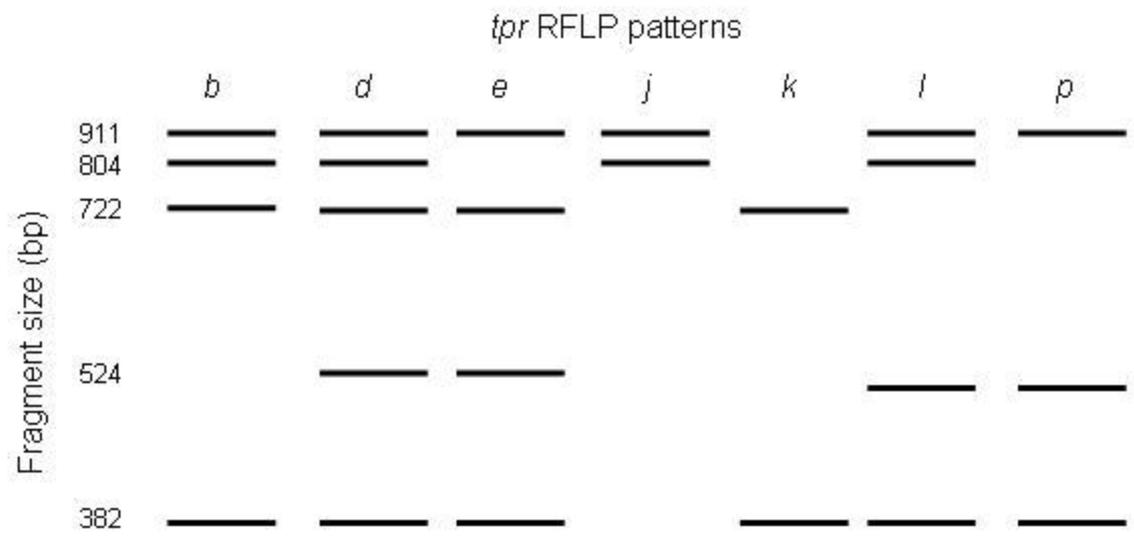


Figure 1. Schematic representation of *tpr* RFLP patterns identified in study