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1 # Metagenomic 16S rRNA gene sequencing
2 # survey of *Borrelia* species in Irish samples
3 # of *Ixodes ricinus* ticks
4
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Abstract

Lyme borreliosis is a systemic infection caused by tick-borne pathogenic borreliae of the *Borrelia burgdorferi* sensu lato complex or of the more heterogeneous relapsing fever borrelia group. Clinical distinction of the infections due to different borrelia species is difficult. Accurate knowledge of the prevalence and the species of borreliae in the infected ticks in the endemic areas is valuable for formulating appropriate guidelines for proper management of this infectious disease. The purpose of this research was to design a readily implementable protocol to detect the divergent species of borreliae known to exist in Europe, using Irish samples of *Ixodes ricinus* ticks as the subject for study. Questing *I. ricinus* nymph samples were taken at six localities within Ireland. The crude DNA of each dried tick was extracted by hot NH_4OH and used to initiate a same-nested PCR with a pair of borrelial genus-specific primers to amplify a highly conserved 357/358 bp segment of the 16S rRNA gene for detection and as the template for Sanger sequencing. To distinguish *B. garinii* from *B. burgdorferi* and to discriminate the various strains of *B. garinii*, a second 282 bp segment of the 16S rRNA gene was amplified for Sanger sequencing. A signature segment of the DNA sequence excised from the computer-generated electropherogram was submitted to the GenBank for BLAST alignment analysis. A 100% ID match with the unique reference sequence in the GenBank was required for the molecular diagnosis of the borrelial species or strain. We found the overall rate of borrelial infection in the Irish tick population to be 5%, with a range from 2% to 12% depending on the locations of tick collection. At least 3 species, namely *B. garinii, B. valaisiana* and *B. miyamotoi,* are infecting the ticks collected in Ireland. The isolates of *B. garinii* were confirmed to be strain BgVir, strain Bernie or strain T25. Since antigens for diagnostic serology tests may be species- or even strain-specific, expanded surveillance of the species and strains of the borreliae among human-biting ticks in Ireland is needed to ensure that the antigens used for the serology tests do contain the epitopes matching the antibodies elicited by the borrelial species and strains in the ticks cohabitating in the same environment.

Introduction

Lyme borreliosis (or Lyme and related borreliosis) can be caused by many members of the *Borrelia burgdorferi* sensu lato (B.b.s.l) complex [1] and the more heterogeneous group of relapsing fever borreliae [2] which are transmitted to humans through the bite of infected ticks of the *Ixodes* genus, including *I. ricinus, I. scapularis, I. pacificus* and *I. persulcatus*. In Europe, the pathogenic B.b.s.l. species include *Borrelia afzelii, B. garinii, B. bavariensis, B. spielmanii, B. valaisiana, B. lusitaniae* and rarely *B. burgdorferi* [1, 3-6]; the major hard tick relapsing fever borrelia is *B. miyamotoi* [7].

Lyme borreliosis at its early stage of infection can usually be effectively treated with timely, appropriate antibiotics to prevent deep tissue damage along with the associated clinical manifestations resulting from host immune response to various spirochetal products or components. However, if not treated early, within days to weeks, the borrelial spirochetes disseminate from the site of the tick bite to other regions of the body [8].

Since Lyme borreliosis only occurs in the endemic areas where the tick population is infected with pathogenic borrelial species, accurate surveys of the borrelial infections of the ticks

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69 collected in the potential endemic areas can provide valuable information on the possible
70 existence of any of these Lyme and related borrelioses, and can serve as guidelines for selection
71 of the proper antigens for diagnostic serology tests which may be species- or even strain-specific
72 [9-11].

73 In the past, the methods used for borrelial surveys were either designed to detect *B. burgdorferi*
74 sensu lato [1] or to detect *B. miyamotoi* [7, 12] in ticks; the PCR primers used for these
75 metagenomic assays were unable to amplify a conserved segment of DNA shared by all species
76 of borreliae. Only one study used a pair of general PCR primers which can amplify a segment of
77 16S rRNA gene of both *B. burgdorferi* sensu lato and *B. miyamotoi* for real-time PCR screening
78 [13]. But the interprimer DNA segment of the latter real-time PCR product is only 25 bp long
79 which is too short for automated Sanger sequencing that is required to confirm the molecular
80 diagnosis of *B. miyamotoi* [7, 12, 13]. In addition, the dye-labeled probe 6FAM-
81 TTCGGTACTAACTTTTAGTTAA used for the real-time PCR screening [13] may miss the
82 species of *B. valaisiana* and *B. lusitaniae* whose corresponding complementary sequences in this
83 segment are TTAACTGAAAGTTAGTACCGAA (Sequence ID: NR_036807) and
84 TTAACTAACAGTTAGTACCGAA (Sequence ID: AB091822), respectively, not fully matched
85 with the sequence of the probe designed for other species of the *B. burgdorferi* sensu lato
86 complex.

87 In the current study, we used a pair of genus-specific PCR primers to amplify a highly conserved
88 segment with single-nucleotide polymorphisms of the borrelial 16S rRNA gene shared by all
89 known pathogenic borrelial strains to survey the borrelial infections among the *I. ricinus* ticks
90 collected in Ireland. Since this PCR amplicon is 357/358 bp long, the PCR products can be used
91 as the template for direct Sanger sequencing for amplicon validation and for speciation [14]. This
92 metagenomic 16S rRNA gene sequencing assay is most suitable for molecular diagnosis of
93 borrelial infections in human-biting ticks and in clinical specimens in Europe because of the
94 great diversity of causative agents in European Lyme borreliosis which needs a broad-spectrum
95 tool to detect the target DNA from various borrelial strains and to prepare the template for
96 Sanger sequencing to ensure diagnostic accuracy.

97 The value of routine metagenomic 16S rRNA gene sequencing is well recognized for detecting
98 the presence of pathogenic bacteria in the environment and in clinical specimens because single
99 nucleotide polymorphisms in the 16S rRNA gene may discriminate closely related bacterial
100 species or indicate a mutant expressing different patterns of synthesized proteins with potential
101 implication in antibody epitopes, virulence and drug resistance of the bacteria [15-19]. However,
102 the usefulness of 16S rRNA gene sequencing as a tool in microbial identification is dependent
103 upon deposition of the complete unambiguous 16S rRNA gene nucleotide sequences into a
104 public database, such as that in the GenBank and applying the correct base labels to each
105 sequence detected for alignment analysis [20]. Indeed, the validity of the results of studying
106 borrelia distribution in *I. scapularis* ticks using PCR and DNA sequencing has been questioned
107 because the DNA sequences claimed to have been detected were questionable [21]. Good-quality
108 sequence traces in Sanger sequencing are essential for molecular diagnosis and typing of
109 borreliae [5] in the era of precision medicine.

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110 In the present study, we aimed to develop a protocol for using a single pair of genus-specific
111 PCR primers to amplify a highly conserved segment with hypervariable regions of the borrelial
112 16S rRNA gene for detection of all species of *Borrelia* infecting the *I. ricinus* ticks collected in
113 Ireland and to use the positive crude nested PCR products as the templates for direct Sanger
114 sequencing to determine the species of the borrelia detected.

115

116 Materials and Methods

117 Unfed, questing *I. ricinus* nymphs were collected by "flagging" which involves brushing the
118 vegetation with a white towel from which the ticks can then be removed. In late May and early
119 June 2018, samples were taken at six localities within Ireland, designed to provide a
120 representative view of tick borrelial infection across the country. The following areas were
121 sampled (county in parenthesis): Killarney (Kerry), Kilmacthomas (Waterford), Clifden
122 (Galway-West), Portumna (Galway-South), Glendalough (Wicklow), and Glenveagh (Donegal)
123 and their locations are shown on the accompanying map of Ireland (Fig 1).

124 **Fig 1. Sampling locations in Ireland for *Ixodes ricinus* ticks in May & June 2018.**

125 Each individual sample was made up of at least 5 sub-samples taken at different points within a
126 locality to minimise any sampling bias. Distance between sub-sampling points was never less
127 than 100 m.

128 The collected ticks were disinfected in 70% ethanol, air-dried on filter paper and sent to Milford
129 Molecular Diagnostics Laboratory in Milford, Connecticut, U.S.A. to be tested. The general
130 procedure for extraction of the crude DNA from archived ticks and for Sanger sequencing
131 detection of the borrelial 16S rRNA gene previously published [22] was followed. Initially, 300
132 ticks were analysed, 50 from each of the six sampled locations listed above.

133 On the day of testing, each dried tick was placed in a 1.5 mL plastic tube and immersed in 300
134 µL of 0.7 mol/L NH_4OH overnight at room temperature. On the following day, the test tubes
135 were heated at 95°C to 98°C for 20 minutes with closed caps, followed by 10 minutes with open
136 caps. After the test tubes were cooled to room temperature and the carcass of the tick was
137 discarded, 700 µL of 95% ethanol and 30 µL of 3 mol/L sodium acetate (Sigma) were added to
138 each NH_4OH digestate. The precipitated crude DNA was spun down in the pellet after
139 centrifugation at ~ 16,000 x g for 5 minutes, washed in 1 mL of 70% ethanol, air dried, and re-
140 dissolved in 100 µL of tris(hydroxymethyl)aminomethane hydrochloride–EDTA (TE) buffer, pH
141 7.4 (Sigma) by heating the DNA extract at 95°C to 98°C for 5 minutes. After centrifugation, 3µL
142 of the crude DNA extract in the supernatant was used to initiate a primary PCR, followed by a
143 same-nested PCR using a pair of M1/M2 borrelial genus-specific primers in a total 25 µL low-
144 temperature PCR mixture for a 30-cycle amplification at primary PCR, followed by another 30-
145 cycle amplification at the same-nested PCR. In carrying out the same-nested PCR, a single pair
146 of M1 (5'-ACGATGCACACTTGGTGTTAA-3') and M2 (5'
147 TCCGACTTATCACCGGCAGTC-3') primers were used for both primary PCR and nested PCR
148 so that a small amount of the primary PCR products was re-amplified with the same pair of PCR

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149 primers in a new PCR mixture [14]. An original target DNA segment in the PCR mixture might
150 have been amplified for 60 cycles exponentially by the same pair of primers to increase the
151 sensitivity of detection. The PCR amplicons of the 357-bp 16S rRNA gene segment of the *B.*
152 *burgdorferi* sensu lato complex and the corresponding 358-bp 16S rRNA gene segment of the
153 relapsing fever borreliae, both defined by the M1 and M2 PCR primer pair, were visualized by
154 agarose gel electrophoresis of the nested PCR products. The same-nested PCR products were
155 used as the template for Sanger reaction without purification.

156 For DNA sequencing, the positive nested PCR products were transferred by a micro-glass rod
157 into a Sanger reaction tube containing 1 μL of 10 μmolar sequencing primer, 1 μL of the
158 BigDye® Terminator (v 1.1/Sequencing Standard Kit), 3.5 μL 5× buffer, and 14.5 μL water in a
159 total volume of 20 μL for 20 enzymatic primer extension/termination reaction cycles according
160 to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA). After
161 a dye-terminator cleanup with a Centri-Sep column (Princeton Separations, Adelphia, NJ, USA),
162 the reaction mixture was loaded in an automated ABI 3130 four-capillary Genetic Analyzer for
163 sequence analysis [14].

164 Sanger sequencing of positive 357/358 bp M1/M2 same-nested PCR products is capable of
165 accurate identification of many species including, *B. valaisiana*, *B. afzelii*, *B. mayonii*, *B.*
166 *spielmanii*, *B. lusitaniae*, *B. recurrentis*, *B. miyamotoi*, *B. hermsii*, *B. lonestari*, *B. coriaceae* and
167 several other members in the relapsing fever group based on known species-specific single-
168 nucleotide polymorphisms in the gene segment [14]. However, DNA sequencing of this 16S
169 rRNA gene segment cannot distinguish *B. garinii* and *B. bavariensis* from *B. burgdorferi* sensu
170 stricto or discriminate the heterogeneous strains within the species of *B. garinii* because their
171 base sequences defined by the M1 and M2 PCR primers are identical.

172 Ireland is an island state geographically at the periphery of Europe. It is important at least for the
173 current study to accurately determine the species or strains of the *B. burgdorferi* sensu lato
174 detected in the ticks because the causative agents of Lyme borreliosis are highly heterogeneous
175 in Europe. [1, 3-6] In North America, species differentiation among members of the *B.*
176 *burgdorferi* sensu lato complex was initially considered unnecessary in a routine diagnostic
177 laboratory because practically all *B. burgdorferi* sensu lato isolates detected in ticks and in
178 clinical specimens in the United States were presumed to be *B. burgdorferi* sensu stricto [23].
179 Nucleic acid-based diagnostic tests had been designed to detect *B. burgdorferi* sensu stricto only
180 [24]. However, it is now recognized that the borrelia strains causing clinical Lyme disease in the
181 United States are in fact quite diverse, especially after *B. miyamotoi* infection in humans was
182 reported in 2013 [25]. According to the current *Viewpoints* of a group of Lyme disease experts, a
183 "core genome" shared by all isolates capable of causing clinical Lyme borreliosis is needed for
184 direct detection diagnosis even in the United States [26].

185 The "genus-specific" M1/M2 PCR primer pair can amplify a "core genome" of all pathogenic
186 borreliae for the purpose of detection. However, to design a pair of reliable PCR primers to
187 amplify a segment of borrelial 16S rRNA gene with single-nucleotide polymorphisms among
188 various borrelial strains for further species or strain differentiation without co-amplification of
189 the unwanted DNA extracted from other bacteria also present in the ticks turned out to be

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190 challenging. In molecular diagnosis, the size of the PCR amplicons for detecting a small quantity
191 of bacterial target DNA in a pool of non-target bacterial DNAs are usually below 300 bp in size
192 [27-30] to avoid loss of sensitivity. It took several weeks of experimental work before we found
193 3 PCR primers to generate a 282-bp heminested PCR amplicon useful as the template for Sanger
194 sequencing to distinguish *B. garinii* from *B. burgdorferi* and to discriminate among the various
195 *B. garinii* strains. The sequences of these 3 heminested PCR primers are listed as follows.

196 Primary PCR Forward Primer Bg1: 5'- GACGTTAATTTATGAATAAGC -3'

197 Primary PCR Reverse Primer Bg 6: 5'- TTAACACCAAGTGTGCATCGT – 3'

198 Heminested PCR Forward Primer Bg5: 5'- CGGGATTATTGGGCGTAAAGGGTGAG-3'

199 Heminested PCR Reverse Primer Bg 6: 5'- TTAACACCAAGTGTGCATCGT – 3'

200 Based on the reference sequences retrieved from the GenBank, the Bg5/Bg6 heminested PCR
201 primer pair defines a 282 bp segment of the borrelial 16S rRNA gene with single-nucleotide
202 polymorphisms which can be used to discriminate *Borrelia burgdorferi* strain B31
203 (ID# CP019767), *Borrelia garinii* BgVir (ID# CP003151), *Borrelia garinii* strain Bernie
204 (ID# D89900), *Borrelia garinii* strain T25 (ID# AB035388) and *Borrelia garinii* strain L20
205 (ID# X85198) for the purpose of routine molecular diagnosis.

206 However, after the first 300 ticks were tested, it was realized that the free DNA of the borrelia
207 16S rRNA gene in the crude DNA extract from the ticks was not stable on storage even at -20°
208 C. By the time when the Bg5 and Bg6 heminested PCR primers were readied to be put into
209 routine practice, the borrelial 16S rRNA gene DNA in 9 of the 12 samples initially found to be
210 positive for *B. burgdorferi* sensu lato already degraded and were no longer amplifiable with any
211 PCR primers. Therefore, an additional series of 50 ticks from each of the Portumna and
212 Kilmacthomas samples were analysed for the specific purpose of species confirmation and strain
213 determination of the *B. garinii* isolates.

214 Results

215 Multiple borrelial species found in *I. ricinus* ticks in Ireland

216 The same-nested PCR amplification of a 357/358 bp segment of borrelial 16S rRNA gene by the
217 M1/M2 genus-specific PCR primers followed by Sanger sequencing of the nested PCR products
218 [14] provided metagenomic evidence of *B. burgdorferi* sensu lato (B.b.s.l.), *B. valaisiana* and *B.*
219 *miyamotoi* infection in the ticks collected in Ireland. Samples of the 16S rRNA gene sequencing
220 with the M2 primer in support of these molecular diagnoses are illustrated by the 3 selected
221 electropherograms presented in Fig 2.

222 **Fig 2. Samples of computer-generated electropherograms of 16S rRNA gene sequences**
223 **representing the 3 borrelial groups detected in the ticks collected in Ireland.**
224 (A) 221 bases Portumna area – *B. burgdorferi* sensu lato (GenBank ID# CP019767).
225 (B) 221 bases Killamey Area – *B. valaisiana* (GenBank ID# AB091815).
226 (C) 222 bases Portumna Area – *B. miyamotoi* (GenBank ID# KM007554).
227

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Strain diversity of *B. garinii*

The *B. burgdorferi* sensu lato isolates other than *B. valaisiana, B. spielmanii, B. mayonii* or *B. lusitaniae* cannot be speciated by the DNA sequence illustrated in Fig 2A, based on single-nucleotide polymorphisms of the 16S rRNA gene DNA sequences retrieved from the GenBank [14]. Sanger sequencing of the PCR amplicon with the reverse M1 primer proved that none of the *B. burgdorferi* sensu lato isolates were *B. afzelii*. A 177-base sequence of the 282-bp amplicon defined by the Bg5 and Bg6 heminested PCR primers distinguished the heterogeneous strains of *B. garinii* from *B. burgdorferi* and *B. bavariensis* due to the presence of single-nucleotide polymorphisms among strain BgVir {1}, strain 25 {2} and strain Bernie {3} of the *B. garinii* species and distinguished these strains from *B. burgdorferi* sensu stricto {4}. Alignment of these four 177-base reference sequences retrieved from the GenBank showing single-nucleotide polymorphisms is presented below with the ending 26-base G5 primer site underlined.

```
{1}CCTTCGCCTCCGGTATTCTTTCTGATATCAACAGATTCCACCCTTACACCAGAAATTCTAACTTCCTCTATCAGACTCTAGAC
{2}···································································································
{3}··································································································T
{4}···································································································

{1}ATATAGTTTCCAACATAGTTCCACAGTTGAGCTGTGGTATTTTATGCATAGACTTATATATCCGCCTACTCACCCTTTACGCC
{2}································C····C·············································
{3}···································································································
{4}··················G················································································

{1}CAATAATCCCG B. garinii Strain BgVir Sequence ID: CP003151 (Range:447801-447977)
{2}··········· B. garinii Strain T25 Sequence ID: AB035388 (Range: 684-508)
{3}··········· B. garinii Strain Bernie Sequence ID: D89900 (Range:692-516)
{4}··········· B. burgdorferi strain B31 Sequence ID: CP019767 (Range: 444582-444758)
```

The corresponding electropherograms of these four 177-base sequences (1-4), using the Bg6 sequencing primer, are illustrated in Fig 3, arranged from top to bottom in numerical order of sequences 1-4 as Strain BgVir, Strain T25, Strain Bernie and *B. burgdorferi* strain B31.

Fig 3. DNA sequencing electropherograms for distinguishing *B. garinii strains*. (A) Strain BgVir. (B) Strain T25. (C) Strain Bernie. (D) *B. burgdorferi* strain B31.

A reverse sequencing with Bg5 primer of the 282-bp PCR amplicon confirmed that the isolates identified as strains BgVir were not strain SL20 (Sequence ID: X85198) because there is no single-nucleotide polymorphism located near the Bg6 primer site of the amplicon which is unique for strain SL20.

Tick infection rates varied with locations

As stated above, we conducted two surveys. Survey 1 consisted of 300 ticks collected in six locations showing 15 of the 300 ticks (5%) infected with *B. burgdorferi* sensu lato of which 3 were confirmed to be *B. garinii* (2 being strain Bernie and 1 strain BgVir), 2 were *B. valaisiana* and 10 were not speciated. Survey 2 consisted of 100 ticks collected in two of the six locations previously surveyed, showing 5 of the 100 ticks (5%) infected by borrelial species, including 4

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271 isolates of *B. garinii* (3 being strain BgVir and 1 being strain T25) and 1 isolate of *B. miyamotoi*.
272 The borrelial infection rates varied from 2% to 12 % depending on the locations of the tick
273 collection. It is noteworthy that all *B. burgdorferi* sensu lato isolates in Survey 2 were confirmed
274 to be *B. garinii* as the Bg5/BG6 PCR followed by Sanger sequencing for speciation was carried
275 out without delay after initial detection by M1/M2 PCR. The final species distributions in
276 different locations were summarized in Table 1.

277 **Table 1. Incidence of *Borrelia* species in Irish *Ixodes ricinus* tick samples (Nymphs).**

Location	Sample size	No. Positive	% Positive	CI 95%	*Borrelia* species
Killarney (Kerry)	50	1	2%	(0% - 6%)	*B. valaisiana*
Killmacthomas (Waterford)	50	3	6%	(0% - 13%)	*B.b s.l*
Killmacthomas (Survey 2)	50	2	4%	(0% - 9%)	1x *B. garinii* strain T25 1x *B. garinii* strain BgVir
Portumna (Galway-South)	50	6	12%	(3% - 21%)	*B.b s.l*
Portumna (Survey 2)	50	3	6%	(0% - 13%)	1x *B. miyamotoi* 2x *B. garinii* strain BgVir
Gelendalough (Wicklow)	50	1	2%	(0% - 6%)	*B. valaisiana*
Glenveagh (Donegal)	50	3	6%	(0% - 13%)	1x *B. garinii* strain BgVir 2x *B. garinii* strain Bernie
Clifden (Galway-West)	50	1	2%	(0% - 6%)	*B.b s.l*
Total	400	20	5%	(3% - 7%)	
Of 20 infected ticks					
B. garinii			35%	(7/20)	
B. miyamotoi			5%	(1/20)	
B. valaisiana			10%	(2/20)	
Percent other *B.b s.l* not speciated			50%	(10/20)	

278 *B.b s.l = Borrelia burgdorferi sensu lato.*

279 *CI 95% = Confidence Interval for 95% probability.*

280

281 **Selection of PCR primers for 16S rRNA gene PCR and sequencing**

282 For routine metagenomic DNA sequencing diagnosis of borrelial infections, it would be ideal to
283 use one single pair of broad-spectrum PCR primers which can amplify a highly conserved
284 segment of the 16S rRNA gene of all species in the *Borrelia* genus and also can discriminate
285 between different borrelial species, and does not amplify the DNAs of other environmental
286 bacteria which may co-exist in the sample, in a nested PCR setting to prepare templates for DNA
287 sequencing. However, such an ideal specific PCR primer pair is difficult to design. We use the

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288 “genus-specific” M1/M2 primer pair to generate a PCR amplicon for Sanger reaction to detect all
289 species of the B.b.s.l. complex and the relapsing fever borreliae, in particular *B. miyamotoi*. But
290 the amplicons generated by the M1/M2 PCR primers do not contain the sequence
291 polymorphisms for discriminating between certain species in the B.b.s.l. complex and between
292 certain species of the relapsing fever borreliae. Hence, we chose a heminested PCR system to
293 amplify an adjacent 282 bp segment defined by the Bg5 and Bg6 primers which is known to
294 have single-nucleotide polymorphisms to prepare the template for further species differentiation
295 by DNA sequencing (Fig 3). However, when the Bg5/Bg6 nested PCR products were used as the
296 DNA sequencing template for confirmation of *B. miyamotoi* in tick samples, the
297 electropherograms showed numerous ambiguous base calling peaks as a result of co-
298 amplification of unwanted DNAs in the sample extract (Fig 4) whereas the sequencing
299 electropherogram generated by the M1/M2 primer PCR products from the same tick extract
300 showed no ambiguous base calling labels (Fig 2C).

301 **Fig 4. DNA sequencing of Bg5/Bg6 primer nested PCR products of *B. miyamotoi* 16S rRNA**
302 **gene showing many ambiguous bases, compared to sequencing of M1/M2 primer nested**
303 **PCR products of the same sample (see Fig 2C)**

304

305 It is well known that in using metagenomic 16S rRNA gene sequencing for target bacterial
306 identification in samples containing a mixture of bacteria, the selected target variable regions of
307 the 16S rRNA gene defined by different PCR primers have a major impact on the analysis results
308 [31, 32].

309 Degradation of 16S rRNA gene DNA in samples

310 We also observed that free borrelial 16S rRNA gene DNA in crude extracts from the ticks was
311 unstable even in TE buffer stored at -20°C for an extended period of time. For example, when
312 the NH_4OH extracts of the 50 ticks from the Portumna area were processed for M1/M2 same-
313 nested PCR screening on July 20, 2018, 3 samples were positive for borrelial 16S rRNA gene
314 DNA, with a robust 357/358 bp band on lanes 11, 15 and 25 (Fig 5) which eventually proved to
315 be *B. garinii* strain BgVir, *B. miyamotoi* and *B. garinii* BgVir, respectively. When the NH_4OH
316 extracts of the 50 ticks from the Kilmacthomas area were processed for M1/M2 same-nested
317 PCR screening on July 24, 2018, 2 samples were positive for borrelial 16S rRNA gene DNA,
318 with a robust 357/358 bp band on lanes 372 and 382 (Fig 5) which eventually proved to be *B.*
319 *garinii* strain BgVir and *B. garinii* strain T25, respectively. However, when the same-nested
320 PCR was repeated on these 5 NH_4OH extracts after 7 days and 3 days storage in a -20°C freezer,
321 respectively, the 16S rRNA gene DNA in sample 11 was no longer detectable and the intensities
322 of the nested PCR bands using the same extracts of samples 15, 25, 372 and 382 as primary PCR
323 templates for amplification under identical experimental conditions decreased markedly over a
324 period of 3-7 days, as demonstrated on the agarose gel dated July 27, 2018 (Fig 5). The image of
325 the gel electrophoresis dated July 27, 2018 also showed that nested PCR is generally required for
326 detection of borrelial infections by 16S rRNA gene analysis. Primary PCR products are usually
327 invisible after gel electrophoresis.

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Fig 5. Images of agarose gel electrophoresis. Images on July 20 and July 24, 2018 (A-C) show positive 357/358 bp borrelial 16S rRNA gene nested PCR amplicons in five positive tick samples on the day of DNA extraction (Lanes 11, 15, 25, 372 and 382). After storage of the DNA extracts at -20°C, the same DNA preparations were used to repeat primary and nested PCR on July 27, 2018 (D), showing no nested PCR products for sample 11; the amount of nested PCR products on samples 15, 25, 372 and 382 was significantly reduced. N=negative control; P= positive *Borrelia coriaceae* control. M=molecular ruler. Note numerous non-specific PCR amplifications on panel for samples 371-390.

Discussion

DNA sequencing of the 16S ribosomal RNA gene is a well-established method to compare and identify bacteria [34]. Sequence analysis of the 16S rRNA gene is a reliable method for species determination of the *B. burgdorferi* sensu lato complex and the relapsing fever borreliae [34-38].

Routine metagenomic 16S rRNA gene sequencing test for spirochetemia was initially developed for the diagnosis of human Lyme disease in the United States [22, 39] where all isolates of pathogenic *B. burgdorferi* sensu lato detected were presumed to be *B. burgdorferi* sensu stricto [23]. Subsequently, a pair of broad-spectrum genus-specific M1/M2 primers was introduced to amplify a highly conserved 357/358 bp segment of the borrelial 16S rRNA gene as the template for Sanger sequencing to include *B. miyamotoi* as the target for detection [14] after *B. miyamotoi* was found in ticks [13] and in a patient [25]. In the era of precision medicine, microbiological diagnosis of bloodstream infections, such as borrelial spirochetemia, may rely on using accurate bacterial nucleic acid analysis to establish if blood culture is not an option [40]. Base calling electropherograms generated in the automated Sanger sequencing process as those shown in Fig 2 and 3 may offer objective physical evidence for visual analysis in all nucleic acid-based diagnostic tests for infectious diseases, as proposed by some bacteriologists who are seriously concerned about the accuracies of molecular diagnosis for health care improvement [41,42].

Our study shows that *I. ricinus* ticks in Ireland are infected by a diversity of pathogenic borreliae. The degree of species and strain diversity may prove to be greater if the number of ticks being surveyed is increased. For example, in our two surveys *B. miyamotoi* and *B. garinii* strain T25 were demonstrated only during Survey 2, but not in Survey 1 (Table 1). Importantly, our results demonstrate that *Borrelia*-infected tick populations exist in the east and south-east of Ireland, areas hitherto not considered to be significantly tick-infested and hence not areas of risk to humans for contraction of Lyme borreliosis.

Historically, the only surveys of *B. burgdorferi* sensu lato in ticks collected in Ireland were carried out more than 20 years ago, using reverse line blot (RLB) for detection and reporting infection rates to be between 3.5% and 26.7% depending on the locations of tick collection at that time [43, 44]. In the current study, we used metagenomic 16S rRNA gene sequencing to survey the *I. ricinus* ticks collected from the wooded areas in six counties of Ireland and found the overall rate of borrelial infection to be 5%, with a range from 2% to 12% depending on the locations of tick collection (Table 1). Comparing our results with those published 20 years ago is

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368 difficult because there is a significant difference between the RLB and Sanger sequencing
369 technologies in DNA test accuracy. Nevertheless, we found that 6% of the nymphs collected in
370 Glenveagh were infected by two different strains of *B. garinii*, compared to 3.6% (5/141)
371 infected of the nymphs collected in Glenveagh National Park which were reported to be positive
372 for *B. valasiana* (labeled as strain VS116) 20 years earlier [44]. As DNA dot-hybridization assay
373 is prone to false positive results when no DNA sequencing confirmation is performed for
374 validation [45], it is not possible to determine whether the local strains of *B. valaisiana* in the
375 ticks in Glenveagh have been replaced by *B. garinii* over the past 20 years. As reported here,
376 with Sanger sequencing it is beyond reasonable doubt that at least 3 pathogenic borrelial species,
377 namely *B. garinii, B. valaisiana* and *B. miyamotoi*, are infecting the ticks collected in Ireland.
378 The isolates of *B. garinii* are strain BgVir strain, strain Bernie or strain T25. This is the first time
379 that *B. miyamotoi* was detected in in a tick collected in Ireland although it was previously
380 reported in *I. ricinus* ticks in Britain [46].

381 Extracellular bacterial 16S rRNA gene DNA is prone to degradation in the presence of
382 environmental substances [47], a phenomenon which was recognized when borrelial 16S rRNA
383 gene DNA in human clinical specimens was studied [48] and was also observed in the current
384 study on borrelial DNA extracts from infected ticks (Fig 5). However, this kind of DNA
385 degradation seems to be not inevitable. For example, as shown in Table 1, all of the 3 positive
386 extracts of the ticks collected from Glenveagh in Survey 1 contained adequate preserved 16S
387 rRNA gene DNA to be further speciated as *B. garinii* after initially being grouped under the *B.*
388 *burgdorferi* sensu lato complex whereas all the extracts from the ticks collected from other
389 locations lost their amplifiable 16S rRNA gene DNA under identical storage conditions. For
390 comparison, all four extracts of the ticks in Survey 2 found to be positive for *B. burgdorferi*
391 sensu lato contained adequate 16S rRNA gene DNA for speciation which confirmed that the
392 isolates initially diagnosed as *B. burgdorferi* sensu lato were in fact strains of *B. garinii* when the
393 speciation PCR was carried out within 24 hours. To avoid DNA degradation when using
394 metagenomic sequencing of borrelial 16S rRNA gene for molecular diagnosis, PCR
395 amplification should be carried out without delay after NH_4OH extraction.

396 For the purpose of patient care, it is probably not necessary to determine the species or strain of
397 the borrelia detected in the blood sample of a patient suffering from acute *B. burgdorferi* sensu
398 lato or *B. miyamotoi* infection confirmed by DNA sequencing in order to initiate timely
399 antibiotic treatment. But, for serological diagnosis a knowledge of the borrelial species and
400 strains carried by the human-biting ticks collected in the endemic areas is crucial since
401 polymorphism of ospC [9-11] and variation of the VlsE antigens among species and strains of *B.*
402 *burgdorferi* sensu lato [49] have been well documented in the literature. There is limited
403 information regarding the sensitivity of commercial tests for different species of borrelia. The
404 manufacturers of Western Blot antibody tests specify the target species. In Europe these are
405 typically *B. afzelii, B. burgdorferi* and *B. garinii*. A study of the most frequently used C6
406 synthetic peptide ELISA test for initial screening does appear to have variable sensitivity for
407 some species [50] but with no data available for many of the European species including *B.*
408 *valaisiana* identified as prevalent in Ireland in this study.

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409 In summary, our study confirms that the genus-specific M1/M2 PCR primers can amplify a
410 highly conserved segment of the borrelial 16S rRNA gene for Sanger sequencing-based
411 molecular diagnosis of tick-borne borreliae. Three species of *borrelia* were identified with *B.*
412 *garinii* the most common (60% of those speciated), followed by *B. valaisiana* (20%), and for the
413 first time in Ireland *B. miyamotoi* (10%) has been identified. *Borrelia* DNA was identified in
414 12% of the ticks collected in the Portumna area from the first survey, and in up to 6% of the ticks
415 in Glenveagh and Kilmathomas areas. Expanded surveillance of the species and strains of the
416 borreliae among human-biting ticks in Ireland is needed to ensure that the antigens used for the
417 serology tests do contain the epitopes matching the antibodies elicited by the borrelial species
418 and strains in the ticks cohabitating in the same environment.

419

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421

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435

436 References

437 1. Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH Jr. Updates on Borrelia burgdorferi sensu
438 lato complex with respect to public health. Ticks Tick Borne Dis. 2011 Sep;2(3):123-8.
439 2. Bunikis J, Tsao J, Garpmo U, Berglund J, Fish D, Barbour AG. Typing of Borrelia relapsing
440 fever group strains. Emerg Infect Dis. 2004 Sep;10(9):1661-4.
441 3. Rijpkema SG, Tazelaar DJ, Molkenboer MJ, Noordhoek GT, Plantinga G, Schouls LM,
442 Schellekens JF. Detection of Borrelia afzelii, Borrelia burgdorferi sensu stricto, Borrelia garinii

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443 and group VS116 by PCR in skin biopsies of patients with erythema migrans and acrodermatitis
444 chronica atrophicans. Clin Microbiol Infect. 1997 Feb;3(1):109-116.
445 4. Rudenko, N. et al., 2009. Molecular detection of Borrelia bissettii DNA in serum samples from
446 patients in the Czech Republic with suspected borreliosis. FEMS microbiology letters, 292(2),
447 pp.274–81
448 5. Wang G, Liveris D, Mukherjee P, Jungnick S, Margos G, Schwartz I. Molecular Typing of
449 Borrelia burgdorferi. Curr Protoc Microbiol. 2014 Aug 1;34:12C.5.1-31
450 6. Ivanova, L.B. et al., 2014. Borrelia chilensis, a new member of the Borrelia burgdorferi sensu lato
451 complex that extends the range of this genospecies in the Southern Hemisphere. Environmental
452 microbiology, 16(4), pp.1069–80
453 7. Crowder CD, Carolan HE, Rounds MA, Honig V, Mothes B, Haag H, Nolte O, Luft BJ,
454 Grubhoffer L, Ecker DJ, Schutzer SE, Eshoo MW. Prevalence of Borrelia miyamotoi in Ixodes
455 ticks in Europe and the United States. Emerg Infect Dis. 2014 Oct;20(10):1678-82.
456 8. Steere AC, Strle F. 2016 Dec 15;2:16090, Wormser GP, Hu LT, Branda JA, Hovius JW, Li X,
457 Mead PS. Lyme borreliosis. Nat Rev Dis Primers. 2016 Dec 15;2:16090.
458 9. Barbour AG, Restrepo BI. Antigenic variation in vector-borne pathogens. Emerg Infect Dis. 2000
459 Sep-Oct;6(5):449-57. Review.
460 10. Theisen M, Frederiksen B, Lebech AM, Vuust J, Hansen K. Polymorphism in ospC gene of
461 Borrelia burgdorferi and immunoreactivity of OspC protein: implications for taxonomy and for
462 use of OspC protein as a diagnostic antigen. J Clin Microbiol. 1993;31:2570-6.
463 11. Wang IN, Dykhuizen DE, Qiu W, Dunn JJ, Bosler EM, Luft BJ. Genetic diversity of ospC in a
464 local population of Borrelia burgdorferi sensu stricto. Genetics. 1999 Jan;151(1):15-30.
465 12. Han S, Hickling GJ, Tsao JI. High Prevalence of Borrelia miyamotoi among Adult Blacklegged
466 Ticks from White-Tailed Deer. Emerg Infect Dis. 2016 Feb;22(2):316-8.
467 13. Barbour AG, Bunikis J, Travinsky B, Hoen AG, Diuk-Wasser MA, Fish D, Tsao JI. Niche
468 partitioning of Borrelia burgdorferi and Borrelia miyamotoi in the same tick vector and
469 mammalian reservoir species. Am J Trop Med Hyg. 2009 Dec;81(6):1120-31.
470 14. Lee SH, Vigliotti JS, Vigliotti VS, Jones W, Moorcroft TA, Lantsman K. DNA sequencing
471 diagnosis of off-season spirochetemia with low bacterial density in Borrelia burgdorferi and
472 Borrelia miyamotoi infections. Int J Mol Sci. 2014 Jun 25;15(7):11364-86.
473 15. Prescott CD, Dahlberg AE. A single base change at 726 in 16S rRNA radically alters the pattern
474 of proteins synthesized in vivo. EMBO J. 1990 Jan;9(1):289-94.
475 16. Prescott CD, Göringer HU. A single mutation in 16S rRNA that affects mRNA binding and
476 translation-termination. Nucleic Acids Res. 1990 Sep 25;18(18):5381-6.
477 17. Shi X, Chiu K, Ghosh S, Joseph S. Bases in 16S rRNA important for subunit association, tRNA
478 binding, and translocation. Biochemistry. 2009 Jul 28;48(29):6772-82.
479 18. Nessar R, Reyrat JM, Murray A, Gicquel B. Genetic analysis of new 16S rRNA mutations
480 conferring aminoglycoside resistance in Mycobacterium abscessus. J Antimicrob Chemother.
481 2011 Aug;66(8):1719-24.
482 19. Watanabe S, Matsumura K, Iwai H, Funatogawa K, Haishima Y, Fukui C, Okumura K, Kato-
483 Miyazawa M, Hashimoto M, Teramoto K, Kirikae F, Miyoshi-Akiyama T, Kirikae T. A Mutation
484 in the 16S rRNA Decoding Region Attenuates the Virulence of Mycobacterium tuberculosis.
485 Infect Immun. 2016 Jul 21;84(8):2264-2273.
486 20. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic
487 laboratory: pluses, perils, and pitfalls. J Clin Microbiol. 2007 Sep;45(9):2761-4.

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488 21. Norris SJ, Barbour AG, Fish D, Diuk-Wasser MA. Response to Esteve-Gassent et al.: flaB
489 sequences obtained from Texas PCR products are identical to the positive control strain Borrelia
490 burgdorferi B31. Parasit Vectors. 2015 Jun 9;8:310.
491 22. Lee SH, Vigliotti VS, Vigliotti JS, Jones W, Pappu S. Increased sensitivity and specificity of
492 Borrelia burgdorferi 16S ribosomal DNA detection. Am J Clin Pathol. 2010 Apr;133(4):569-76.
493 23. Feder HM Jr, Johnson BJ, O'Connell S, Shapiro ED, Steere AC, Wormser GP; Ad Hoc
494 International Lyme Disease Group, Agger WA, Artsob H, Auwaerter P, Dumler JS, Bakken JS,
495 Bockenstedt LK, Green J, Dattwyler RJ, Munoz J, Nadelman RB, Schwartz I, Draper T,
496 McSweegan E, Halperin JJ, Klempner MS, Krause PJ, Mead P, Morshed M, Porwancher R,
497 Radolf JD, Smith RP Jr, Sood S, Weinstein A, Wong SJ, Zemel L. A critical appraisal of "chronic
498 Lyme disease". N Engl J Med. 2007 Oct 4;357(14):1422-30.
499 24. Marconi RT Garon CF . Development of polymerase chain reaction primer sets for diagnosis of
500 Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA
501 signature nucleotide analysis. J Clin Microbiol . 1992;30:2830–2834.
502 25. Gugliotta JL, Goethert HK, Berardi VP, Telford SR 3rd. Meningoencephalitis from Borrelia
503 miyamotoi in an immunocompromised patient. N Engl J Med. 2013 Jan 17;368(3):240-5.
504 26. Schutzer SE, Body BA, Boyle J, Branson BM, Dattwyler RJ, Fikrig E, Gerald NJ, Gomes-Solecki
505 M, Kintrup M, Ledizet M, Levin AE, Lewinski M, Liotta LA, Marques A, Mead PS, Mongodin
506 EF, Pillai S, Rao P, Robinson WH, Roth KM, Schriefer ME, Slezak T, Snyder JL, Steere AC,
507 Witkowski J, Wong SJ, Branda JA. Direct Diagnostic Tests for Lyme Disease. Clin Infect Dis.
508 2018 Oct 11. doi: 10.1093/cid/ciy614. [Epub ahead of print]
509 27. Way JS, Josephson KL, Pillai SD, Abbaszadegan M, Gerba CP, Pepper IL. Specific detection of
510 Salmonella spp. by multiplex polymerase chain reaction. Appl Environ Microbiol. 1993
511 May;59(5):1473-9.
512 28. Abbaszadegan M, Huber MS, Gerba CP, Pepper IL. Detection of enteroviruses in groundwater
513 with the polymerase chain reaction. Appl Environ Microbiol. 1993 May;59(5):1318-24.
514 29. Bej AK, Steffan RJ, DiCesare J, Haff L, Atlas RM. Detection of coliform bacteria in water by
515 polymerase chain reaction and gene probes. Appl Environ Microbiol. 1990 Feb;56(2):307-14.
516 30. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of lyme borreliosis. Clin
517 Microbiol Rev. 2005 Jul;18(3):484-509. Review.
518 31. Rintala A, Pietilä S, Munukka E, Eerola E, Pursiheimo JP, Laiho A, Pekkala S, Huovinen P. Gut
519 Microbiota Analysis Results Are Highly Dependent on the 16S rRNA Gene Target Region,
520 Whereas the Impact of DNA Extraction Is Minor. J Biomol Tech. 2017 Apr;28(1):19-30.
521 32. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. Evaluation of
522 general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-
523 based diversity studies. Nucleic Acids Res. 2013 Jan 7;41(1):e1.
524 33. CDC. MicrobeNet. https://www.cdc.gov/microbenet/about.html
525 34. Fukunaga M, Hamase A, Okada K, Nakao M. Borrelia tanukii sp. nov. and Borrelia turdae sp.
526 nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted
527 PCR analysis. Microbiol Immunol. 1996;40(11):877-81.
528 35. Le Fleche A, Postic D, Girardet K, Peter O, Baranton G. Characterization of Borrelia lusitaniae
529 sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol. 1997 Oct;47(4):921-5.
530 36. Marconi RT, Garon CF. Identification of a third genomic group of Borrelia burgdorferi through
531 signature nucleotide analysis and 16S rRNA sequence determination. J Gen Microbiol. 1992
532 Mar;138(3):533-6.

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533 37. Wang G1, van Dam AP, Le Fleche A, Postic D, Peter O, Baranton G, de Boer R, Spanjaard L,
534 Dankert J. Genetic and phenotypic analysis of Borrelia valaisiana sp. nov. (Borrelia genomic
535 groups VS116 and M19). Int J Syst Bacteriol. 1997 Oct;47(4):926-32.
536 38. Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, Maleev VV, Fish D,
537 Krause PJ. Humans infected with relapsing fever spirochete Borrelia miyamotoi, Russia. Emerg
538 Infect Dis. 2011 Oct;17(10):1816-23.
539 39. Lee SH, Vigliotti VS, Vigliotti JS, Jones W, Williams J, Walshon J. Early Lyme disease with
540 spirochetemia - diagnosed by DNA sequencing. BMC Res Notes. 2010 Nov 1;3:273.
541 40. Rello J, van Engelen TSR, Alp E, Calandra T, Cattoir V, Kern WV, Netea MG, Nseir S, Opal
542 SM, van de Veerdonk FL, Wilcox MH, Wiersinga WJ. Towards precision medicine in sepsis: a
543 position paper from the European Society of Clinical Microbiology and Infectious Diseases. Clin
544 Microbiol Infect. 2018 Mar 24. pii: S1198-743X(18)30221-0. doi: 10.1016/j.cmi.2018.03.011.
545 [Epub ahead of print]
546 41. Hakovirta JR, Prezioso S, Hodge D, Pillai SP, Weigel LM. Identification and Analysis of
547 Informative Single Nucleotide Polymorphisms in 16S rRNA Gene Sequences of the Bacillus
548 cereus Group. J Clin Microbiol. 2016 Nov;54(11):2749-2756. Epub 2016 Aug 31.
549 42. Peacock S. Health care: Bring microbial sequencing to hospitals. Nature. 2014 May
550 29;509(7502):557-9.
551 43. Kirstein F, Rijpkema S, Molkenboer M, Gray JS. Local variations in the distribution and
552 prevalence of Borrelia burgdorferi sensu lato genomospecies in Ixodes ricinus ticks. Appl
553 Environ Microbiol. 1997 Mar;63(3):1102-6.
554 44. Kirstein F, Rijpkema S, Molkenboer M, Gray JS. The distribution and prevalence of B.
555 burgdorferi genomospecies in Ixodes ricinus ticks in Ireland. Eur J Epidemiol. 1997;13:67–72.
556 45. Diegutis PS, Keirnan E, Burnett L, Nightingale BN, Cossart YE. False-positive results with
557 hepatitis B virus DNA dot-hybridization in hepatitis B surface antigen-negative specimens. J Clin
558 Microbiol. 1986 Apr;23(4):797-9.
559 46. Hansford KM, Fonville M, Jahfari S, Sprong H, Medlock JM. Borrelia miyamotoi in host-seeking
560 Ixodes ricinus ticks in England. Epidemiol Infect. 2015 Apr;143(5):1079-87. doi:
561 10.1017/S0950268814001691. Epub 2014 Jul 14.
562 47. Corinaldesi C, Danovaro R, Dell'Anno A. Simultaneous recovery of extracellular and
563 intracellular DNA suitable for molecular studies from marine sediments. Appl Environ Microbiol.
564 2005 Jan;71(1):46-50.
565 48. Persing DH, Rutledge BJ, Rys PN, Podzorski DS, Mitchell PD, Reed KD, Liu B, Fikrig E,
566 Malawista SE. Target imbalance: disparity of Borrelia burgdorferi genetic material in synovial
567 fluid from Lyme arthritis patients. J Infect Dis. 1994 Mar;169(3):668-72.
568 49. Wang D, Botkin DJ, Norris SJ. Characterization of the vls antigenic variation loci of the Lyme
569 disease spirochaetes Borrelia garinii Ip90 and Borrelia afzelii ACAI. Mol Microbiol. 2003
570 Mar;47(5):1407-1
571 50. Tjernberg I, Sillanpää H, Seppälä I, Eliasson I, Forsberg P, Lahdenne P. Antibody responses to
572 borrelia IR6 peptide variants and the C6 peptide in Swedish patients with erythema migrans. Int J
573 Med Microbiol. 2009;299: 439–446

574

575

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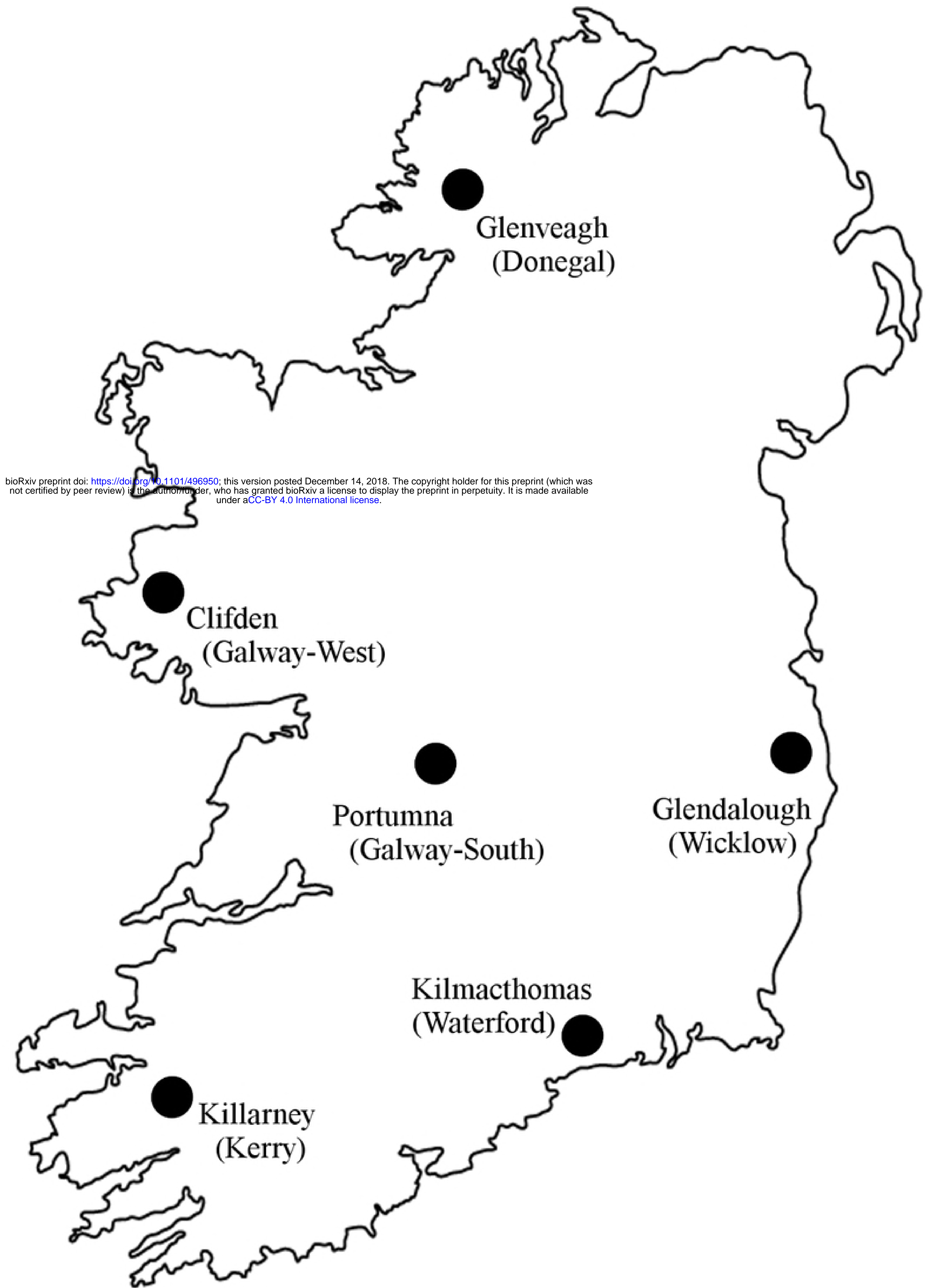
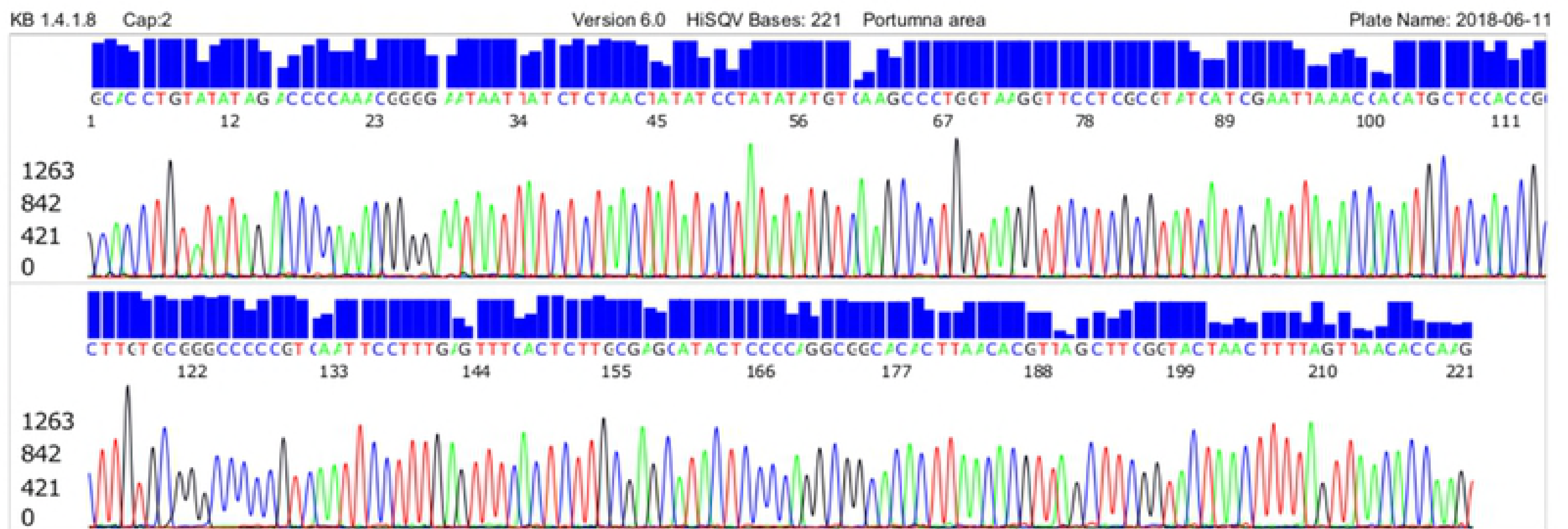


Fig 1

A



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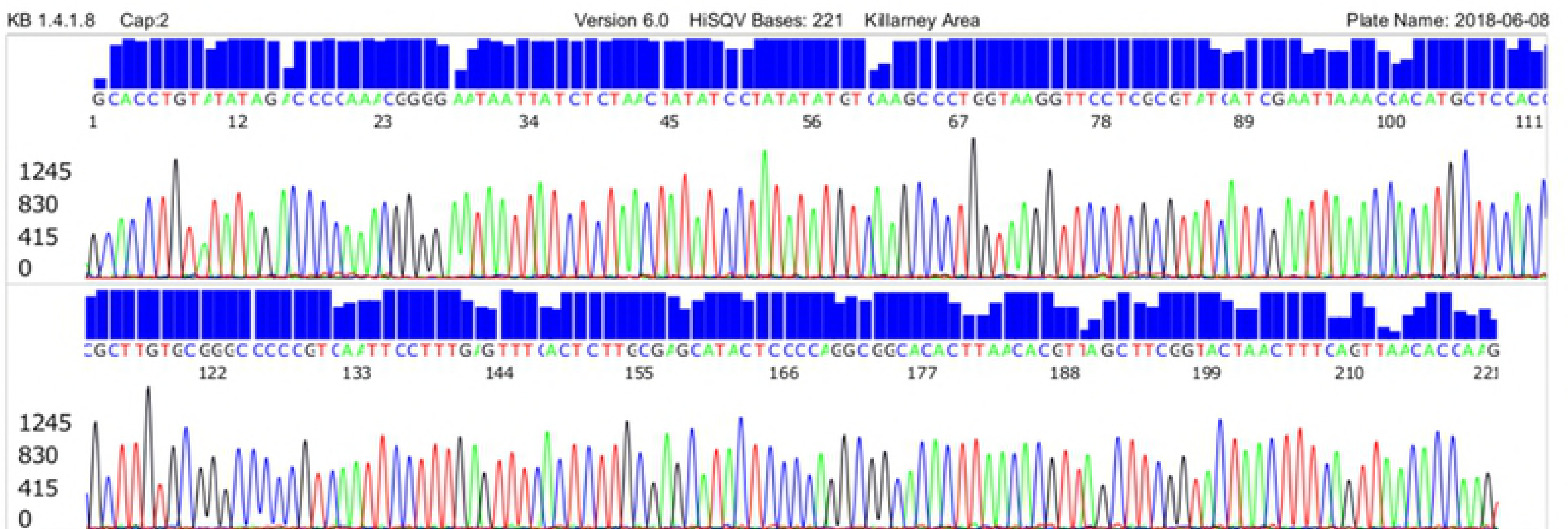
B



C

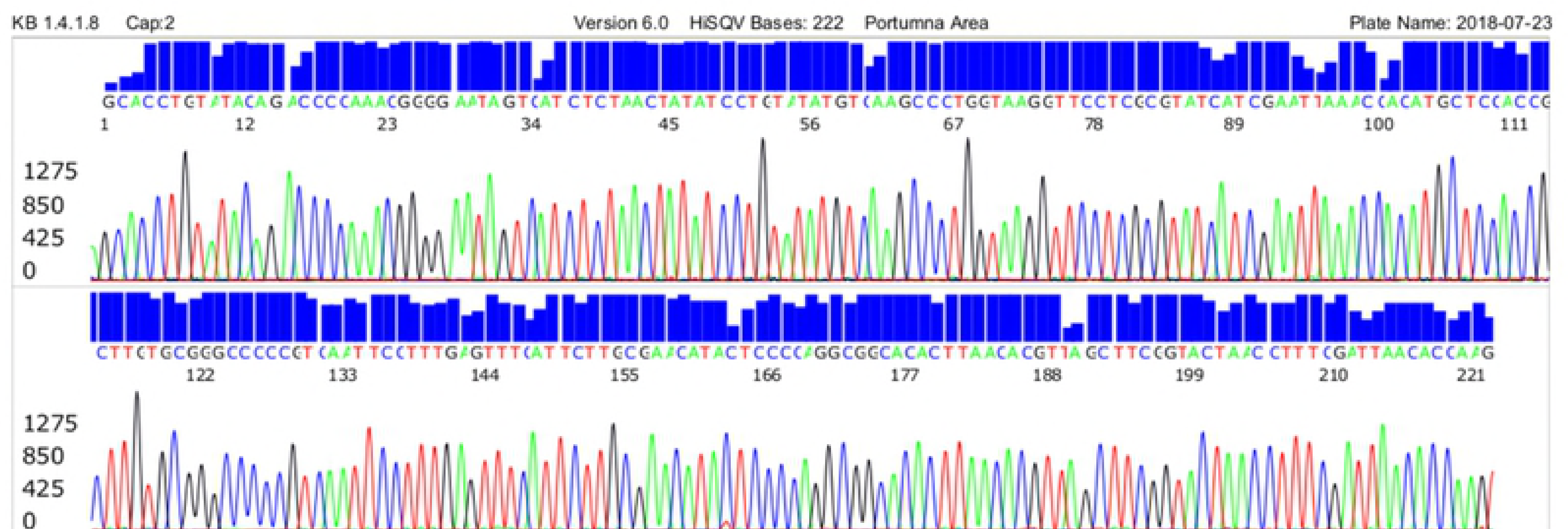
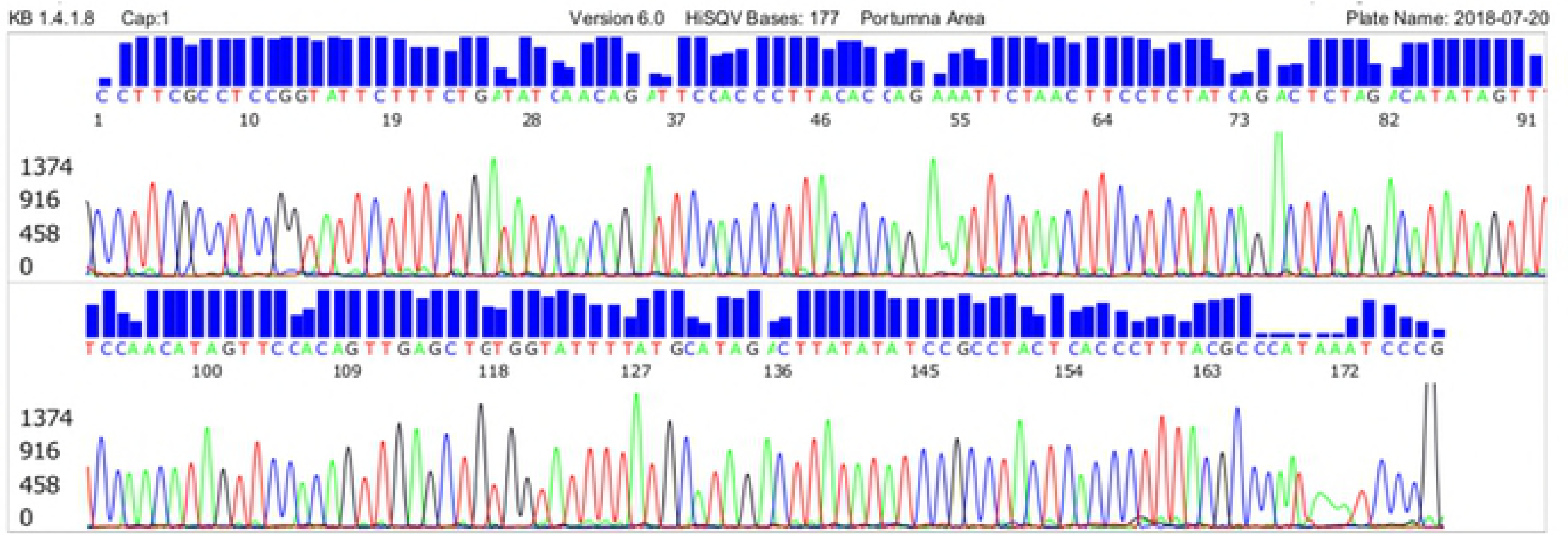
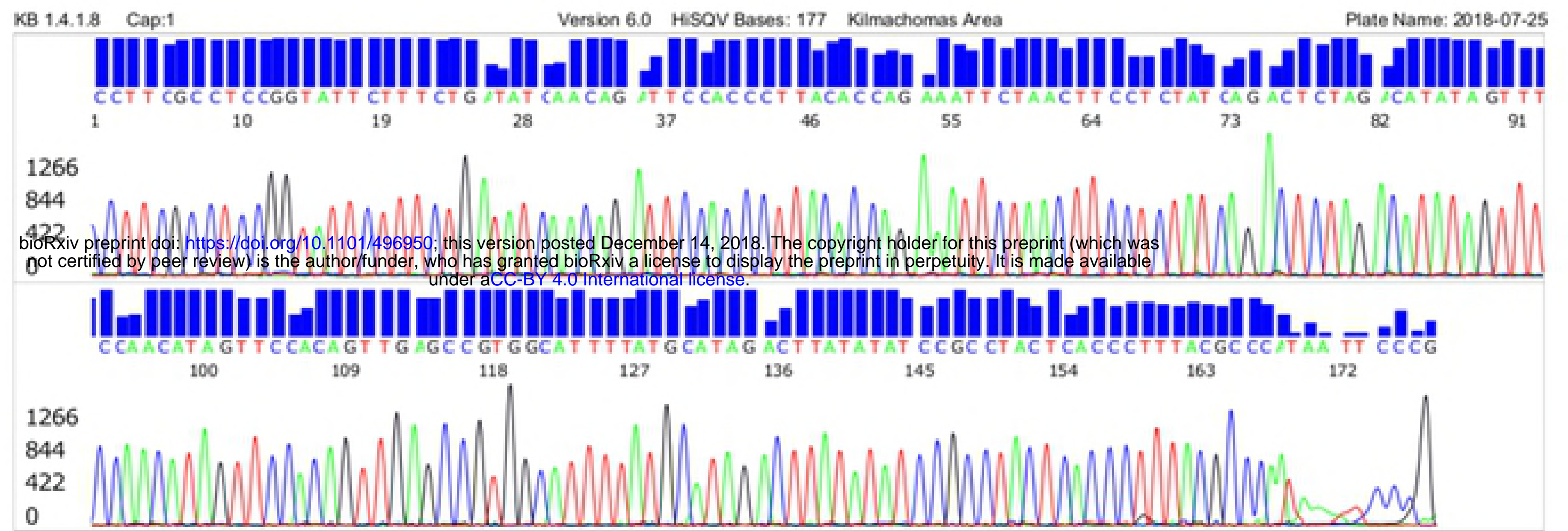


Fig 2

A



B



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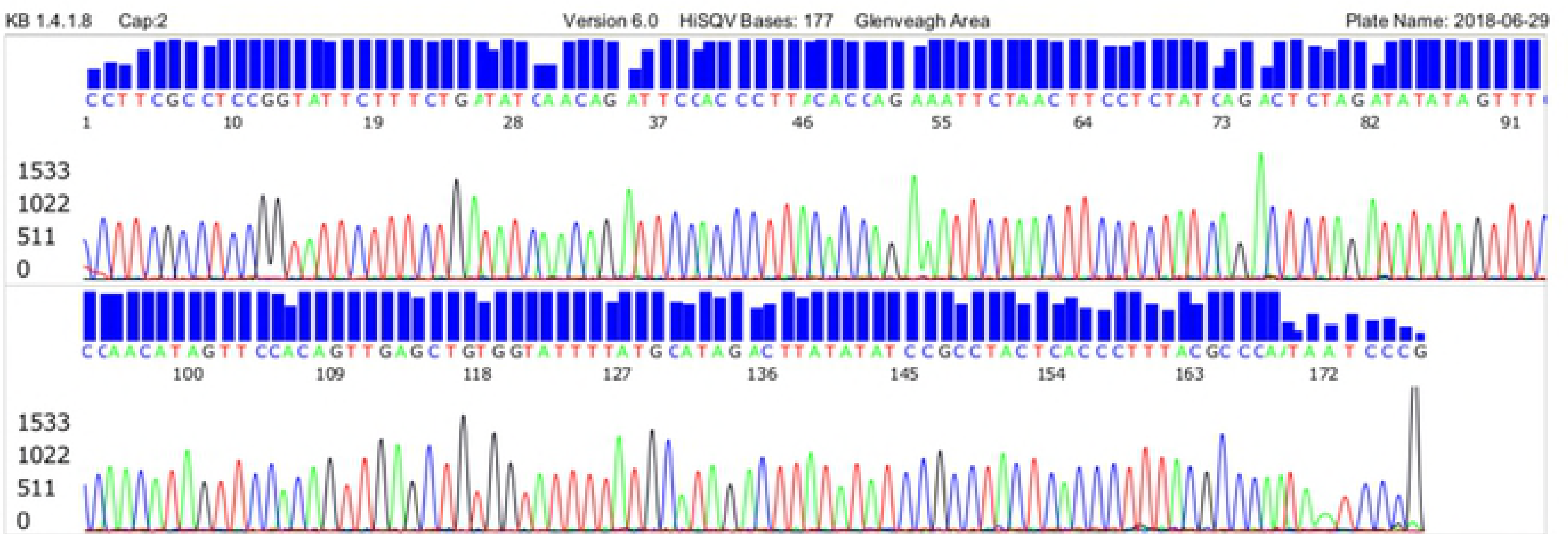
C



D

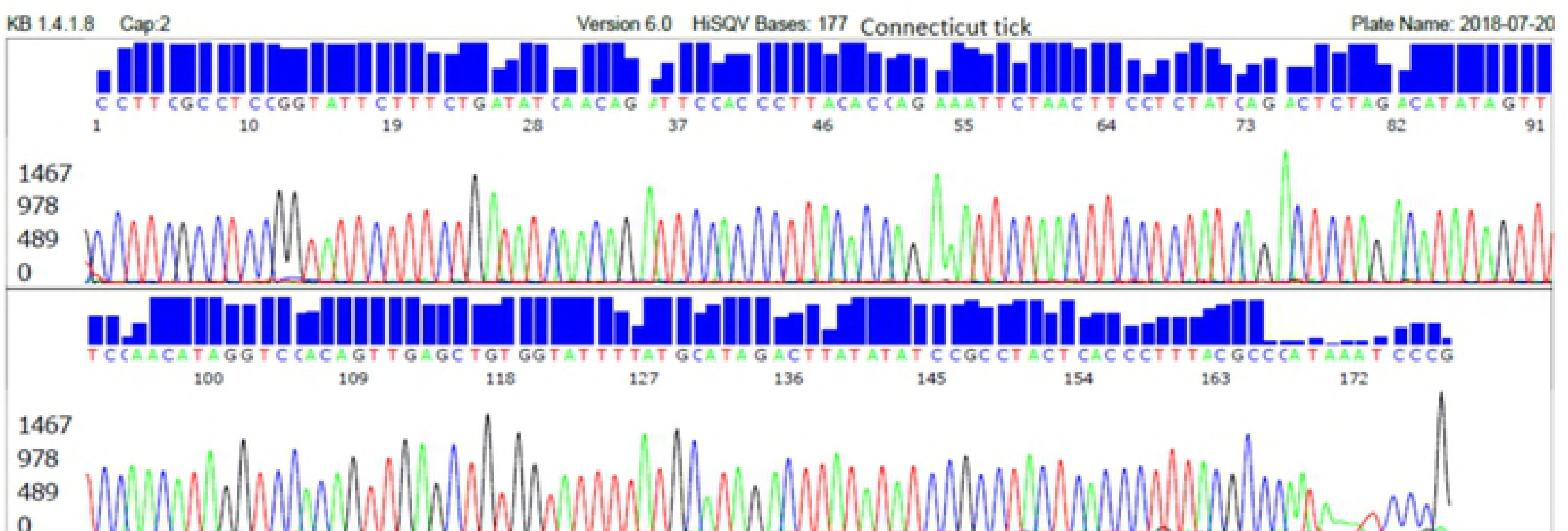


Fig 3

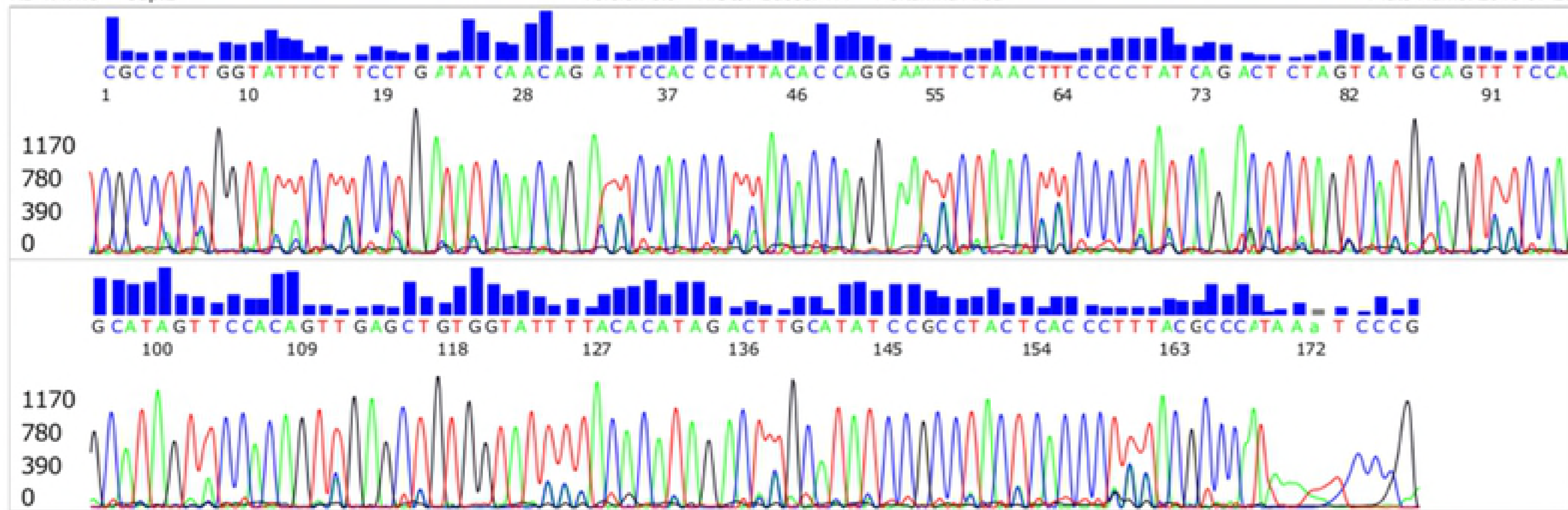


Fig 4

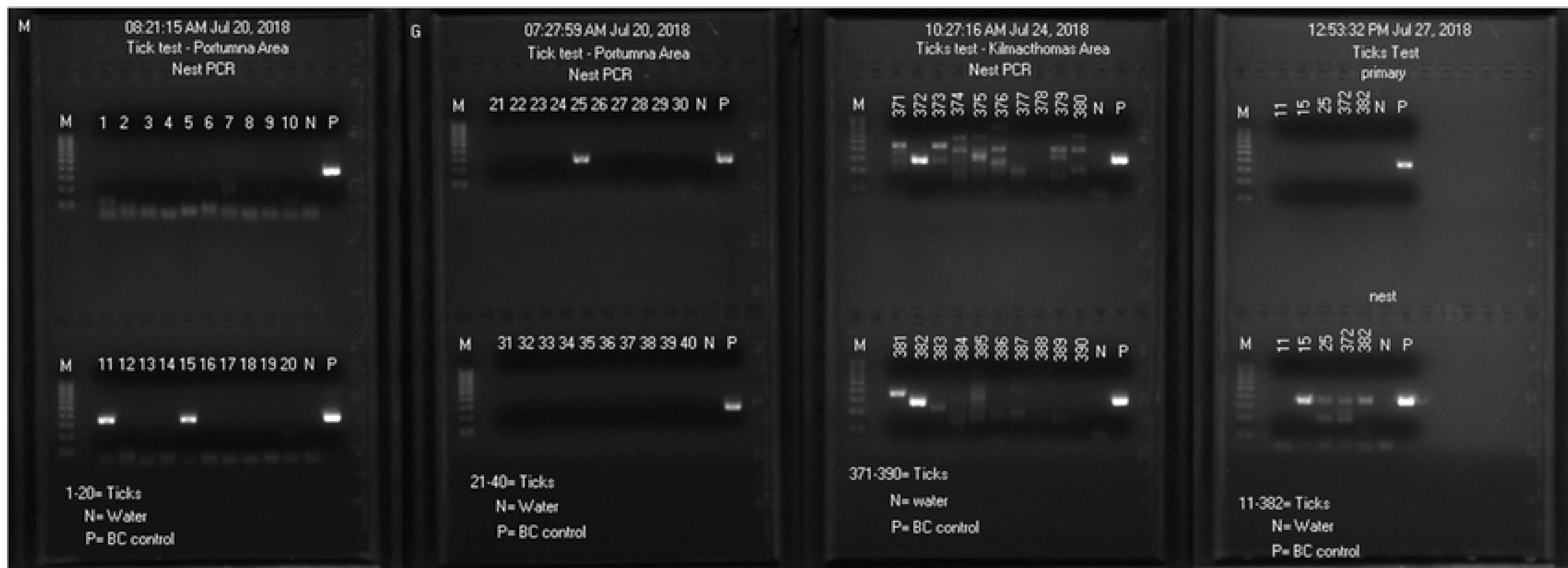


Fig 5