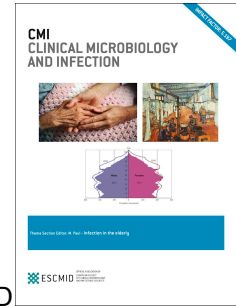# Accepted Manuscript

Detection of the NS3 Q80K polymorphism by Sanger and deep sequencing in hepatitis C virus (HCV) genotype 1a strains in the United Kingdom

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1 **Detection of the NS3 Q80K polymorphism by Sanger and deep sequencing in hepatitis C**

2 **virus (HCV) genotype 1a strains in the United Kingdom**

3

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ABSTRACT

The Q80K polymorphism in the HCV NS3 enzyme reduces susceptibility to simeprevir and other novel protease inhibitors. The study aimed to determine the prevalence of Q80K in treatment-naïve HCV-1a carriers in the North-West (NW) and South-East (SE) of England, investigate occurrence of Q80K as a minority variant, and characterise viral phylogeny. Plasma samples from subjects that were naïve to anti-HCV therapy underwent conventional (Sanger) and deep (Illumina-Miseq, 1% interpretative cut-off) sequencing of NS3. Q80K occurred in 44/238 subjects (18.5%, 95% confidence interval 13.6%-23.4%), including 19/70 (27.1%) in the NW and 25/168 (14.9%) in the SE (p=0.0425), with no difference by HCV RNA load or HIV status. **Q80K frequencies in reads of samples that underwent Illumina sequencing were >40% in all cases. Among subjects with Q80K, 5/44 (11.4%) showed one additional major resistance-associated mutation in NS3 detected at mutant frequency above (V36L, V55A) or below (V36M) 10%.** Phylogenetic analyses identified the two recognised HCV-1a lineages with (clade I) and without (clade II) Q80K. Overall, 148/238 (62.2%) sequences occurred within regional or inter-regional clusters, **each comprising 3-20 sequences**. There was no unique clustering of English sequences relative to strains from continental Europe and North America. In conclusion, Q80K was found at high prevalence among treatment-naïve HCV-1a carriers in England, and was reliably detected by conventional sequencing, **with no increased detection by deep sequencing.** English sequences were highly interspersed with sequences from elsewhere in Europe (clade II) and North America (clade I), and their phylogeny was consistent with multiple introductions from different areas.

INTRODUCTION

The HCV NS3 protease enzyme exhibits a high degree of genetic variability and only 47% of its amino acids are conserved among circulating HCV genotypes.[1] NS3 genetic heterogeneity and the associated molecular and structural differences influence HCV susceptibility to protease inhibitors (PIs), including licensed first-generation (telaprevir, boceprevir) and second-generation (simeprevir) compounds. HCV variants with reduced PI susceptibility have been observed in treatment-naive patients.[1-10] Among these, the glutamine to lysine substitution at codon 80 (Q80K) reduces susceptibility to simeprevir *in vitro* [2, 4, 11] and was also seen to reduce virological responses to simeprevir plus pegylated interferon alpha and ribavirin (P/R) in clinical studies.[12-14] Screening for Q80K is therefore recommended before starting simeprevir.[15]

The prevalence of Q80K among PI-naïve subjects varies by HCV genotype and subtype. An analysis of global NS3 sequences deposited in GenBank reported Q80K in 42% of HCV-1a strains, and also detected a high prevalence in HCV-5 and HCV-6 strains.[1] Among HCV-1a strains, prevalence of Q80K ranges between 3% and 47% by geographical location, exceeding 40% in North America.[2, 4] Reported prevalence is 20% overall across Europe, although differing markedly by country.[7-9] Most studies to date have produced prevalence estimates using conventional (Sanger) sequencing, which allows detection of virus variants present at a frequency ≥20% within a patient's sample.

This study had three aims. Firstly, to determine the prevalence of Q80K in NS3 sequences obtained from HCV-1a carriers attending for care in two regions of England in the United Kingdom(UK). A further aim was to investigate the occurrence of Q80K as a low frequency variant by deep sequencing, and thereby both gain insights into the viral quasispecies and determine whether conventional sequencing offers sufficient sensitivity for screening simeprevir candidates for the presence of Q80K. The third aim was to determine the phylogeny of NS3 sequences in relation to publically available sequences from the rest of Europe and North America.

METHODS

Study population

The study was performed retrospectively using stored plasma samples from consecutive adults that in 2006-2014 attended for care at the Royal Liverpool University Hospital in Liverpool (North-West region) and at King's College Hospital, Chelsea & Westminster Hospital, and Charing Cross Hospital in London (South-East region). Eligible patients were infected with HCV-1a **based on the genotyping method available at the local National Health System diagnostic laboratory; the assignment was confirmed by analysis of the NS3 sequences produced in this study.** Patients were naïve to all anti-HCV therapy. HIV status and HCV RNA load were retrieved from the clinics' databases. Ethics permission was granted by the South Berkshire Regional Ethics Committee to conduct the study after removing personal identifiers from the samples.

Sanger sequencing

HCV RNA was extracted with Nuclisens easyMAG (bioMérieux, Netherlands) and cDNA synthesis was performed with the Qiagen OneStep RT-PCR Kit (Hilden, Germany) with forward primer 3278 [GGAGACCAAGMTCATCACSTGG] and reverse primer 4032 [GCTCTTRCCGCTGCCRGTGGG]. The first-round amplicon was subjected to nested PCR using the Qiagen HotStarTaq Kit with forward primer 3307 [ACACCGCSGCGTGYGGKGACAT] and reverse primer 4014 [GGRGCRTGYAGRTGGGCCAC]. The second-round 727bp amplicon (aa180 of NS2 to aa204 of NS3) was purified with the Qiagen QIAquick PCR Purification Kit and sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 on the ABI Prism 3730 Genetic Analyser (Applied Biosystems®, USA). Consensus sequences were assembled using SeqScape (v2.7) and analysed with the geno2pheno system (http://hcv.geno2pheno.org/index.php; March 2015 version) for the presence of HCV resistance-associated mutations (RAMs).

Deep sequencing

HCV RNA was extracted with QiasymphonySP (Qiagen) using the Qiagen DSPVirus/Pathogen Midi Kit and the CellFree_500_V4 protocol; cDNA was produced using the ImProm II Reverse Transcription System (Promega, USA) and random primers. NS3 was amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) and the above primers. PCR products were purified using AMPure XP Beads (Beckman Coulter, USA) before quantification with the Quant-iT dsDNA High Sensitivity Kit (Life Technologies, USA)

4

111 on a Qubit 2.0 Fluorometer (Life Technologies). The Nextera XT Sample Preparation Kit

112 (Illumina, USA) was used for library preparation and pooled amplicon libraries were

113 sequenced on Illumina MiSeq using v2 reagents. Data generation and initial analysis were

114 carried out at the Centre for Genomic Research at the University of Liverpool. Adapter

115 sequences were trimmed from the Illumina reads using Cutadapt v1.2.1[16]; further trimming

116 was done using Sickle v1.2 (http://github.com/najoshi/sickle) applying a minimum window

117 quality score of 20. BAMStats (http://sourceforge.net/projects/bamstats/files/) was used to

118 calculate the read statistics. Variants were analysed using the VirVarSeq pipeline,[17] which

119 utilises the quality of the run and individual bases to filter poor quality bases and reduce false

120 positive rates. A 727bp region of a HCV-1a molecular clone encoding full-length NS3 was

121 amplified and sequenced in four replicates in order to estimate the assay error rate. The plasmid

122 control yielded an average of 174,799 reads, with a mean coverage of 46,268 reads per

123 nucleotide. The error rate, calculated by counting **false substitution reads** at the codon level of

124 the molecular clone [18], was mean 0.6% (SD 0.2%) over NS3 amino acids 1-181.

125

126 *Phylogenetic analysis*

127 Reference HCV-1a sequences and all available HCV-1a NS3 sequences from North America

128 and Europe with confirmed genotype assignment and country of origin were retrieved from the

129 Los Alamos HCV database (hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html; accessed

130 on 8 March 2015) **(supplementary file). All sequences, including those produced in this**

131 **study (Sanger sequences and NGS consensus sequences with 1% interpretative cut-off),**

132 were aligned pairwise against the HCV-1a reference genome H77 (accession no. NC_004102)

133 using MEGA v.6. The alignment **was trimmed** to codon positions 1-194 of NS3 (nucleotides

134 3421-4014 of H77). Sequences with gaps or ambiguous nucleotides affecting ≥50% of the

135 targeted region or where codon 80 was missing or ambiguous were removed **(n=3)**, yielding a

136 total of 1162 NS3 sequences for analysis. Two maximum-likelihood (ML) phylogenies were

137 estimated with and without the 80 codon, using a general-time reversible nucleotide

138 substitution model with gamma-distributed among-site rate variation and the ML method as

139 implemented in PHYML v.3.0.[19, 20] Phylogenetic support was evaluated using a bootstrap

140 approach and a total of 1000 bootstrap replicates were generated using the FastTree v.2.1.7

141 software;[21] local branch support was calculated by the Shimodaira–Hasegawa-like (SH-like)

142 test. Phylogenies were visualized and annotated in FigTree (http://tree.bio.ed.ac.uk). Clusters

143 (≥3 sequences) were identified by a bootstrap support >75% and were defined as regional if

144 comprising sequences unique to either the NW or the SE, and inter-regional if including
145 sequences from both regions.

146

147 *Statistical analysis*

148 Descriptive statistics were used to analyse the prevalence of mutants in the study population
149 (as the proportion of subjects showing the mutation) and the frequency of mutants in each
150 patient's sample (as the proportion of deep sequencing reads showing the mutation). Proportion
151 of subjects with Q80K by geographic location and HIV status were compared by Fisher's exact
152 test. The Mann-Whitney U test was used to compare the HCV RNA load of patients with and
153 without Q80K. The analysis was performed using SPSS v.21.

154

155 **RESULTS**

156 *Study population*

157 Overall 238 adults (median age 44 years, with no difference by region) infected with HCV-1a
158 were studied, including 70/238 (29.4%) in the North-West (NW) and 168/238 (70.6%) in the
159 South-East (SE). At the time of testing, HCV RNA load was median 6.3 $\log_{10}$ IU/ml
160 (interquartile range,IQR 5.8-6.8), without differences between the two regions. Subjects were
161 naïve to all anti-HCV therapy. A total of 61/238 (25.6%) subjects, all from the SE, were co-
162 infected with HIV.

163

164 *Q80K prevalence by Sanger sequencing*

165 Overall, Q80K was detected in 44/238 subjects, yielding a prevalence of 18.5% (95%
166 confidence interval, CI 13.6%-23.4%)(Table 1). Samples from two of the 44 subjects had a
167 mixed first base at codon 80 (Q80Q/K). **The median HCV RNA load was 6.4 (IQR 5.8-6.7)**
168 **vs. 6.3 (IQR 5.8-6.8) $\log_{10}$ IU/ml in samples with vs. samples without Q80K, respectively**
169 **(p=0.63)**. The prevalence of Q80K was 19/70 (27.1%) in the NW and 25/168 (14.9%) in the
170 SE (p=0.043). When comparing results by HIV status in the SE, Q80K prevalence was 18/107
171 (16.8.%) in HCV mono-infected subjects and 7/61 (11.5%) in HCV/HIV co-infected subjects
172 (p=0.379). Table 1 shows other major and minor NS3 RAMs detected by Sanger sequencing.

173

174 *Q80K prevalence by deep sequencing*

175 A total of 178/238 (74.8%) samples underwent deep sequencing, comprising 28/178 (15.7%)
176 with and 150/178 (84.3%) without Q80K by Sanger sequencing. An average of 58,585 reads
177 per sample was obtained (median 53,413; IQR 40,740-70,255) with an average coverage per

6

178   nucleotide of 16,107. Prevalence of Q80K was 27/178 (15.2% 95% CI 9.9%-20.5%). All 28

179   samples showing Q80K by Sanger sequencing also showed the mutation by deep sequencing,

180   usually (26/28 samples) with mutant frequencies of ≥98%. Of the two samples showing

181   Q80Q/K by Sanger sequencing, one had a Q:K ratio of 54:46, and the other showed Q, K and L

182   at a ratio of 57:41:2. Of 150 samples lacking Q80K by Sanger sequencing, none showed the

183   mutation by deep sequencing when applying an interpretative cut-off of ≥1% for the frequency

184   of mutants within the reads, as usually recommended.[22] Prevalence of Q80K increased when

185   the interpretative cut-off was lowered, and was 3/150 (2%) for Q80K occurring at a frequency

186   between ≥0.5% and <1%, and 15/150 (10%) at a frequency ≥0.2% and <0.5%. These cut-offs

187   however fell within the estimated error rate of the assay (0.6%, SD 0.2%).

188

189   Deep sequencing (interpretative cut-off ≥1%) identified additional NS3 RAMs that had not

190   been observed by Sanger sequencing and which occurred at frequencies between 1% and 6%

191   (Table 1). **Among the 44 subjects with Q80K, 4/44 (9.1%) had one additional major NS3**

192   **RAM at frequency >10% including V36L in 3/44 (6.8%) and V55A in 1/44 (2.3%). In**

193   **addition 1/44 (2.3%) subjects had V36M detected only by Illumina at a frequency of 3%**.

194

195   *Phylogeny of NS3 sequences*

196   HCV-1a strains separated into the two recognised distinct lineages with and without Q80K.[23,

197   24] The sequences harbouring Q80K at frequency <1% did not align with the Q80K lineage

198   (data not shown). There was no unique clustering of the study English sequences (Figure 1),

199   rather the sequences were interspersed with sequences from the rest of Europe and North

200   America, and the phylogeny was consistent with multiple introductions from different areas.

201   With the English sequences, four NW, eight SE, and 11 inter-regional clusters were identified,

202   each consisting of 3-20 sequences (Figure 2). In total, 148/238 (62.2%) sequences (49/70,

203   70.0% in the NW and 99/168, 58.9% in the SE) were circulating as regional or inter-regional

204   clusters.

205

206   **DISCUSSION**

207   This study detected the NS3 polymorphism Q80K at high prevalence among treatment-naïve

208   HCV-1a carriers attending for care in England, particularly in the North-West where

209   prevalence reached 27.1%. Previously published data from the UK include a small cohort of 38

210   HIV-positive patients with acute HCV-1a infection, of which 16% had Q80K.[6] A larger

211   study analysed 159 subjects with HCV-1a that were enrolled in Phase II/III studies of

telaprevir and simeprevir, of which 23% showed Q80K[8]; these sequences are not available for analysis however, as to date they have not been deposited in the GenBank or the Los Alamos database. These data indicate that HCV-1a strains circulating in the UK have the highest prevalence of Q80K observed in Europe.

In this study the prevalence of Q80K varied by geographical region, while showing no difference by HIV status. Prevalence rates also show marked geographical variability in the rest of Europe.[7-9] The variable prevalence has not been linked to race or ethnicity [8], but rather is consistent with the circulation of two distinct HCV-1a lineages with and without Q80K.[23, 24] The Q80K-carrying lineage is believed to have originated in the United States in the 1940s.[23] In this study, the maximum likelihood phylogenetic analysis confirmed the two HCV-1a lineages, and detected multiple introductions of the two lineages in the UK, with wide interspersing of the English strains with other European and North American strains. Most HCV-1a sequences occurred as regional or mixed regional clusters, however, suggesting that for this cohort the majority of transmission events occurred within the UK.

The deep sequencing analysis showed that Q80K mutants occurred at high frequency (>40%) in the patients' samples, allowing detection by conventional sequencing in all cases. This finding is consistent with the observation that Q80K mutants have replication capacity similar to that of wild-type virus, which allows transmissibility and persistence at high frequency within the viral quasispecies despite the absence of drug selective pressure. There were no samples showing Q80K at a frequency below the detection threshold of Sanger sequencing (≥10-20%) and above the typical ≥1% interpretative cut-off for deep sequencing, which is recommended to differentiate biologically meaningful mutations from those caused by methodological errors.[22] When applying less stringent cut-offs (0.5% or 0.2%) the proportion of samples showing Q80K by deep sequencing increased. A previous study similarly found that among 21 subjects with Q80K detected by deep sequencing, four showed the mutation at a frequency <1%.[10] The estimated error rate of the assay (0.6%) and the observation that sequences showing Q80K at a frequency <1% did not align within the Q80K lineage suggest that the detection of Q80K at frequency <1% was spurious. **Furthermore, it remains to be determined whether any Q80K variant occurring at low frequency has clinical relevance.**

244 Q80K reduces HCV-1a susceptibility to simeprevir by 8-11 fold *in vitro*.[4, 8, 11] In HCV-1a

245 infection, sustained virological responses (SVR) to combination therapy with simeprevir plus

246 P/R are reduced by the presence of baseline Q80K in both treatment-naïve subjects (from 84%

247 to 58%) and prior relapsers (from 79% to 47%).[11-13] The impact of Q80K on the

248 combination of simeprevir plus sofosbuvir was not immediately apparent in some reported

249 studies.[25] **However, a phase III, open-label, single-arm study with simeprevir plus**

250 **sofosbuvir in cirrhotic patients with HCV-1 recently reported that SVR rates were higher**

251 **in subjects without Q80K (92%) versus those with the mutation (74%).[26]** Q80K has no

252 effects on susceptibility to telaprevir and boceprevir [4], but may have an effect on responses to

253 asunaprevir.[27] Q80K also confers small reductions (~3-fold) in susceptibility to faldaprevir

254 and paritaprevir *in vitro*, without necessarily affecting response rates.[28] The Q80L and Q80R

255 mutations were detected in some subjects, usually at low frequency. Their significance is

256 unclear. *In vitro*, the mutations confer 2-fold and 7-fold reductions in susceptibility to

257 simeprevir, respectively.[11] Q80R has also been observed in a small number of patients

258 experiencing failure of simeprevir therapy, when it occurred alongside major resistance

259 mutations at position 155 and/or position 168.[29] Other major NS3 RAMs occurred at codons

260 36, 54, 55, 168, and 170, which is consistent with previous observations.[1-6, 9, 30]. **A small**

261 **subset of five subjects had Q80K plus one other major RAM in NS3**.

262

263 In summary, we found a high prevalence of Q80K in HCV-1a carriers with and without HIV

264 accessing care in two regions of the UK, thus complementing estimates from elsewhere in

265 Europe. While deep sequencing increased detection of Q80K, mutants missed by Sanger

266 sequencing occurred at frequency ≤0.5% and detection seemed a likely technical artefact.

267 There was evidence of a high degree of interspersing of UK sequences with sequences from

268 elsewhere in Europe and North America, although the phylogeny indicated that most

269 transmission events occurred in the UK. Investigation of full viral genomes will elucidate

270 HCV-1a transmission networks and gain data to inform control strategies.

Table 1. Prevalence of NS3 resistance-associated mutations (RAMs) in treatment-naïve HCV-1a carriers **according to modality of detection (Sanger or Illumina) and frequency of the mutant as determined by Illumina[a]**

| RAMs	Sanger/Illumina >10% n (%)	Illumina 1-10% n (%)
Total tested	238 (100)	178 (100)
36 M	1 (0.4)	2 (1.1)
36 L	5 (2.1)	0 (0)
54 S	9 (3.8)	0 (0)
55 A	10 (4.2)	0 (0)
80 K	44 (18.5)	0 (0)
80 L	1 (0.4)	4 (2.2)
80 R	0 (0)	3 (1.7)
117 H	0 (0)	1 (0.6)
168 E	1 (0.4)	0 (0)
170 A	1 (0.4)	0 (0)
170 T	0 (0)	2 (1.1)
174 S	97 (40.8)	10 (5.6)

[a]RAMs were classified according to the geno2pheno mutation list (March 2015 version) with the addition of Q80L (reference 11). Bold indicates major mutations for genotype 1/1a.

10

Figure 1. Phylogenetic analysis of HCV-1a NS3 sequences from the United Kingdom, where green indicates sequences from the North-West and yellow indicates sequences from the South-East of England. **The global reference dataset was derived from Los Alamos HCV database (March 2015; see supplementary file for accession numbers)** and comprises sequences from the rest of Europe (in red) and North America (in blue). Nodes with bootstrap support values ≥90% are indicated with an asterisk.
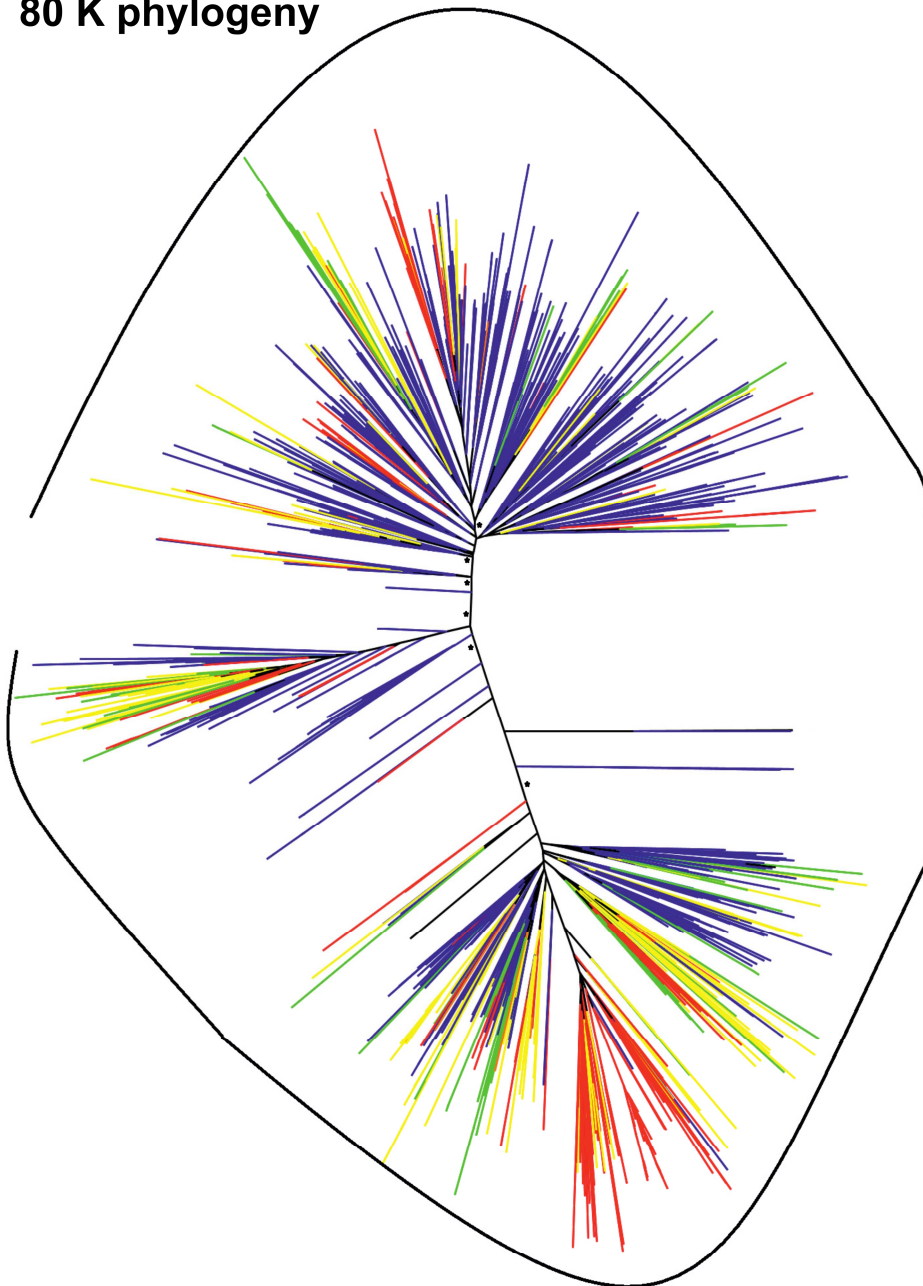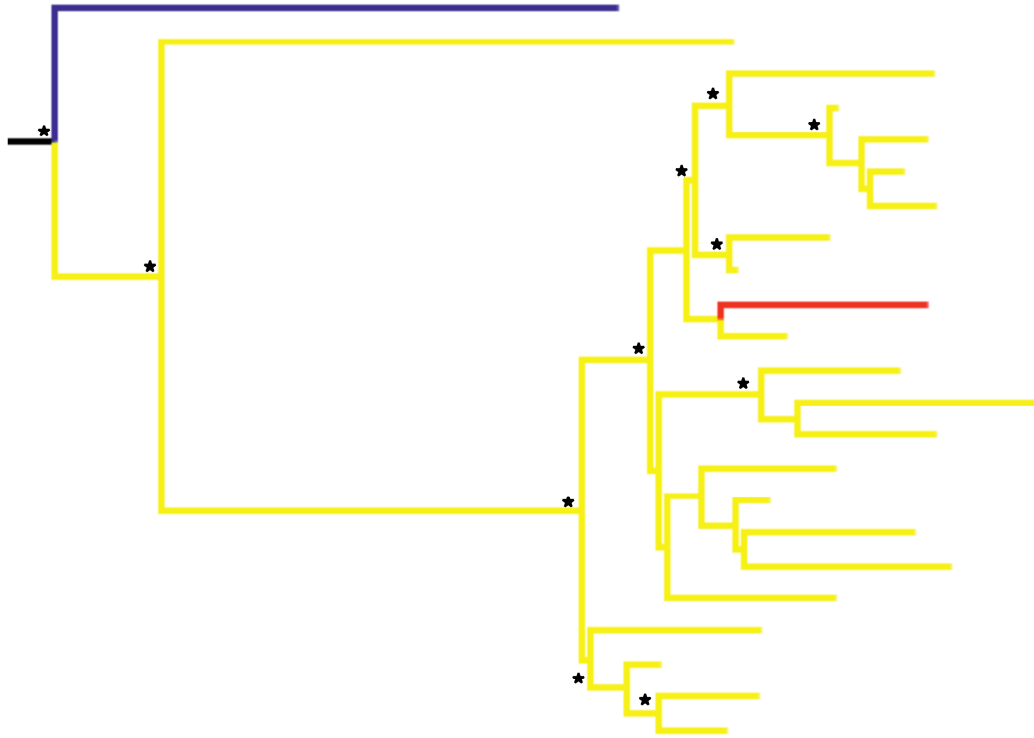

80 K phylogeny

80 Q phylogeny

284 **Figure 2**. A HCV transmission cluster in the South-East of England, comprising 20 HCV-1a

285 NS3 sequences (in yellow). Sequences from the rest of Europe and North America are shown

286 in red and blue respectively. Nodes with bootstrap support values ≥90% are indicated with an

287 asterisk.

288

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