

Influenza C incidence and herd immunity in Lancaster, UK, in the winter of 2014-2015

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28 **Abstract**

29 Influenza C is not included in the annual seasonal influenza vaccine, and has historically
30 been regarded as a minor respiratory pathogen. However, recent work has highlighted its
31 potential role as a cause of pneumonia in infants. We performed nasopharyngeal or nasal
32 swabbing and/or serum sampling ($n=148$) in Lancaster, UK, over the winter of 2014-2015.
33 Using enzyme-linked immunosorbent assay (ELISA), we estimated a seropositivity of 77%.
34 By contrast, only 2 individuals, both asymptomatic adults, were influenza C-positive by
35 polymerase chain reaction (PCR). Deep sequencing of nasopharyngeal samples produced
36 partial sequences for 4 genome segments in one of these patients. Bayesian phylogenetic
37 analysis demonstrated that the influenza C genome from this individual is evolutionarily
38 distant to those sampled in recent years and represents a novel genome constellation,
39 indicating that it may be a product of a decades-old reassortment event. Although we find
40 no evidence that influenza C was a significant respiratory pathogen during the winter of
41 2014-2015 in Lancaster, we confirm previous observations of seropositivity in the majority
42 of the population. We calculate that this level of herd immunity would be sufficient to
43 suppress epidemics of influenza C and restricts the virus to sporadic endemic spread. (194
44 words)

45 **Key words**

46 herd immunity, flu, ELISA, RT-PCR, deep sequencing, respiratory pathogen

47 **Introduction**

48 *Clinical presentation*

49

50 Influenza C (family *Orthomyxoviridae*, genus *Influenzavirus C*, species *Influenza C virus*)
51 produces malaise and coryza when administered to susceptible healthy adult volunteers,
52 with fever in a minority of cases [Joosting et al., 1968]. Historically, influenza C has been
53 regarded as the least serious of the three species of influenza infecting humans, and
54 seasonal vaccination programmes have been confined to influenzas A and B. More recent

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55 studies in Finnish army recruits confirmed influenza C's production of a mild respiratory
56 illness in healthy adults, with only occasional complications [Kauppila et al., 2013].

57

58 However, in a paediatric context, acute respiratory illness and/or pneumonia have been
59 reported as a consequence of influenza C infection [Calvo et al., 2006; Matsuzaki et al.,
60 2007; Matsuzaki et al., 2003; Moriuchi et al., 1991; Peng et al., 1996; Principi et al., 2012;
61 Shimizu et al., 2015] especially in those under 2 years old [Matsuzaki et al., 2006], as well as
62 vomiting, diarrhoea, acute otitis media [Laxdal et al., 1966], a high rate of hospitalization
63 [Gouarin et al., 2008] and even acute encephalopathy [Takayanagi et al., 2009]. There is
64 increased recognition that under-reporting of influenza C in children is a problem [Pabbaraju
65 et al., 2013]. This growing awareness of the paediatric clinical importance of influenza C
66 raises the issue of its inclusion in the annual seasonal influenza vaccine, or its position as a
67 candidate for vaccine development specifically for infants.

68

69 *Epidemiology*

70

71 Nearly 40% of adult volunteers were susceptible to administered influenza C [Joosting et al.,
72 1968]. The 60% who did not develop disease after experimental exposure is neatly
73 consonant with observation of seropositivity levels of 59% in Spain [Manuguerra et al.,
74 1994], 61% in France [Manuguerra et al., 1992] and 57% in Brazil [Motta et al., 2000], and
75 suggests that seropositivity may possibly confer resistance. By contrast, other studies have
76 suggested that antibodies against influenza C tend to be more universal: 100% in an isolated
77 Philippine village [Nishimura et al., 1987] and in US adults and children [Hilleman et al.,
78 1953], 90% in Czechoslovakia, 86% in the Soviet Union [Vasil'eva et al., 1985] and 70% in
79 East Germany [Tumova et al., 1983]. Antibody titre levels among those classed as
80 seropositive, varied widely. Some studies have also found age-structured variability: in
81 California, seropositivity of 64% in children under 5 but 98% in adults [Dykes et al., 1980]; in
82 Japan, 40-50% in early childhood to nearly 100% in adulthood [Kaji et al., 1983]; in
83 Louisiana, 47% in children to 96% in younger adults, but then a decline to 18% in the over-
84 65s [O'Callaghan et al., 1980]; in France, 46% seropositivity in children, 76% in younger
85 adults, but only 44% in the over-50s [Manuguerra et al., 1992].

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86

87 Influenza C does not appear to be seasonal, based on contemporaneous two-year surveys of
88 its occurrence in Bucharest and Japan from 1988-1990 [Ionita et al., 1992; Moriuchi et al.,
89 1991]. Using this observation together with the seropositivity data, it is possible to propose
90 several epidemiological scenarios. The first of these is that influenza C is essentially an
91 endemic virus in human populations, with more or less lifelong immunity conferred by
92 exposure but, in the absence of a vaccination programme, a sufficient supply of newborns
93 and unexposed adults to provide a host population. The decline in seropositivity in later life,
94 found in at least two studies [Manuguerra et al., 1992; O'Callaghan et al., 1980], potentially
95 due to immunosenescence, would then provide the virus with opportunities to infect
96 individuals for a second time. The second scenario is that the virus is only intermittently
97 epidemic. The variation in seropositivity according to place, time and individual age is
98 therefore a reflection of previous epidemic history in different locations. The third scenario
99 is that the virus is endemic but antigenically variable over time. Seropositivity would
100 therefore be an unreliable guide to the true immune status of any individual. Individuals
101 may acquire immunity, but this will eventually disappear as its value is eroded by antigenic
102 drift, for which there is some evidence in influenza C [Chakraverty, 1978].

103

104 *Phylogenetics and molecular evolution*

105

106 The rate of nucleotide substitution is lower in influenza C than in A and B [Buonagurio et al.,
107 1986; Gatherer, 2010; Muraki et al., 1996; Yamashita et al., 1988]. Like the other influenza
108 viruses, influenza C has a segmented RNA genome, and reassortment has been detected
109 [Buonagurio et al., 1986; Gatherer, 2010; Matsuzaki et al., 2003; Moriuchi et al., 1991; Peng
110 et al., 1994; Racaniello and Palese, 1979]. There is also evidence of positive selection for
111 evasion of the host immune system at two residues in the receptor-binding domain of the
112 haemagglutinin-esterase (HE) protein, but the overall ratio of non-synonymous to
113 synonymous substitutions (ω) across the genome is low, individual proteins ranging
114 from 0.05 to 0.13 [Gatherer, 2010]. The low levels of ω indicate a virus that is well
115 adapted to its host, but the presence of positive selection in the HE receptor-binding
116 domain also indicates selective pressure from the host immune system. This provides a

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117 molecular explanation for the observed antigenic drift [Chakraverty, 1978] and some
118 evidence against the scenario that humans are likely to acquire lifelong immunity. Influenza
119 C would therefore resemble influenza A and B in that a new vaccine would be required
120 every time the antigenic drift had reached a certain extent, potentially annually.

121

122 The issue of endemicity versus sporadic epidemics also remains unresolved. Only one
123 candidate epidemic surge has been identified, in Japan in 2004 [Matsuzaki et al., 2007]. The
124 existence of reassorted strains indicates that double infection with two or more strains
125 cannot be very infrequent, implying that it ought to be possible to detect numerous (or at
126 least >1) strains co-circulating both temporally and geographically, previously demonstrated
127 in Japan [Matsuzaki et al., 2007]. Indeed, a continually shifting pattern of segment
128 combinations, referred to as genome constellations [Gatherer, 2010], is observed when full
129 genomes are studied, a phenomenon also seen in influenza B [Chen and Holmes, 2008].
130 Eight genome constellations circulating in the 1990s differed from the genome
131 constellations present in a set of reference genomes from the 1940s to the 1980s [Gatherer,
132 2010].

133

134 **Methods**

135 *Patient recruitment*

136 Lancaster (54.05°N 2.80°W) is a small city with a population of 45,000 rising to 141,000
137 when surrounding towns and villages are included. The permanent resident population is
138 >95% white and 18% are over age 65. 3 cohorts of participants were approached: 1) staff
139 and students at Lancaster University, 2) patients attending a general practitioner (GP)
140 consultation, 3) patients attending hospital clinics. After informed consent was given,
141 patients with coryza and/or other symptoms consistent with respiratory infection, were
142 classified as the symptomatic group ($n=71$) and the remainder as asymptomatic ($n=77$).
143 Nasopharyngeal (or nasal) swabbing, blood sampling, or both, were performed on the
144 patients, according to consent. Sample collection was performed from November 2014 to
145 May 2015.

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147 *Sample processing*

148 Nasopharyngeal swabs (MW951SENT, Medical Wire) were used to remove cells and mucus
149 from the rear wall of the nasopharynx or nose (according to consent) of patients, and the
150 tips then snapped off directly into Sigma Virocult® medium.

151

152 Blood was drawn from forearm veins into Beckton Dickinson Vacutainer® tubes containing
153 clot activator or from a finger prick, according to consent, using Beckton Dickinson Serum
154 Separator® tubes (SST™). Serum was separated at 1000-2000g for 10 minutes (for arm
155 samples) or at 6000-15000g for 90s (for finger-prick samples) and then stored at -80°C.

156

157 RNA was extracted from the nasopharyngeal swabs using a MagMAX™ Viral RNA Isolation
158 Kit (Ambion). The quality and quantity of RNA extracted from samples was assessed by
159 spectrophotometry using the NanoDrop® 1000 Spectrophotometer V3.3.0 (Thermo Fisher
160 Scientific). cDNA was prepared using a High-Capacity RNA-to-cDNA™ Kit (Applied
161 Biosystems®, Life Technologies™) and a Veriti® Thermal Cycler (Applied Biosystems®, Life
162 Technologies™). The samples were incubated at 37°C for 60 minutes, before stopping the
163 reaction at 95°C for 5 minutes and then holding at 4°C. Once completed, the plates were
164 stored at -20°C.

165

166 Polymerase chain reaction (PCR) was then performed using a 7500 FAST Real-Time PCR
167 system (Applied Biosystems®, Life Technologies™) with thermo-cycling carried out as
168 follows: one cycle of 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR
169 primers for influenza C were as used previously [Salez et al., 2014]. Samples judged positive
170 after quantitative PCR were processed using the Illumina Nextera XT library kit and deep
171 sequenced in 2x126bp format using an Illumina HiSeq2500 system.

172

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173 Enzyme-linked immunosorbent assay (ELISA) was performed on the serum samples using
174 influenza C antigen as previously described [Salez et al., 2014] and goat anti-human HRP-
175 conjugated secondary antibody (ab6858, Abcam®) with SureBlue™ TMB Microwell
176 Peroxidase Substrate solution. Absorbance was measured at 450nm using a Wallac
177 Victor2™ (Perkin Elmer) plate reader. Anti-influenza C IgG was quantified by calibration of
178 the peroxidase reaction against a standard dilution series of IgG concentrations. The
179 threshold for seropositivity was placed at 2 standard deviations above the mean level of the
180 negative control serum.

181

182 *Genome segment sequence assembly*

183 Illumina reads were trimmed of adapters and other non-genomic elements using CutAdapt
184 1.1 [Martin, 2011: <https://pypi.python.org/pypi/cutadapt>], fastq-mcf 0.11.3 [Aronesty,
185 2013: <https://expressionanalysis.github.io/ea-utils>], and trim_galore
186 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), within the
187 Read_cleaner pipeline (Gatherer, unpublished, see Supplementary Data Pack). Ethical
188 approval required that no genetic material remain within the samples which could enable
189 identification of patients. Therefore, human genome and transcriptome sequences were
190 removed by iterative alignment onto the NCBI, Ensembl and UCSC human iGenomes
191 (http://support.illumina.com/sequencing/sequencing_software/igenome.html), first using
192 bowtie 1.1.1 [Langmead et al., 2009: <http://bowtie-bio.sourceforge.net/index.shtml>], then
193 BWA 0.7.12-r1039 [Li and Durbin, 2010: <http://bio-bwa.sourceforge.net>] within the Valet
194 pipeline (Gatherer, unpublished, see Supplementary Data Pack). Following each alignment,
195 extraction of unaligned reads was achieved using samtools 0.1.19 [Li et al., 2009:
196 <http://samtools.sourceforge.net/>] and the next alignment commenced. Bowtie, BWA and
197 samtools were co-ordinated using the Vanator pipeline [Jarrett et al., 2013:
198 <https://sourceforge.net/projects/vanator-cvr>]. The resulting trimmed and cleaned reads
199 are available from the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>:
200 BioSamples SAMN05954290 and SAMN05954291, Runs SRR4733498 and SRR4733494)

201

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202 Influenza C genome C/Victoria/2/2012 (Genbank ref. KM504282) was selected as a
203 representative of recently circulating influenza C and alignment of cleaned reads carried out
204 using bowtie within the Valet pipeline. Consensus sequences were constructed using
205 samtools 0.1.19 (bcftools and vcfutils functions). C/Victoria/2/2012 was used to fill gaps in
206 the consensi and the bowtie alignment repeated. This cycle was performed until a stable
207 consensus was obtained for each genome segment. The same process was repeated using
208 BWA and combined consensi obtained. Alignment of reads to the final consensi was
209 examined with Tablet [Milne et al., 2013: <https://ics.hutton.ac.uk/tablet>]. Resulting
210 assemblies of more than 200 bases were submitted to GenBank (references KY075640 -
211 KY075642). The remaining smaller fragments, along with composite partial segments used
212 in phylogenetic analysis, are available in the Supplementary Data Pack. The new strain of
213 influenza C identified was designated C/Lancaster/1/2015.

214

215 *Phylogenetics and genome constellations*

216 Sequence alignments of composite partial segments with full influenza C genomes from
217 GenBank, were performed using Muscle [Edgar, 2004] in MEGA [Kumar et al., 2008:
218 <http://www.megasoftware.net>] and neighbour joining trees [Saitou and Nei, 1987]
219 constructed. Clock-like behaviour in sequence evolution on those trees was checked using
220 TempEst [Rambaut et al., 2016: <http://tree.bio.ed.ac.uk/software/tempest>]. Bayesian
221 phylogenetic analysis was performed in BEAST v.1.8.3 [Drummond and Rambaut, 2007:
222 <http://tree.bio.ed.ac.uk/software/beast/>]. A Tamura [1992] 3-parameter (T93+G)
223 substitution model, coalescent constant size tree prior and relaxed lognormal clock were
224 run for 100 million iterations in BEAST, as previously [Gatherer, 2010]. A burn-in of 25% of
225 all trees was used to create the consensus tree. Genome constellations were determined by
226 establishing the clade, as defined by Gatherer [2010], in which each genome segment was
227 located.

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228

229 **Results**

230 *Participants*

231 Of the 148 participants, 69 were male and 79 female. 71 were symptomatic and 77
232 asymptomatic. Distribution of male and female participants within symptomatic and
233 asymptomatic groups was assessed by a 2x2 chi-square test and was not statistically
234 significant. Except for a relative excess of age group 20-29 participants (mostly from the
235 university), age approximated a normal distribution. The summary clinical presentation
236 within the symptomatic group, graph of age distribution and details of the chi-square tests
237 are available in the Supplementary Data Pack.

238

239 *Influenza C seropositivity*

240 Of the 148 participants, 129 consented to donate serum. Of these 99 were seropositive and
241 30 negative, giving a figure of 77% seropositivity. Figure 1 shows the anti-influenza C IgG
242 concentration by age. Gender differences in seropositivity were also nearly absent (male
243 2.5mg/dl, female 2.3mg/dl) with no statistical significance on t-test, but symptomatic
244 individuals had slightly more IgG (symptomatic 2.6mg/dl, asymptomatic 2.2mg/dl),
245 significant on a t-test at $p < 0.05$. A Mann Whitney U-test was performed on the distribution
246 of seropositive individuals between each age group, and was not statistically significant (see
247 Supplementary Data Pack).

248

249 *Incidence of detectable virus*

250 Two participants out of 148 (1.4%) were detected as positive for influenza C using PCR. Both
251 were asymptomatic. On deep sequencing (SRR4733498 and SRR4733494), only one patient
252 showed sufficient levels of influenza C reads for genome assembly to be attempted

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253 (SRR4733498). If the other individual is a false positive by PCR, the population incidence
254 may therefore be 0.7%. This figure is compromised by the fact that the sample is not
255 randomly selected, but is deliberately enriched for symptomatic individuals (71/148, 48%).
256 Since incidence is also often given as positive individuals per symptomatic case, and neither
257 positive individual was symptomatic; on that strict formulation the incidence is 0%. In view
258 of this, we can say little other than that influenza C incidence is low and probably accounted
259 for <1% of coryza and other respiratory disease in Lancaster during the winter of 2014-2015.

260

261 *Genetic relationships of isolated influenza C genome segments*

262 Partial genome segment sequences were obtained from deep sequencing for segments 1, 5,
263 6 and 7, encoding PB2, NP, M1/CM2 and NS1/NS2 respectively. Those greater than 200
264 bases are deposited in GenBank, accession numbers KY075640 - KY075642 and the
265 remainder are available in the Supplementary Data Pack. Insufficient reads were available
266 to assemble the other segments. Although breadth of coverage across segments is low
267 (ranging from 22% in segment 5 to 32% in segment 6), there is sufficient genetic information
268 to assign each fragment to a clade as defined by Gatherer [2010], using Bayesian
269 phylogenetics. Plotting of the root-to-tip genetic distance on a neighbour-joining tree using
270 TempEst showed that molecular clocks apply best to segments 2 and 7 (PB2 and NS1/NS2),
271 but that both segments 5 and 6 (NP and M1/CM2) have lower root-to-tip distances for
272 C/Lancaster/1/2015 than expected. Figures 2 and 3 shows the TempEst plots for segments
273 1 and 6 (PB2 and M1/CM2), giving examples of clock-like and non-clock-like behaviour,
274 respectively. The TempEst plots for segments 5 and 7 (NP and NS1/NS2) are Supplementary
275 Figures 3 and 4 respectively.

276

277 Clade memberships were determined by examination of Bayesian phylogenetic trees
278 produced in BEAST, following the classificatory scheme of [Gatherer, 2010] and then
279 annotated onto the neighbour-joining trees used for the molecular clock analysis. Figure 4
280 shows the tree for segment 5 (encoding NP), demonstrating that C/Lancaster/1/2015
281 belongs to the C/Miyagi/1/93 clade, and not to the C/Greece/79 and C/pig/Beijing/81 clades
282 circulating in recent isolates. Figure 5 shows the tree for segment 7 (encoding NS1/NS2) has

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283 an even more distant relationship to recent genomes, being part of the C/Sapporo/71 clade
284 last seen in 1979. The phylogenetic trees for PB2 and MP are given in Supplementary
285 Figures 1 and 2, and further confirm the genetic distance between C/Lancaster/1/2015 and
286 other recently sequenced genomes. Clade memberships are then synthesised to derive the
287 relationship between C/Lancaster/1/2015 and defined genome constellations (Table 1).

288

289 **Discussion**

290 *Herd immunity to influenza C*

291 Our participant group were 77% seropositive to influenza C. This is slightly higher than the
292 57-61% levels from studies in western Europe and Brazil [Manuguerra et al., 1992;
293 Manuguerra et al., 1994; Motta et al., 2000], within the range of the 70-90% found in
294 eastern Europe [Tumova et al., 1983; Vasil'eva et al., 1985] but still considerably short of
295 those studies reporting universal seropositivity in the USA and east Asia [Hilleman et al.,
296 1953; Nishimura et al., 1987]. As in previous studies, our antibody titre levels were widely
297 variable among those classed as seropositive, and our choice of threshold is purely
298 statistical. However, we also found no statistically significant age-structured or gender-
299 structured variability in seropositivity (Figure 1). This is at variance with some previous
300 studies in the USA, Japan and Europe [Dykes et al., 1980; Kaji et al., 1983; Manuguerra et al.,
301 1992; O'Callaghan et al., 1980]. It should also be noted that many serological studies on
302 influenza C are now some decades old and techniques have varied over the years, so
303 individual studies are not necessarily directly comparable. We also cannot exclude the
304 possibility of some cross-reactivity of our influenza C antigen with antibodies to other
305 influenza viruses, but this is also an issue in all previous studies.

306

307 Whatever the source of the initial antigenic stimulus for the production of anti-influenza C
308 IgG, such seropositivity may be equivalent to immunity to influenza C, even if of a
309 temporary or partial nature, and this may have implications for the epidemiology of the
310 virus. We are not aware of any study on the reproductive number (R_0) of influenza C,
311 although extensive studies have been performed for influenza A [reviewed by Biggerstaff et

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312 al., 2014]. If we assume that the R_0 of 1.28 for seasonal influenza A, calculated by
313 Biggerstaff et al. [2014] as a median of 47 published values, also applies to influenza C, then
314 the herd immunity threshold (HET) – at which R_t would be reduced to 1, and an epidemic
315 would be unsustainable – is:

316

$$317 \text{ HET} = (R_0 - 1) / R_0 = (1.28 - 1) / 1.28 = 0.5$$

318

319 This implies that 50% immunity in the population would be sufficient to suppress any
320 epidemic outbreak of influenza C. Our level of 77% seropositivity may therefore explain
321 why the influenza C virus was so difficult to detect in our participant group. We also note
322 that it is not relevant to this calculation if our 77% partially represents cross-reactive
323 antibodies against influenza A and B. Regardless of the virus type that initially produced the
324 antibodies, their binding to influenza C antigen in ELISA suggests that they may be
325 effectively protective *in vivo* and would contribute to herd immunity.

326

327 *Influenza C evolution*

328 Neither of the two participants who were identified as influenza C-positive by PCR
329 generated sufficient deep sequencing reads for complete genomes to be assembled. Our
330 deep sequencing of the nasopharyngeal swabs of both of our PCR-positive participants,
331 produced much fuller genome sequence results for other RNA viruses apart from influenza
332 C, as well as sequences from a range of bacterial species (Atkinson *et al* in preparation). We
333 therefore do not think that the difficulty in detecting influenza C, or in generating complete
334 genomes, is due to RNA degradation or other technical failure, but rather a true reflection of
335 the rarity of the virus and a low viral titre in infected individuals.

336

337 In the individual with the 4 partial genome segment sequences, it is evident that
338 C/Lancaster/1/2015 is a reassortant that does not fall into any of the genome constellations
339 previously classified [Table 1 and Gatherer, 2010]. It contains a rare NS1/NS2 segment of
340 the C/Sapporo/71 clade, related to sequences that were last observed in the late 1970s.
341 Influenza C genomes sequenced since 2010 all have the C/Shizuoka/79 clade in the NS1/NS2

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342 segment (Figure 5). C/Lancaster/1/2015 also has a rare NP segment of the C/Miyagi/1/93
343 clade, related to sequences that were last observed around 2000 (Figure 4) and typical of
344 genome constellation 4a (Table 1). The other segments are within clades found more
345 recently, although C/Lancaster/1/2015's position within these clades is never close to any of
346 the recent genome sequences (Supplementary Figures 1 and 2). The exact position of
347 C/Lancaster/1/2015 on each segment's phylogenetic tree is rarely well supported by
348 Bayesian phylogenetics posterior probability density, but its location within each of the
349 broader clades is well supported (see Supplementary Data Pack). We therefore conclude
350 that its apparent reassortant nature is unlikely to be simply an artefact of partial sequence
351 information.

352

353 Tentative reconstruction of the reassortment event may be attempted. Gatherer [2010]
354 defines genome constellation 4a as consisting of C/Sapporo/71, C/Miyagi/1/93,
355 C/Sapporo/71 and C/Shizuoka/79 in segments 1, 5, 6 and 7 respectively. The corresponding
356 clades for C/Lancaster/1/2015 are C/Sapporo/71, C/Miyagi/1/93, C/Sapporo/71 and
357 C/Sapporo/71 (Table 1), suggesting that a strain of constellation 4a reassorted with one
358 containing a C/Sapporo/71-clade segment 7. Since no strain containing a segment 7 of this
359 clade has been seen since the 1970s and constellation 4a was only seen in the 1990s, it
360 seems likely that the reassortment event occurred in the 1990s. This would also explain the
361 dissimilarity of C/Lancaster/1/2015 in all of its segments, to other recently sequenced
362 strains. We are tempted to speculate that this reassortant occurred locally in Lancaster, but
363 in the absence of any other British genomes since C/England/892/1983 [Matsuzaki et al.,
364 2016], which is itself incomplete, it is impossible to come a conclusion.

365

366 If this scenario is common in small isolated populations, influenza C diversity in terms of
367 shifting genome constellations may be even greater than suggested from the available
368 genomes. Rare strains may persist at low levels in small urban/rural locations, such as
369 Lancaster. Our detection rate, at 0%, 0.7% or 1.4% depending on whether both samples, or
370 merely one sample, is scored as positive, or whether asymptomatic individuals are included,
371 is broadly similar to the 0.2% (frequency per symptomatic individual) found in another

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372 recently sampled British population [Smith et al., 2016]. The M1/CM2 (Figure 3) and NP
373 segments (Supplementary Figure 3) for C/Lancaster/1/2015 have lower root-to-tip distances
374 than expected under the assumption of molecular clock-like evolution. When this method
375 is used on database-derived sequences, it is often taken as indicative of incorrect dating.
376 However, given that we know precisely when our samples were collected, it is more likely to
377 reflect a genuinely slower rate of evolution in these samples. The M1/CM2 segment of
378 C/Lancaster/1/2015 is positioned in the phylogenetic tree near segments from the 1980s
379 (Supplementary Figure 1) and the NP segment near segments from the 1990s and 2000
380 (Figure 4). This same phenomenon of slowed molecular clock, and aberrant positioning with
381 the phylogenetic tree, has been seen in some strains of Zaire ebolavirus [Lam et al., 2015]
382 and also in the 1977 “Russian Flu” H1N1 outbreak [Wertheim, 2010], and is thought to be a
383 consequence of the virus entering a host population where the serial interval – the time
384 between infection of one host and the next in a transmission chain – is reduced and the
385 virus therefore spends longer in a non-replicative state. For ebolavirus, this is assumed to
386 be a non-typical animal reservoir host, and for Russian Flu possibly a laboratory freezer.
387 Neither of these options would seem to be possible for influenza C, so it may simply be a
388 cumulative result of low transmission rates within relatively small populations slightly
389 delaying the average serial interval, conditions which could apply in Lancaster.

390

391 *Implications for vaccination strategy*

392 We began this study with the premise that influenza C might be a candidate for inclusion in
393 the seasonal influenza vaccine. Our results do not provide any support for the proposition
394 that vaccination of adults is appropriate, a conclusion also reached by Smith et al. [2016].
395 Although we recruited 71 symptomatic individuals with a range of cold/flu-like symptoms,
396 none of these was influenza C-positive, and none of the respiratory disease burden in
397 Lancaster during our study period can be attributed to influenza C.

398

399 There may still be a case for vaccination of children in the light of published reports of
400 serious respiratory disease caused by influenza C in that age group. [Calvo et al., 2006;
401 Gouarin et al., 2008; Laxdal et al., 1966; Matsuzaki et al., 2007; Matsuzaki et al., 2006;

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402 Matsuzaki et al., 2003; Moriuchi et al., 1991; Peng et al., 1996; Principi et al., 2012; Shimizu
403 et al., 2015; Takayanagi et al., 2009]. We recruited 6 participants in the <9 years age group
404 but none were consented to allow serum sampling. In the single participant in the 10-19
405 year age group, anti-influenza C IgG levels were at <1 mg/dl and this individual is classified
406 as seronegative (Figure 1). Whether the higher levels of anti-influenza C IgG found in our
407 adults are as a consequence of a single exposure, or limited number of exposures, during
408 childhood, or are maintained by recurrent possibly sub-clinical infections (as in our 2
409 positive participants) in adulthood, remains a matter of debate. The apparent slowing of
410 evolutionary rate in the M1/CM2 and NP segments of C/Lancaster/1/2015, if caused by
411 reduced average serial interval due to reduced number of infections in small isolated
412 populations, possibly suggests the former.

413

414 (Text 4082 words)

415

416 **Figure Legends**

417 **Table 1: Clade membership of segments of C/Lancaster/1/2015** based on Bayesian
418 phylogenetic analysis and the prior clade and genome constellation classifications of
419 Gatherer [2010]. The rightmost column lists those clades found in other segments
420 sequenced from 2010 onwards. Segments 1 and 6 of C/Lancaster/1/2015 are outliers within
421 clades found in other recent genomes, but segments 5 and 7 are not.

422 **Figure 1: Anti-influenza C IgG concentration** (mg/dl), plotted for each individual against age.
423 Blue: >2 standard deviations above negative control; green: 1-2 standard deviations above
424 negative control; red: <1 standard deviation above negative control.

425 **Figure 2: Root-to-tip distance in a neighbour joining tree for segment 1** (encoding PB2) of
426 the influenza C genome. 100 full-length or near full-length genome segments (2365 bases)
427 are used plus the 724 discontinuous bases of segment 1 derived from deep sequencing.
428 C/Lancaster/1/2015 has a degree of divergence from the root consistent with molecular
429 clock-like behaviour in its lineage.

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430 **Figure 3: Root-to-tip distance in a neighbour joining tree for segment 6** (encoding
431 M1/CM2) segment of the influenza C genome. 86 full-length or near full-length genome
432 segments (1180 bases) are used plus the 380 discontinuous bases of segment 6 derived
433 from deep sequencing. C/Lancaster/1/2015 is less divergent from the root than it should
434 be given its known sampling date, consistent with a perturbation of molecular clock-like
435 behaviour in its lineage.

436 **Supplementary Figure 1: Neighbour joining tree rooted on C/Taylor/1233/1947 for**
437 **segment 6** (M1/CM2), annotated with clades derived from Gatherer [2010] and confirmed
438 by BEAST analysis (see Supplementary Data Pack), demonstrating the closer relationship of
439 C/Lancaster/1/2015 (red) to M1/CM2 segments of the C/Sapporo/71 clade from the 1980s
440 than to recent isolates. Scale: substitutions per site.

441 **Supplementary Figure 2: Neighbour joining tree rooted on C/Taylor/1233/1947 for**
442 **segment 1** (PB2), annotated with clades derived from Gatherer [2010] and confirmed by
443 BEAST analysis (see Supplementary Data Pack), demonstrating the closer relationship of
444 C/Lancaster/1/2015 (red) to PB2 segments of the C/Sapporo/71 clade from the 1970s and
445 1980s than to recent isolates. Scale: substitutions per site.

446 **Supplementary Figure 3: Root-to-tip distance in a neighbour joining tree for segment 5**
447 (encoding NP) of the influenza C genome. 96 full-length or near full-length genome
448 segments (1809 bases) are used plus the 397 discontinuous bases of segment 5 derived
449 from deep sequencing. C/Lancaster/1/2015 is less divergent from the root than it should be
450 given its known sampling date, consistent with a perturbation of molecular clock-like
451 behaviour in its lineage.

452 **Supplementary Figure 4: Root-to-tip distance in a neighbour joining tree for segment 7**
453 (encoding NS1/NS2) segment of the influenza C genome. 134 full-length or near full-length
454 genome segments (935 bases) are used plus the 288 discontinuous bases of segment 7
455 derived from deep sequencing. C/Lancaster/1/2015 has a degree of divergence from the
456 root consistent with molecular clock-like behaviour in its lineage.

457

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463

464 **Ethics Statement**

465 Informed consent was obtained from adult volunteers and supported assent from juveniles
466 with prior informed parental consent. Ethical approval was granted by the UK National
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470 Portfolio, ID 17799.

471

472 **Data Accessibility Statement**

473 The Supplementary Data Pack containing statistical analyses on volunteers and ELISAs, BAM
474 files and reference genomes for genome assemblies, genome fragments too short for
475 inclusion in GenBank, BEAST inputs and outputs, TempEst inputs and outputs and pipeline
476 Perl scripts, are available from: [doi://10.17635/lancaster/researchdata/111](https://doi.org/10.17635/lancaster/researchdata/111)

477

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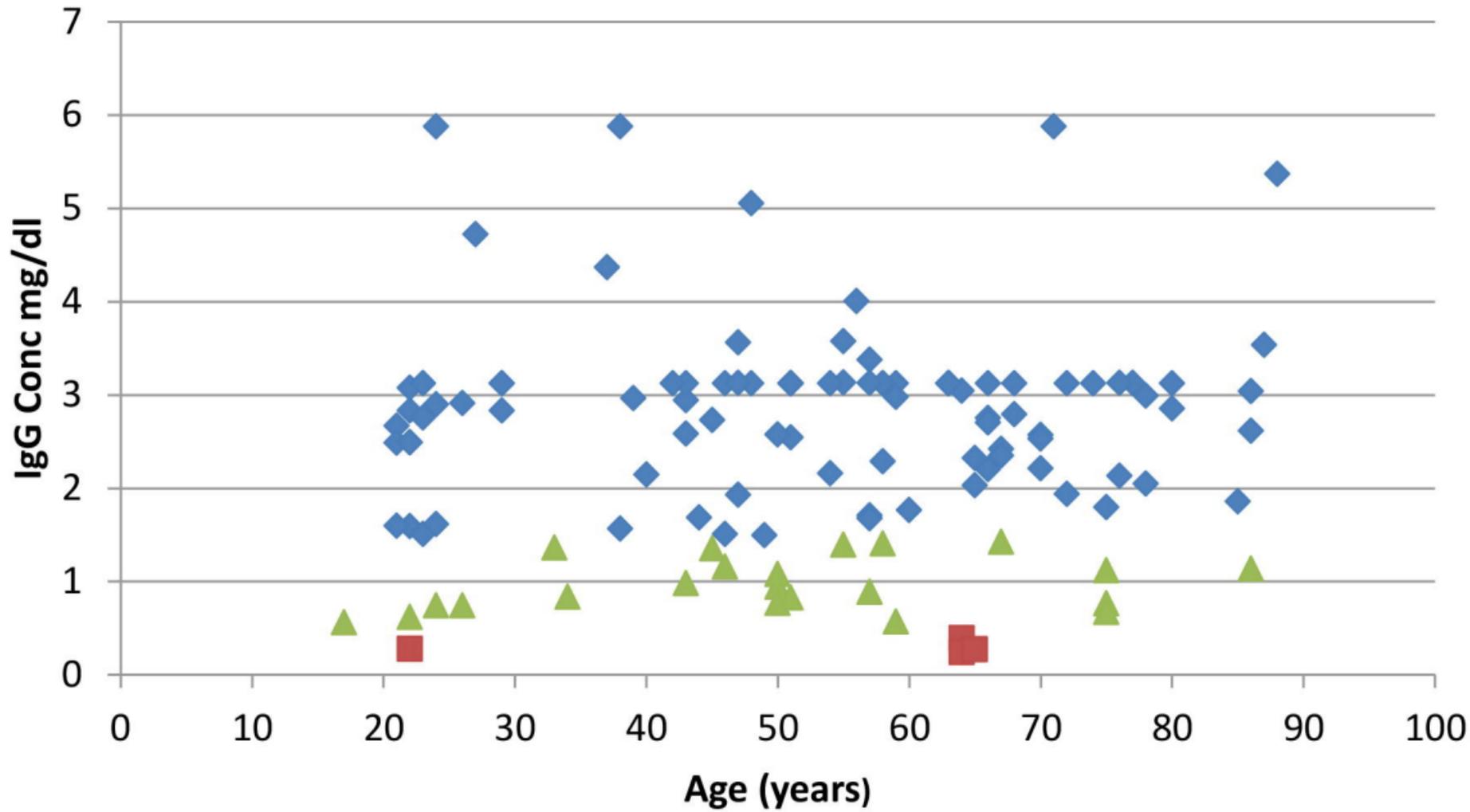
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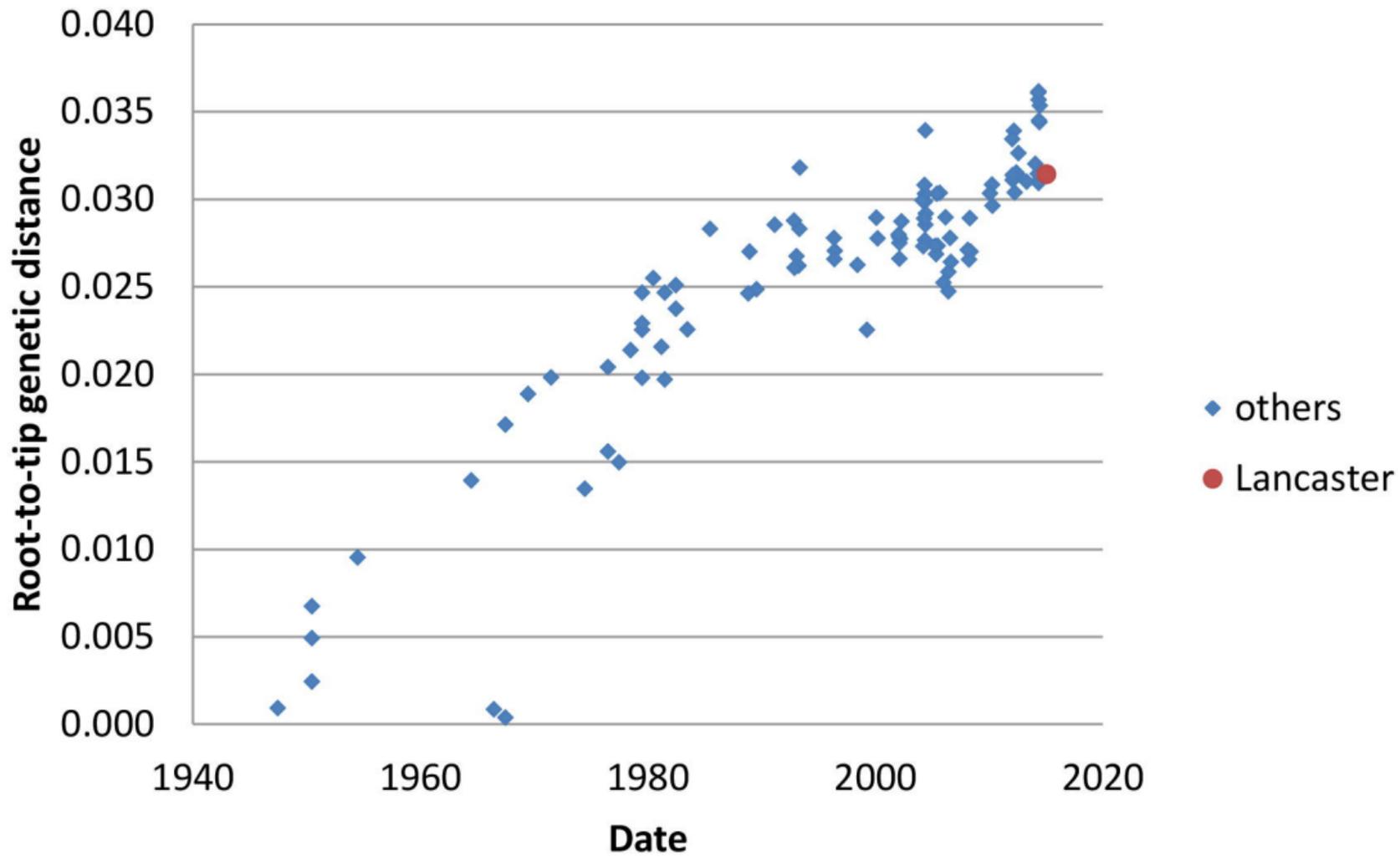
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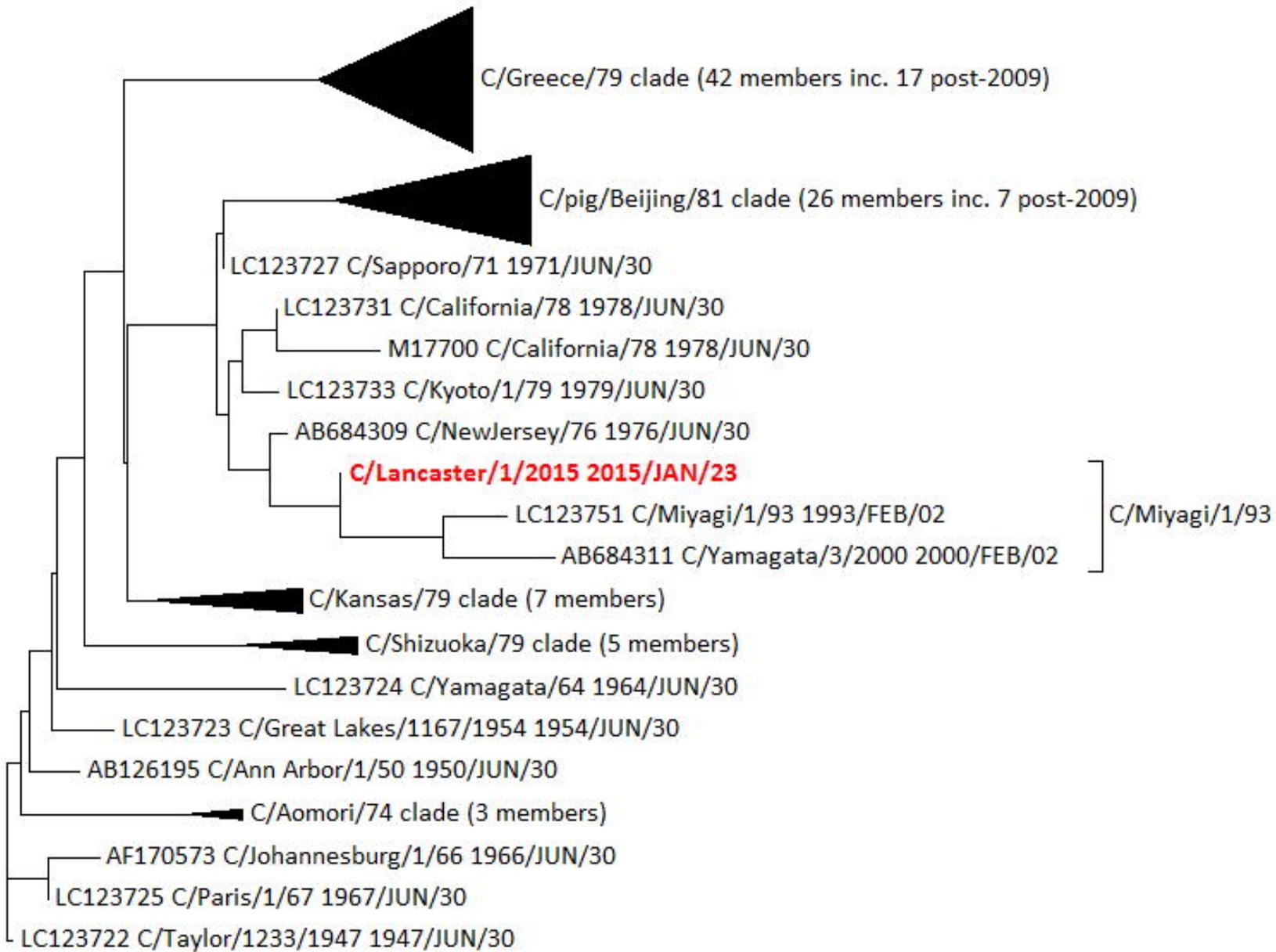
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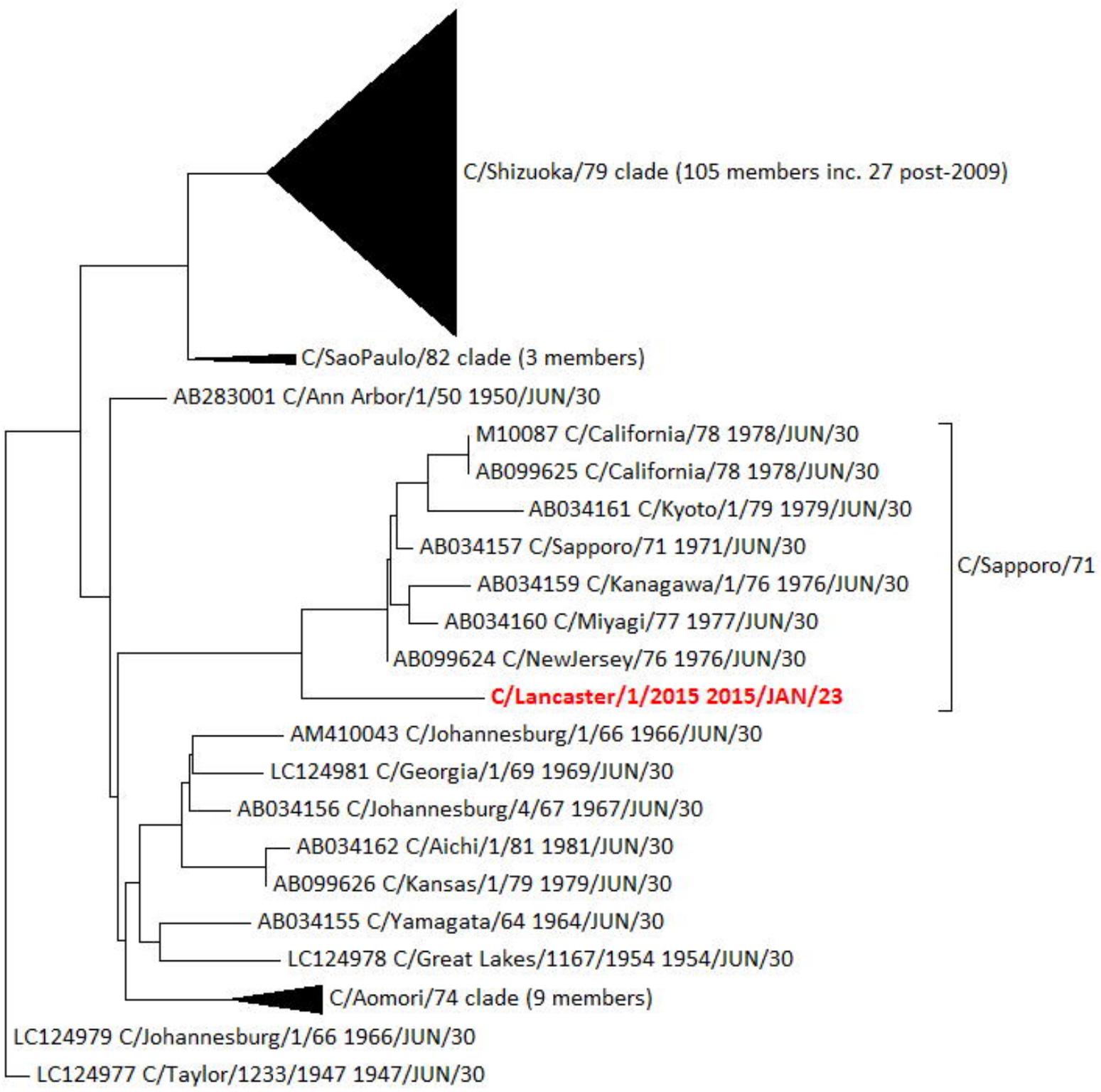
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- 598
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0.002



0.0020

Genome segment (encoded protein)	Clade of segment in Lancaster consensus, as defined by Gatherer [2010]	Genome constellation in which that clade is present	Clade(s) of other post 2009 genomes
1 (PB2)	C/Sapporo/71	All, except 5	C/Greece/79; C/Sapporo/71
5 (NP)	C/Miyagi/1/93	4a	C/pig/115/Beijing/81; C/Greece/79
6 (M1/CM2)	C/Sapporo/71	All, except 2 & 3	C/Sapporo/71
7 (NS1/NS2)	C/Sapporo/71	None: clade not seen since 1970s	C/Shizuoka/79

Table 1: Clade membership of segments of C/Lancaster/1/2015 based on Bayesian phylogenetic analysis and the prior clade and genome constellation classifications of Gatherer [2010]. The rightmost column lists those clades found in other segments sequenced from 2010 onwards. Segments 1 and 6 of C/Lancaster/1/2015 are outliers within clades found in other recent genomes, but segments 5 and 7 are not.