Highly diverse MLVA-*omp*A genotypes of rectal *Chlamydia trachomatis* amongst men who have sex with men in Brighton, UK and evidence for an HIV-related sexual network

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ABSTRACT

Objectives In this prospective study we aimed to determine the distribution of genotypes by multi-locus variable number tandem repeat (VNTR) analysis plus analysis of the *omp*A gene (MLVA-*omp*A) of rectal *Chlamydia trachomatis* (CT) among MSM attending Brighton GUM Clinic and to examine any correlations with clinical variables including HIV status, and to isolate rectal CT cultures maximising the possibility of obtaining complete genotyping data.

Methods Samples were assigned genotypes by PCR and sequencing of the markers of the MLVA-*omp*A genotyping system. Rectal CT was isolated in cell culture using McCoy cells. Data regarding demographics, HIV status, rectal symptoms, past history of STIs including CT were collected.

Results 1809 MSM attending the clinic between October 2011 and January 2013 took part in the study, 112 (6.2%) of whom had rectal samples which tested positive for CT. 85/112 (75.9%) CT positive rectal samples were assigned 66 different genotypes. Two distinct genotype sub-clusters were identified: Sub-cluster 1 consisted of more HIV-negative men than sub-cluster 2 (p=0.025), and the MLVA-*omp*A genotypes in these sub-clusters reflected this. Isolates were successfully cultured from 37 of the 112 specimens, from which 27 otherwise unobtainable (from direct PCR) MLVA-*omp*A genotypes were gained.

Conclusions The most prevalent genotypes were G, E and D representing some overlap with the heterosexual distribution in UK. Sub-cluster 1 consisted of more “heterosexual genotypes” and significantly more HIV-negative men than sub-cluster 2, associated with “MSM genotypes”. There was a higher diversity of CT strains among MSM in Brighton than observed in other cities.

INTRODUCTION

Rectal *Chlamydia trachomatis* (CT) remains one of the most commonly diagnosed bacterial sexually transmitted infection (STI) among men who have sex with men (MSM) attending GUM clinics in the UK[1]. There is strong evidence for the role of rectal STIs as a risk factor in both the

acquisition and transmission of HIV[W1-3] and routine rectal testing in MSM is recommended in national guidelines[2].

Different CT strains determine disease outcomes (for example there is linkage of lymphogranuloma venereum (LGV) strain L2b to proctitis in MSM[3 4]. The majority of infections occur within transmission networks[5 6], therefore knowledge of different genotypes is useful for mapping transmission within sexual networks and surveillance of emerging trends. In the past two decades, genotyping of CT has relied on PCR and sequence analysis of the *omp*A gene[7-12]. But within each *omp*A genotype, very little variation exists with respect to *omp*A sequences [6 13 14]. Furthermore, genome sequencing data reveals that *omp*A must not to be relied upon solely as a marker for genotyping, as recombination of all or parts of this gene within and between biovars is evident[15]. There are now several very highly discriminating multi-*locus* molecular typing systems including multi-locus sequence typing (MLST) for CT that allow differentiation and sub-grouping of isolates within a genotype[13 16 17 W4-6]. The multi-locus variable number tandem repeat (VNTR) analysis plus analysis of the *omp*A gene (MLVA-*omp*A) high resolution genotyping system of Pedersen et al (2008)[13], has been successfully evaluated[18] and recent work has shown that the VNTR marker sequences are stable and therefore ideal for genotyping[19]. The system consists of short, easy to amplify markers (the longest being part of the *omp*A gene) which are simple to manipulate and yield a high efficiency return of results as demonstrated in the quoted surveys . It also has an optimal ability to differentiate between genotypes[20], as measured by the Simpson's discriminatory index[13 18].

The diversity of CT in the heterosexual population has already been characterized and thus the selection of genotypes that form this population are well-established and set the baseline of known strains[13 21 22]. Previous work has reported differences in the distribution of CT genotypes between heterosexual and MSM populations[6 9] and sexual behaviour factors, social and sexual network movement, and tissue tropism may account for these differences. According to current literature, the distribution of genotypes in rectal infections among MSM is dominated by D, G and J[6W7-10].

However, little is known with regards to the more detailed molecular analysis of the CT genotypes that inhabit the rectum, although a study in Amsterdam attempted to compare anorectal samples from MSM and women[23], searching for evidence of rectal tropism.

The purpose of our work was to evaluate whether the MLVA-*omp*A genotyping scheme could be adapted and applied to rectal specimens in a MSM population looking for correlations with clinical variables including HIV status. A unique and additional aspect of our project was to isolate rectal CT in cell culture. This component was included to maximise the possibility of obtaining complete genotyping data and to provide a fully characterisable collection of live rectal CT isolates for future studies into tropism *in vitro*.

The aims of this study were to determine the distribution of CT genotypes found in rectal infection in MSM attending a GUM clinic in the UK city of Brighton, to compare this between HIV-positive and HIV-negative men and to examine any clinical correlations with certain genotypes.

METHODS

Participants

Participants were men reporting sex with other men, undergoing rectal testing for CT at the GUM clinic between October 2011 to January 2013. Males <16 years old were not eligible. Participants could be recruited more than once if the period between consecutive visits was greater than three months. If more than one sample was collected from the same patient in less than three months (e.g. on the same day), one of these was excluded from the study.

Specimen collection and storage

Men were only eligible for our study if they underwent proctoscopy i.e. had direct visualisation of the rectum and swabs taken directly from the rectal mucosa and not sweeping the perianal area on the way in so the provenance of our specimens as rectal was assured. In addition to the routine nucleic acid amplification test (NAAT) with the BD Probetec™ assay (Becton-Dickinson, Sparks, MD, US), clinicians took a separate rectal swab (Σ-VCM™; Sigma, Poole, UK) for cell culture of CT, which was stored at -80°C. If a participant was found to have rectal CT on local testing, stored

supplementary rectal samples were transported on dry ice to The University of Southampton Molecular Microbiology Group for tissue cell culture and genotyping. Stored specimens from patients subsequently found to be CT-negative were discarded. All patient identification information was removed and samples anonymised prior to transport to Southampton.

Patient demographic and clinical data

Data regarding demographics, HIV status and clinical parameters: rectal signs and symptoms, history of unprotected sex in the previous 3 months and history of previous STIs were retrieved from patient case notes and recorded on a Microsoft Excel spreadsheet.

DNA sequence analysis of MLVA-*ompA* markers and tissue culture of *C. trachomatis*

Methodological details of DNA extractions from samples, PCR, sequence analysis and tissue culture isolation of *C. trachomatis* can be found in Web Appendix 1.

Statistical methods

Fisher's exact test (using the statistical software package SPSS version X21) was used for statistical analysis of associations between CT sub-clusters and clinical parameters.

Ethics

This study was approved by Brighton East Research Ethics Committee (REC reference 11/LO/0745).

RESULTS

Participants

1809 men consented to participate in this study (the clinic tests approximately 3000 MSM for STIs per year), from which 112 CT-positive rectal samples were obtained (6.2% positivity for rectal CT). Seven of these men also had concurrent urethral CT. Complete paired MLVA-*omp*A genotypes were only obtained for three of these seven participants. These three paired results were: for

participant 6 rectal swab: 8.5.5-L2, urine: 8.5.1-E; for participant 50 rectal swab 3.5.3-G, urine 3.6a.3-G; for participant 79 rectal swab 3.6.4-D, urine 3.6a.4-G. Of the four incomplete paired MLVA-*omp*A genotypes (where one or more *loci* were not sequenced), two included individual paired *loci* which differed between the rectal swab and urine and therefore these two samples could also represent different MLVA-*omp*A genotypes.

Mean age of men with rectal CT was 39 years (median 40, interquartile range 31-46), 104 (93%) were Caucasian and 47(42%) were HIV-positive.

85/112 (76%) of CT-positive rectal samples were assigned full MLVA-*omp*A profiles. All of the other samples had types assigned to at least two markers based on direct patient samples.

Genotypes identified among MSM in Brighton

Amongst the 85 fully-genotyped samples there were 66 different MLVA-*omp*A sequence types (Table 1).

Table 1 MLVA-*omp*A genotypes according to HIV status

*omp*A	MLVA*	HIV positive (number of CT positives)	HIV negative (number of CT positives)
D/IC-CAL8†	3.6.4		1
	3.8.1	1	
	6.13.3	1	
	8.5.1		3
	8.6.4	1	
	8.6.5	1	
	8.7.2	1	
	8.8.1		1
	8.8.3	1	
	14.7.3		1
D/UW-3/CX†	3.6.4		1
	8.2.4	1	
	8.4.1		1
	8.6.1		1
	8.6.3		1
E	3.7.2		1

	8.2.1		1
	8.3.1	1	
	8.3.2		1
	8.5.1	1	4
	8.5.3	1	1
	8.6.1		1
	8.7.1	1	2
	8.7.2	1	
	8.7.4	1	1
	8.9.1	1	
	8.9.3		1
F	8.2.4	1	
	8.4.5		1
	8.5.1	2	1
	8.7.3	1	
	8.8.2	1	
G	1.3.3		1
	2.5.3	1	
	2.5.4		1
	2.11.4		1
	3.2.4		1
	3.3.3	1	
	3.4.3	1	1
	3.5.3		1
	3.6.3		2
	3.6.4		1
	3.7.3	3	
	8.5.1		1
	8.5.2	1	
	8.5.4	1	
	8.5.5		2
	8.6.2		1
	8.6.3	1	
I	8.7.5		1
J	1.6.3		1
	2.7.3	1	
	3.2.4		1
	3.5.2	1	
	3.5.3		1
	8.3.4	1	
	8.9.2		1
L2	1.9.4	1	
	8.5.5		1

L2b	1.5.2	1	
	1.5.3	1	
	1.9.1	1	
	1.9.2	1	2
	3.5.2	1	
	8.5.4		1
	8.5.5		1

*MLVA genotype is designated in order of *loci*: CT1335; CT1299; and CT1291
†*omp*A genotype D is subdivided into D/IC-CAL8 and D/UW-3/CX

The most prevalent *omp*A genotypes were G, E and D comprising 19.6%, 17.9% and 15.2% of the 112 positive samples respectively (Table 2). There were two D subgroups: D/UW-3Cx and D/IC-CAL8 which comprised of 4.5% and 10.7% respectively (Table S2). The distribution of *omp*A genotypes from most to least numerous was: G, E, D, L2, F/J (both same number of samples), and I (Table 2). Column two of Table 2 shows the total number of rectal specimens per *omp*A genotype, and column three shows the number of these which were culturable.

Table 2 Distribution of MLVA-*ompA* genotypes among CT positive MSM in Brighton

*omp*A genotype	Number rectal specimens (% of 112 CT positive specimens)		Numberrectal specimens culturable	Number different MLVA-*omp*A genotypes
D	17	(15.2)	7	15
E	20	(17.9)	6	12
F	7	(6.3)	3	5
G	22	(19.6)	9	17
I	1	(0.9)	0	1
J	7	(6.3)	4	7
L2	2	(1.8)	1	2
L2b	9	(8.0)	6	7
Not fully genotyped	27	(24.1)	1*	-
Total	112	(100)	37	66

*One cultured isolate was successfully identified as *ompA* genotype G, but the CT1299 sequence analysis did not work on this sample, hence it is categorised as not fully MLVA-*ompA* genotyped.

Six new VNTR repeats were identified in this study (Table 3), in addition to those described in Pedersen *et al* (2008)[13] and Wang *et al* (2011)[18].

Table 3 New types and variants of the variable number tandem repeats (VNTRs) identified in this study

VNTR type*	Variant number of VNTR type and associated repeat sequence							
CT1335	14	14T7A	15	11T9A				
CT1299	10	15C	11	16C	12	18C	13	21C

*No new variants for CT1291 were identified

In most cases the MLVA types were unique to the *ompA* genotypes. However, eleven MLVA genotypes were associated with more than one *ompA* genotype. These MLVA types were; 3.2.4, 3.5.2, 3.5.3, 3.6.4, 8.2.4, 8.5.1, 8.5.4, 8.5.5, 8.6.1, 8.6.3, 8.7.2 (Table S2). The MLVA-*ompA* profile most commonly detected was 8.5.1-E-Bour which was identified in five patients. MLVA type 8.5.1 was also the MLVA type most associated with different *ompA* genotypes: it was identified in four *ompA* genotypes. Genotypes H and K were not detected from any of the samples

Cluster Analysis of genotype distribution among MSM in Brighton

Owing to the variation of genotypes within the highly diverse number of fully genotyped samples in this study (85), it proved necessary to identify possible founder groups within the minimum spanning tree, so as to enable statistical analysis of sub-clusters. Two possible sub-clusters were identified (sub-cluster 1 & sub-cluster 2, Fig. 1) on the basis that the putative sub-cluster founders (A & B, Fig. 1) had five or more single locus variants (SLVs) associated with them. Only SLVs and double locus variants (DLVs) of the sub-cluster founders A and B were included in further analysis. Statistical analysis using the Fisher's exact test showed a significant difference between the sub-

clusters with respect to HIV status (p=0.025). Most genotypes were from either HIV-positive or HIV-negative MSM (27 genotypes were from HIV-positive MSM only; 32 from HIV-negative MSM only). There were seven genotypes that were found in both HIV-positive and -negative MSM, which were *omp*A type E (4 genotypes); F (1 genotype); G (1 genotype); and L2b (1 genotype) (Table 1). Sub-cluster 1 consisted predominantly of HIV-negative men and sub-cluster 2 of HIV-positive men (see also Fig S2). The distribution of *omp*A genotypes in sub-cluster 1 was mainly *omp*A D, E and F (p<0.001), whereas that in sub-cluster 2 consisted mostly of *omp*A G, and J (p<0.001), and there were *omp*A D and L2b genotypes closely associated with this cluster (Fig. 1). There were seven different MLVA-*omp*A genotypes amongst the nine L2bs in this study, five of which were from HIV positive MSM and four were from HIV negative MSM. Of the 85 fully genotyped samples, 66 had unique MLVA-*omp*A genotypes (Tables 2 and 3).

Cultured isolates

33% (37/112) of the samples were successfully isolated in cell culture. The distribution of *omp*A genotypes amongst these isolates was as follows; G (n=10), E (n=6), D (n=8), and L2B (n=6) (Table 2). MLVA-*omp*A genotypes 3.4.3-G, 8.5.1-F, 8.7.1-E and 1.9.2-L2b were found in multiple culture-positive samples. In some of these cases, (8.5.1-F; 8.7.1-E), genotypes were detected in direct patient samples, and post culture (but not from the same patient). Two of the samples with MLVA-*omp*A genotype 1.9.2-L2b were collected on the same day.

Clinical correlations

There were no significant clinical differences between HIV-positive and HIV-negative men.

Overall 75% of individuals were asymptomatic with regards to rectal symptoms (including pain, bleeding, tenesmus, constipation, diarrhoea, discharge and perianal itch). Individuals with *omp*A genotype L2/L2b were more likely to be symptomatic (64% of individuals with this genotype were symptomatic) than any of the other *omp*A genotypes. Further analysis using Fisher's exact test

showed that there was no statistical significant differences with respect to rectal symptoms (p=0.725) or signs (p=1.000) and MLVA-*omp*A genotypes between sub-clusters 1 and 2.

DISCUSSION

This is the first MLVA-*omp*A study of CT in MSM with HIV. Using this method we have seen much greater diversity in CT genotypes than was expected in comparison to other studies using other genotyping schemes[6W10]; however, there are some trends and significant findings.

Our principle finding in this prospective study is that there are distinct sub-clusters of chlamydial genotypes for HIV positive and HIV negative MSM. We have also observed that the distribution of *omp*A genotypes amongst MSM overall differed somewhat from that previously described[6 W10-11]. We found that the three predominant *omp*A genotypes were, in descending order: G, E and D, whereas elsewhere studies have shown predominant MSM genotypes to be G, D and J[6 24 W10]. The Brighton *omp*A distribution bears a close resemblance to that of MSM in Northern Spain[25].

The second most prevalent *ompA* genotype amongst our samples was E which is the predominant *ompA* genotype among heterosexual individuals. This overlap is further represented by MLVA-*omp*A genotype 8.5.1-E, the predominant genotype in both this study and a study of Southampton women[18]. A unique finding is that this genotype could represent a bridging population between heterosexual females and MSM that could be responsible for the transmission of CT between risk groups. Alternatively, this particular genotype may have been introduced the MSM network some time ago and has since been circulating among predominantly HIV-men.

Specifically, whilst there was no significant symptomatic correlation between HIV positive and HIV negative men there was a trend for individuals with *omp*A genotypes L2/L2b to be symptomatic compared to those with any other genotype, in keeping with what is already known of the current LGV epidemic in MSM[26]. Whilst the initial LGV outbreak was characterised by a painful proctitis and, on fewer occasions, an inguinal syndrome, the number of asymptomatic individuals at that time was low. However, the most recent national LGV case-finding exercise found that 22% of

LGV cases were asymptomatic, a quarter of those being pre-symptomatic i.e. men were asymptomatic at first visit but symptomatic by the time they attended for treatment of rectal chlamydia[27]. Despite smaller numbers, our data showed an asymptomatic LGV rate of 36%, slightly higher than the national rate but also consistent with the changing epidemiology of LGV.

In terms of strengths and weaknesses, the sample size was small giving insufficient power to make symptomatic associations with the large diversity of genotypes. Inherent to many epidemiological studies in MSM, the challenges of contact tracing in MSM, who often have multiple and anonymous partners, makes obtaining information for complete clusters and sexual networks difficult.

There is some debate over what influences the site of *C. trachomatis* replication, whether it is tissue tropism or sexual behaviour, or both. However, a recent study comparing MLST types of *C. trachomatis* from women (anorectal, cervical, vaginal, pharyngeal) did not correlate genotypes with these sites[23]. Geisler *et al*. (2008) examined *omp*A genotypes in women showing that rectal CT genotype distribution was similar to that of genital CT[28], notably genotype E. By contrast genotype G, which was found commonly in the rectums of MSM, was seldom seen at either cervix or rectum in women. From this it would appear that sexual behaviour factors and epidemiology play a significant role. Another study used WGS to identify correlations between polymorphisms in specific *loci* and tissue tropism. Strong associations with rectal tropism were found for polymorphisms in serovar G[29].

Of the seven participants from whom we obtained paired samples, five appeared to have different MLVA-*omp*A genotypes in the rectal compared to urine samples. This suggests that these participants may have been in sexual contact with a wider network.

This is a meaningful study since we have shown that the MLVA-*omp*A high resolution genotyping system can be successfully applied to rectal samples in MSM and that the recovery rate of CT in cell culture from rectal swabs (33%) was greater than has previously been reported. The implications for clinicians is that different behaviours amongst HIV positive and HIV negative MSM affect co-infections with chlamydial genotypes, and is encouraging evidence for serosorting

behaviour (a known HIV transmission reduction strategy). Policy makers should note that paying attention to identification of "HIV-positive MSM *C. trachomatis* genotypes" can lead to trapping high risk networks in terms of reducing onward transmission of and avoidable deaths from HIV. Implications from our study also indicate that future vaccines for *C. trachomatis* must encompass all genotypes and cannot be targeted to single groups such as HIV positive MSM.

Our second main finding is based on minimum spanning tree analysis and the derivation of two distinct sub-cluster, suggestive of two separate sexual networks. Sub-cluster 1 consisted of more "heterosexual" CT genotypes, reinforcing the suggestion that linkages between risk groups exist, whereas sub-cluster 2 displayed a genotype distribution more typical of that previously seen in MSM. However, these two sub-clusters are closely related genetically, differing by no more than DLVs, which indicates that there is a circulating pool of closely related strains in a single sexual network, which can only be subdivided into "more MSM-like" and "more MSW-like" sub-groups.

Furthermore, sub-cluster 2 consisted of significantly more HIV-positive men, suggesting that these men choose sexual partners from within risk groups, i.e. serosorting. The LGV epidemic over the last 10 years has largely affected HIV-positive men and, within this, a distinct group of men with specific behaviour patterns has been implicated. It follows that other genotypes may also be found in higher rates among HIV-positive individuals but the fact that they are clinically indistinct from other non-LGV genotypes, being mainly asymptomatic, means that we are less likely to be aware of them. Indeed an alternative explanation would be that HIV-positive individuals who are immune suppressed are less able to clear certain types of CT.

Further larger studies examining rectal genotypes are needed to enable sexual network mapping. Moreover, similar studies in women will provide useful insight into whether certain CT genotypes have a predilection for the rectum because of sexual behaviour factors and tissue tropism.

One unanswered question is whether the 8.5.1-E found in MSM is similar or quite different to the 8.5.1-E found in the cervices of women. Further work includes whole genome sequencing (WGS) which will facilitate comparison between 8.5.1-E isolate(s) observed in the current MSM study and the women's study[18]. Comparison of total single nucleotide polymorphism differences (SNPs)

within the women's 8.5.1-E isolates to those of MSM will reveal if there is a significant difference or not, and therefore support a conclusion as to whether or not the "MSM 8.5.1-E" is the same as the women's "heterosexual 8.5.1-E". Whilst not part of the present study, further work will involve detailed *in vitro* analysis of the tropism of the cultured rectal isolsates.

In conclusion, we have been able to identify an association of MLVA-*omp*A genotypes with HIV status and our results indicate a possible network within Brighton defined by HIV status.

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KEY MESSAGES

- There is a wide diversity of *C. trachomatis* genotypes as defined by MLVA-ompA typing circulating in the MSM population of Brighton.
- High-resolution multi *locus* VNTR analysis – *omp*A genotyping points to discrimination of sub-clusters of MSM who are distinguishable by their HIV-status.
- Heterosexual genotypes circulate within the MSM community in Brighton, but appear to be more associated with HIV negative MSM.

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CONTRIBUTORS

CL conducted the tissue culture and genotyping, as well as data analysis with PM and SG.

SS was involved in the concept of the study and study design with INC and PM, led on running the clinical side of the study, and was involved in writing of all drafts of manuscript and the final version. PM and INC drafted the manuscript with SS. JZ was involved in the running of the study and contributed to the first drafts of the manuscript. AB was involved in running the study from a clinical perspective. INC provided expertise in chlamydial biology, genetics and tissue culture, PM

provided public health, *C. trachomatis* epidemiology and molecular diagnostics expertise, SG provided statistical analysis.

COMPETING INTERESTS

None

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ETHICS APPROVAL

This study was approved by the local Research Ethics Committee

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Additional references can be found in Web Appendix 2.

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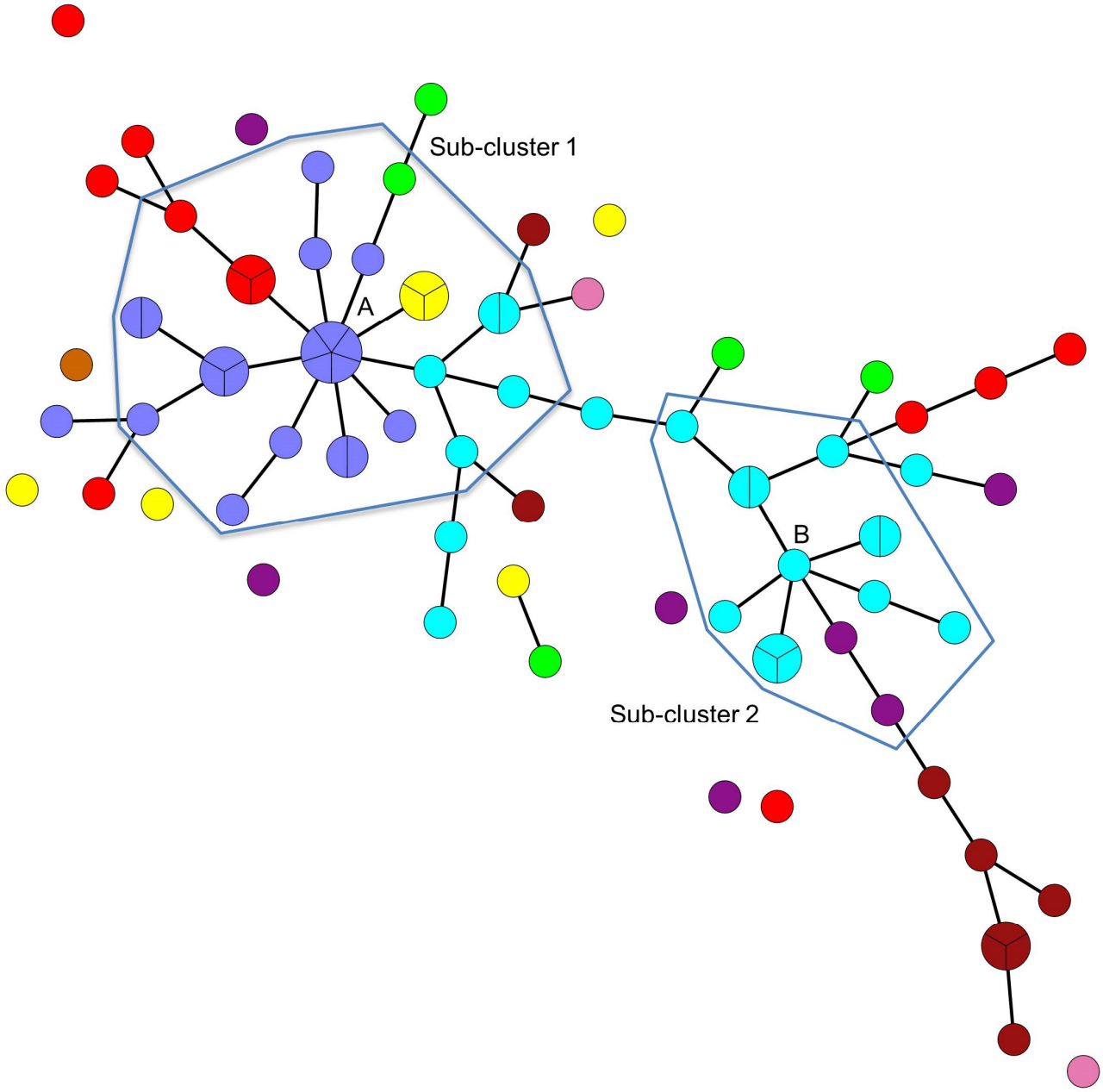
Sub-cluster 1
A
B
Sub-cluster 2

Figure 1 Minimum-spanning tree of 85 MSM samples from Brighton. Each coloured circle represents a MLVA-*omp*A genotype. Segmentation within the circles shows the number of samples per genotype, branches show single-locus variants (SLV). Unlinked circles show genotypes which differ from nearest linked genotype by more than a SLV. Hand-drawn blue lines delineate user-defined sub-clusters based on founder groups A (sub-cluster 1) and B (sub-cluster 2). The circle colour coding shows *omp*A genotypes as follows: green = D (subtype D/UW-3CX); red = D (subtype D/IC-Cal8); lavender = E; yellow = F; blue = G; light brown = I; purple = J; pink = L2; dark brown = L2b.