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Spatio-temporal and risk factor analysis of alleles related to Scrapie resistance in sheep in Great Britain before, during and after a national breeding program

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Abstract

Certain genotypes of sheep have been identified to increase their susceptibility (the VRQ allele) or resistance (the ARR allele) to classical scrapie. This study aim was to assess the spatio-temporal pattern of the ARR and VRQ alleles in GB and to explore the risk factors associated to their presence.

Data was collected from the GB scrapie surveillance program, the sheep and goat inventory survey (GB census survey) and the agricultural survey for the period 2002-2015. Spatio-temporal trends of genotypes were assessed through the use of choropleth maps, spatial cluster and linear regression analyses. Multivariable mix-effect logistic regression analyses were performed to investigate the association between the resistant or susceptible genotypes, and breeds, farm purpose, animal purpose, surveillance stream, country location and herd size.

The results show a significant upward trend in the frequency of most resistant ARR alleles (1.15% per year, 95%CI: 0.76-1.53) and significant downward trend of most susceptible VRQ alleles (-0.40% per year; 95%CI: -0.69 to -0.10]. The trend continue after the termination of the national scrapie control plan in 2009. Breeds such as Herdwick (OR=0,27; 95%CI: 0.15-0.48), Shetland (OR=0.21; 95%CI: 0.12-0.37), Swaledale (OR=0.56; 95%CI: 0.45-0.71), Scottish blackface (OR=0.53; 95%CI: 0.41-0.70) and Welsh Montain (OR: 0.61; 95%CI: 0.45-0.81) were identified with lower odds ratios of having the resistant ARR allele, while Beulah speckled face (OR=1.73; 95%CI: 1.14-2.61), Jacob (OR=2.98; 95%CI: 1.46-6.53), Lleyn (OR=2.91; 95%CI: 1.27-6.66) and Suffolk (OR=2.15; 95%CI: 1.66-2.78) had higher odds ratios of having the ARR allele. Other risk factors associated to presence of ARR allele were finishing farms (OR=1.18; 95%CI:1.06-1.32) and farms in Scotland (OR=0,78; 95%CI: 0.74-0.84) and in Lowland grazing areas (OR=1.52; 95%CI: 1.39-1.67). Risk factors associated with presence the VRQ genotype were farms in Scotland (OR=0,85; 95%CI: 0.77-0.93) and breeds such as Herdwick (OR=2.05; 95%CI: 1.02-4.14), Shetland (OR=4.29; 95%CI: 2.31-7.28) and Sweledale (OR=1.53; 95%CI: 1.11-2.10). For the most resistant genotype, two significant spatial clusters were identified: a high-risk cluster in the south-west of GB (RR=1.51, p<0.001) and a low-risk cluster in northern GB (RR=0.65, p<0.001). For the most susceptible genotypes, one significant high-risk cluster was identified in Wales (RR = 2.89 and p=0.013).

Surveillance for classical scrapie could be improved with a risk-based approach by focussing on those areas and farm types identified to have higher frequency of VRQ alleles and less frequency of ARR alleles. Scrapie control strategies could focus on developing breeding programs on farms with Shetland, Herdwick and Swaledale breeds.

Keywords: Scrapie; PrP genotype; Risk factor; Sheep; Surveillance; cluster analysis

45 **1. Introduction**

46 Long-term national occurrence of scrapie can be reduced by selection of sheep carrying the resistant PrP
47 gene (ARR - encode alanin at codon 136 and arginin at codon 154 and 171) and removal of susceptible genes
48 (VRQ – encode valine at codon 136, arginine at codon 154 and glutamine at condon 171) (Moum et al. 2005;
49 Goldmann, 2008; Fast and Groschup, 2013). Under this axiom, the implementation of classical scrapie (CS)
50 eradication programmes for sheep in European countries proved that it is possible to significantly reduce the
51 prevalence of this disease using polymorphisms of prion protein gene (PrP) approaches in conjunction with
52 herd cull protocols (EFSA, 2014). Similar programs were implemented in several non-EU countries, including
53 Canada and the United States of America (APHIS, 2018, Scrapie Canada, 2018). Commission Regulation (EC)
54 2245/2003, which is an amendment of the (EC) 999/2001 regulation, requires that, in addition to each
55 positive transmissible spongiform encephalopathy (TSE) case in sheep, the prion protein genotype shall be
56 determined for a random subsample of those ovine animals tested negative under active surveillance. In
57 Great Britain (GB), since the adult sheep population accounts for more than 750,000 animals, the active
58 surveillance program requires a total of 20,000 sheep samples to be tested each year, with minimum sample
59 for genotyping of at least 600 animals. The primary objective of genotyping is to estimate the prevalence of
60 the most resistant and most susceptible genes in the national sheep flock.

61 Recent data for the prevalence of scrapie have shown that the number of CS cases in sheep is consistently
62 falling in GB and other EU Member States (EFSA, 2014). The NSP in GB was implemented in July 2001 in a
63 huge effort to change the dominating genotypes of the national herd with the most scrapie resistant
64 genotypes for breeding, and decrease the frequency of the most susceptible animals (Ortiz-Pelaez et al.,
65 2014). This program ended in March 2009. In this regard, sheep with the ARR allele have a significantly
66 reduced risk of developing scrapie compared with other genotypes, while presence of the VRQ allele
67 increases greatly the risk (Hunter et al., 1992, 1994; Belt et al., 1995; Hunter, 1997; Elsen et al., 1999; Baylis
68 et al., 2004a:2004b). Probably, the NSP in GB has been the largest genotyping programme for animal disease
69 control ever implemented in the world (Ortiz-Pelaez et al., 2014), with roughly 3 million sheep from 90
70 different breeds genotyped (Dawson et al., 2008) and with costs of approximately £86 million per year (Ortiz-
71 Pelaez et al., 2014). As a result, CS incidence has decreased by over 90% since 2002 (with incidence of up to

72 0.25% in the fallen stock survey) (Ortiz-Pelaez et al., 2014), and only two cases of CS were detected in the
73 fallen stock survey (incidence <0.01%) between 2013 and 2016 (EFSA 2016). However, since the selection
74 for scrapie resistant genotypes was amde voluntary (in the GB since 2009), active scrapie surveillance has
75 become the main means of controlling and eradicating CS in sheep populations (EFSA, 2014). In GB, farms
76 with positive cases are also monitored for a period of two years through testing of their fallen stock and their
77 sheep at slaughter, and no depopulation is normally required, with few exceptions. However, a CS eradication
78 policy relying solely on current surveillance programs is unlikely to succeed (EFSA, 2014) and there is a risk
79 of a future increase in CS, unless the sensitivity of the surveillance program for detection of CS is improved.
80 It has been argued that "control and prevention of scrapie is beyond reach when knowledge about the
81 pathways of transmission is absent" (Adams, 2016). This is also hindered by the inefficacy of current
82 treatments for the decontamination and disinfection of farms following scrapie outbreaks (Acin, 2015;
83 Hawkins et al., 2015; Gough et al., 2017). Furthermore, it is known that the scrapie prion can persist in the
84 environment for several years (3 to +16 years) (Miller et al., 2004; Georgsson et al., 2006; Genovesi et al.,
85 2007; Wiggins, 2009; Smith et al., 2011). Under this scenario, and given the risk of developing CS again
86 through contamination from the environment, the main threat is considered to be a population level
87 decrease in the ARR allele frequency and a re-emergence of the VRQ allele in the national flock (Ortiz-Pelaez
88 et al., 2014). The minimum ARR allele frequency below which within-flock infection cannot be sustained is
89 uncertain and may depend on the prevalence of local risk factors, such as breed, flock-type heterogeneity,
90 management systems and trading practices (Melchior et al., 2010). Considering that the objective of the TSE
91 surveillance program is to detect and eradicate CS, a continuous adjustment process of this program is
92 needed to increase the detection of cases, especially with the current low incidence levels. Therefore,
93 additional knowledge is required of the spatio-temporal distribution of the resistant and susceptible allele
94 frequencies in the population, together with identification of farm-level factors associated with these alleles,
95 in order to develop and apply further risk-based surveillance strategies.
96 The aim of this study was twofold: firstly, to visualize and explore the spatio-temporal patterns and assess
97 clustering of the resistant (ARR) and most susceptible (VRQ) PRNP alleles in sheep in GB since commissioning

98 the NSP (from 2002 till 2015); and secondly, to identify farm-level factors associated with genotypes in order
99 to assist in the development of targeted risk-based scrapie surveillance programs.
100
101 **2. Materials and methods**
102 **2.1. Data source**
103 Historical data, from 2002 until 2015, were extracted from three national databases: (1) the Scrapie
104 Surveillance database, (2) the annual Agricultural Survey, and (3) the annual Sheep and Goat Inventory
105 survey. The datasets were merged using the county (administrative area) parish holding (CPH) reference
106 which is a unique identification number for each farm in GB.
107 **2.1.1. Scrapie surveillance data**
108 The structure of the scrapie surveillance program in GB is summarized in Figure 1. Passive surveillance (not
109 explored in this study), provides testing of all sheep with clinical suspicion of scrapie (via the scrapie
110 Notification Database; SND). Active surveillance is completed using three different routes: fallen stock survey
111 (FSS), abattoir survey (AS) and dead in transit survey (DTS). With an adult population of over 750,000 sheep
112 in the GB, active surveillance requires sampling of 20,000 sheep per year, and at least 600 animals per year
113 need to be genotyped. However, a derogation in Annex III, Chapter II, paragraph C of the EU TSE Regulation
114 permits Member States to replace up to 50% of their requirement for sheep tested for human consumption
115 with the same number of fallen stock sheep. Animals sampled for active surveillance are selected from 20 to
116 27 fallen stock site per year (number varied per year) and about 14 abattoirs. Only abattoir slaughtering more
117 than 40,000 sheep per year were ask to participate in the surveillance. The selection of abattoirs and fallen
118 stock site was done based on their geographical distribution. From each site, samples are chosen randomly
119 (process not specified), but provided that sheep are over 18 month age, an eartag is present and with the
120 condition not to select more than two animals from the same holdings. The selection of the negative samples
121 for genotyping is also based on a stratification method. Few samples per fallen stock site and abattoir site
122 are selected each month for genotyping (per year 300 samples per each surveillance route are selected).
123 Apart from this, only samples with good quality conditions for testing are selected. In addition, all scrapie
124 positive cases are genotyped. In the years 2002, 2003 and 2012, a large number of sheep (>10,000) were

125 genotyped as part of the NSP and research study (Ortiz-Pelaez et al., 2014). The samples collected were the
126 obex region of the brainstem and the Cerebellum. In the fallen stock survey, the staff from the disposal sites
127 collected the samples after receiving training from Animal and Plant Health Agency (APHA). In abattoirs,
128 samples are collected by trained staff from the Food Standard Agency.

129 Genotyping of the prion protein gene were done for the codons 136, 141, 154 and 171. Genomic DNA for
130 ovine PrP 4 codon genotyping was extracted using a DNeasy Blood & Tissue Kit (Qiagen). To obtain nucleotide
131 sequences for PRNP, 904-bp amplifications including exon 3 ORF were obtained through Polymerase Chain
132 Reaction (PCR). PCR assays were performed in a 25 µL reaction volume containing 1 µL genomic DNA, 0.6 µM
133 of each primer (G30 (5'-catttgatgctgacaccctcttta -3') and G16 (5'-atgagacaccaccactacagggct-3')), and a PCR
134 Master Mix, 2X (Promega) containing: 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction
135 buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2. Amplifications were
136 performed starting with one cycle of 10 min at 95°C, and then followed by 40 cycles of 20 seconds at 95°C,
137 30 seconds at 60°C and 3 minutes at 72°C. A final extension step of 10 min at 72°C was done. PCR Products
138 were checked through electrophoresis on 2% agarose gels containing GelRed Nucleic Acid Gel Stain,10,000x
139 in water. PCR products were then purified using Agencourt AMPure XP (BeckmanCoulter) using a Biomek
140 NXP Laboratory Automation Workstation (Beckman Coulter). Both strands of the PCR products were
141 sequenced by using a forward and reverse primer SWF3 (5'-gtaagccaaaaaccaacatgaagc-3') and SWR6.2 (5'-
142 tcgctccattatcttgatgtcagttt-3'). Sequencing was performed by using BigDye Terminator v3.1 reagents (Applied
143 Biosystems). Cycle-sequencing reactions were undertaken by using thermal-cycler conditions of an initial
144 denaturation at 96 °C for 60 seconds, followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and
145 60 °C for 4 minutes. Prior to loading on an ABI 3130 or ABI 3730 genetic analyser, the sequencing product
146 was purified by using a CleanSEQ kit (Agencourt). The sequence data obtained were compared with the PrP
147 ORF of Ovis aries (sheep) (GenBank accession no. AY350267; Seabury and Derr (2003)) by using SeqScape
148 software v2.5 (Applied Biosystems) to identify DNA polymorphisms. However, for genotyping of samples
149 from 2012, the protocol detailed in Ortiz-Pelaez et al. (2014) was used.

150 All genotypes were grouped in five categories, following the classification completed in the NSP. These were:
151 type 1 (ARR/ARR), type 2 (ARR/AHQ, ARR/ARH, ARR/ARQ), type 3 (AHQ/AHQ, AHQ/ARH, AHQ/ARQ,
152 ARH/ARH, ARH/ARQ, ARQ/ARQ), type 4 (ARR/VRQ) and type 5 (AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ).
153 They established decreasing levels of resistance to CS, with type 1 or genotype ARR/ARR being the most
154 resistant and type 5 or genotypes with VRQ alleles and non-ARR alleles being the most susceptible. Linear
155 regression analysis was carried out to evaluate the trends on the frequency of each group genotype (outcome
156 variable) over the year (exposure variable). For this analysis, data for the year 2005 was excluded due the low
157 number of observations for that year.
158 To assess the spatial distribution and farm-level risk factors, two binary outcome variables were created. The
159 first variable indicates the presence of the resistant allele ARR, and therefore includes homozygous or
160 heterozygous combinations of ARR allele, except the ARR/VRQ genotype (grouped NSP types 1 and 2). The
161 other outcome variable indicates the presence of the most susceptible genotype VRQ, including homozygous
162 or heterozygous combinations of VRQ allele (corresponding with NSP type 4 and 5).
163 For each sample, data was also collected on farm CPH, the country and the year of sampling. Breed
164 information was also collected, but only for sample from the fallen stock survey. Breed information was
165 recorded at the sites through previously trained staff working on the fallen stock site.

166 **2.1.2. Agricultural Survey**

167 The annual agricultural survey provides data on farm purpose (less favoured area (LFA) grazing, lowland
168 grazing, dairy and others) This survey is conducted in June of each year and covers 80% of the farming
169 population in GB (DEFRA, 2017). Data for each year for the period 2002-2015 was used, and farm type was
170 then matched for each animal in the scrapie surveillance system by CPH and year. When matching by year
171 was not possible, if the CPH was present in future or past years in the agricultural survey, the farm
172 type for that CPH in those years was then used.

173 **2.1.3. Sheep and Goat Annual Inventory**

174 The annual inventory is a census survey and a legal requirement that provides estimates of the number of
175 sheep and goats (herd size), together with the geographical coordinates for the location of the holdings,
176 animal purpose (finishing, breeding, grazing, stores, dealer, wool, pet and other) and production type (dairy,

177 meat, wool and others). Data for the period 2002-2015 was collected were match to the sheep genotype
178 dataset by CPH and year, when possible.

179 **2.2. Statistical and spatial analysis**

180 **2.2.1. Spatial analysis**

181 The annual distribution of most resistant and most susceptible alleles were calculated spatially in each
182 administrative area. A choropleth map was generated in ArcMap 10.2.2 (ESRI, USA) showing the frequency
183 of both allele groups for each administrative area. Spatial clusters in GB of NSP genotype group 1 (ARR/ARR)
184 were investigated using Kulldorf's spatial scan statistic implemented using the SaTScan software (version
185 9.4.4) in order to identify potential areas for breeding and replacement of resistant genotypes. In addition,
186 spatial clusters in GB of the NSP genotype group 5 (VRQ/non-ARR) and group 4 (VRQ/ARR) were also
187 investigated in order to identify areas to target for eradication/surveillance schemes. Aggregated data for
188 the period 2012-2015 were used to allow sufficient sample numbers for the identification of possible and
189 current genotype clusters. For all genotype groups, 50% percent of the population must be at risk for a cluster
190 to be detected.

191 **2.2.2. Farm-level risk factor analyses**

192 Two binary outcomes were created to investigate farm level factors associated with presence of ARR allele
193 (outcome 1) and presence of the VRQ (outcome 2) allele. Risk factors considered were: year, breed, herd
194 size, animal purpose, farm purpose, testing stream (fallen stock, abattoir and death in transit), production
195 type and country. For breed, all minor breed present in less than 20 animals in the dataset were removed
196 from the analysis.

197 A univariable analysis was done using multilevel mix-effect logistic regression to identify significant
198 independent associations between the outcome and all predictor variables, and with the variable year
199 included as a fixed effect and the farm id as a random effect. For this, all farms with CPH or flock mark with
200 missing values were assumed to be new farms. All variables significant ($p<0.10$) at the univariable level were
201 then included in a multivariable mix-effect logistic regression model to assess the risk factors associated with
202 the two outcomes. For each outcome, three models were developed: 1) Model with breed included, 2) Model
203 without breed and without farm purpose, and 3) Model without breed and without country. The reason were

204 that data on breed of animals were only collected in the fallen stock survey, reducing dramatically the
205 number of observations in the multivariable models. Data on farm purpose was only collected in England,
206 and therefore a model without country was needed to explore the possible association with this variable. For
207 the multivariable models, a backward stepwise process was completed to retain those variables not
208 significant at the 0.05 level. Models were run using Stata 12 (StataCorp, 2011. College Station, Texas, USA)
209 and the command 'melogit'. To assess goodness of fit of the models, model predictions (probability of having
210 an allele) were compared with actual outcomes. For this, model predictions were transformed into binary
211 variable using 0.5 as the cut-off probability to classify a sheep into positive (having the allele) or negative (not
212 having the allele).

213 **3. Results**

214 **3.1. Descriptive statistics**

215 From 2002 to 2015, a total of 435,159 sheep were TSE tested after the launch of the NSP. Under the active
216 surveillance program, most frequently collected samples were for the abattoir survey (49.0%), followed by
217 the fallen stock survey (41.0%) and death in transit survey (1.0%). The numbers of animals tested over time
218 are shown in Table 1. A change in the trend in the sampling streams was observed over time, going from over
219 90% animals tested through abattoirs in 2002-2003 to over 73% of sheep tested through the fallen stock
220 survey in 2015.

221 A total of 65,666 sheep were genotyped, consisting of 54.3% from the abattoir survey, 42.6% from the fallen
222 stock survey and 3.1% from the death in transit survey (Table 1). These originated from 18,590 different
223 farms (an average of 2.68 samples per farm). However, for 16,944 (25.8%) sheep data on CPH or flock mark
224 was missing. Breed information was obtained for 7,430 (11.3%) of sheep genotyped. A total of 48,194 (73.4%)
225 sheep were matched to the Sheep and Goat inventory dataset. Only 18,795 (28.6%) sheep were matched to
226 the agricultural survey.

227 Type 2 genotypes were the most frequent (63.6%), followed in decreasing order by NSP type 3 (25.2%), type
228 1 (21.5%), type 5 (6.0%) and type 4 (5.0%) (Figure 2). On average, the ARR allele accounted for 63.7%
229 (n=42,133), while the VRQ allele accounted for 11.1% (n=7,312). Significant trends in the frequency of the
230 NSP genotype categories were evident across all the types, except for type 2 (Figure 2). Significant frequency

increase over time was observed for type 1 (b=1.15 95% CI [0.76 to 1.53], R^2=0.79, p<0.001). Significant frequency decrease over time was observed for type 3 (b=-0.72 95% CI [-1.01 to -0.43], R^2=0.73, p<0.01), type 4 (b=-0.24 95% CI [-0.37 to -0.12], R^2=0.62, p<0.001) and type 5 (b=-0.40 95% CI [-0.69 to -0.10], R^2=0.44, p<0.05). It is important to note that the results observed for year 2005 must be considered anecdotal since only 78 animals were genotyped (Table 1). The total number of CS cases from 2002 to 2015 were 53 (0.1%) out of 45,106 sheep with the ARR allele and 253 (3.5%) out of the 7,263 sheep having the VRQ allele. The total number of Atypical scrapie cases were 181 (0.4%) for sheep with the ARR allele and 2 cases (0.03%) for sheep with the VRQ allele.

3.2. Spatial Analysis

3.2.1. Choropleth Maps

The results indicate that the spatial distribution of the most resistant allele (ARR) has increased over time (Figure 3). While in 2002 and 2003, few counties had a proportion of sheep with ARR allele larger than 31%, during the following years this genotype dominated the national population. Conversely, in the first years of the TSE active surveillance program, several counties presented a higher proportion (>10%) of the VRQ allele (Figure 4). However, for the period 2010-2015 after the NSP, the proportion of this allele has decreased in most counties.

3.2.2. Cluster Analysis

For the most resistant genotype (ARR/ARR), two significant clusters were identified through spatial cluster analysis (p < 0.001, Figure 5-A); one high- and one low-risk cluster. The high-risk cluster of ARR/ARR occurrence was in south-west GB (RR=1.51, p<0.001) and the low-risk cluster for ARR/ARR occurrence covered the entirety of northern GB (RR=0.65, p<0.001). For the most susceptible genotypes (VRQ and non-ARR) only one significant high-risk cluster was identified in Wales (RR = 2.89 and p=0.013, Figure 5-B); farms in this region were almost three times as likely to have animals with the susceptible genotypes VRQ and non-ARR, than farms outside Wales .

3.3. Univariable risk factor analyses

Findings from the univariable multilevel mix-effect logistic regression models are summarised in Tables A and B in the supplementary material. Six variables (year, testing route, country, breed, animal purpose and farm purpose) were independently associated with presence of ARR allele, while for presence of VRQ allele four potential risk factors were identified (year, testing route, country and breed). Examining the relationship between both alleles and year of sampling, there was a significantly increased odds from 2008 until 2015 for the most scrapie resistant genotypes, while the odds ratios across time for most scrapie susceptible genotypes have progressively decreased up to 2015.

3.4. Multivariate risk factor analyses

The final multivariable models that accounted for breed resulted in country and breed being associated with the presence of resistant or susceptible genotypes. Country was significant for both alleles, with Wales demonstrating a higher odds of having the resistant ARR allele and Scotland a lower odds of having the susceptible VRQ allele (Table 4). Several breeds were associated with higher odds of having the resistant ARR allele. These were, in decreasing order, Jacob, Lleyn, Suffolk, Beulah speckled face and Suffolk crosses. Breeds associated with lower odds of having the resistant ARR allele were, in increasing order, Shetland, Herdwick, Scottish Blackface, Swaledale and Welsh Mountain (Table 4). Breeds associated with higher odds of having the susceptible VRQ allele were, in decreasing order, Shetland, Border Leicester, Herdwick, Dorset Horn&Poll and Swaledale. Breeds associated with lower odds of having the VRQ allele were, in increasing order, Suffolk and Suffolk cross (Table 4). However, predictions from the multivariable models using VRQ outcome did not seem to predict well which sheep had the allele.

The final multivariable models that did not accounted for breed showed that higher odds of having the ARR allele were found in lowland grazing farms and in farms classified as 'finishing', 'grazing', 'pet' and 'wool'. Lower odds of having ARR where found in farms located in Scotland and in farms with more than 250 sheep. Wales was no longer found as a significant risk factor associated to higher odds of ARR allele. Model predictions were more accurate in models without breed. Lower odds of having VRQ allele was found in Scotland and in farms classified as 'wool' producers, but with model predictions being unable to provide accurate estimates.

4. Discussion

283 The implementation of CS eradication programs for sheep in European countries have demonstrated that it
284 is possible to reduce the prevalence of this disease using PrP genetic approaches in conjunction with herd
285 cull protocols (EFSA, 2014). Recent data for the prevalence of scrapie have shown that the number of CS
286 cases in sheep is consistently falling in GB and other EU Member States (EFSA, 2014). However, at EU level
287 no decreasing trend has been observed (EFSA, 2016). To determine the long-term impacts of the breeding
288 strategies for scrapie implemented in GB, all the available epidemiological data since the beginning of the
289 NSP were used. The NSP was launched in 2001 as a voluntary programme until July 2004 where it became
290 mandatory for all flocks with confirmed cases from that date (Boden et al., 2012), as required by EC
291 Regulations (EC2001/999, EC, 2003a, EC, 2003b). The programme ended in 2009. Results of this study show
292 clearly that over the 14-year study period (2002 to 2015), the impact of the implementation of statutory
293 eradication measures and the use of genetic breeding programs has had a significant effect on the increase
294 in ARR allele frequency and decrease of VRQ allele frequency in the sheep population. These results agree
295 with previous studies (Tongue et al., 2008; Arnold and Ortiz-Pelaez, 2014). Whereas the odds of the
296 occurrence of the ARR allele increased to double of the initial frequency, the VRQ allele was reduced to half.
297 Furthermore, results show that while a higher proportion of VRQ alleles appeared in the vast majority of the
298 counties in the earliest years of the NSP, this CS susceptible allele seems to have disappeared in many
299 counties in the most recent years. It is important to highlight the high number of animals genotyped in 2012
300 and 2013. Specifically, a chi-square test showed that there is a significant upward trend for occurrence of
301 allele ARR (61.5% to 77.2%) and downward trend for allele VRQ (12.0% to 5.7%) between 2002-2003 and
302 2014-2015 (additional file 1), in agreement with observations in other European countries where the actual
303 frequency of the ARR allele is around 80% (the Netherlands: 78.5%, Hagenaars et al., 2010 and Belgium:
304 79.3%, Dobly et al., 2013). In GB, a previous study compared the period between 2002–2003 and 2012–2013,
305 and reported an absolute increase of 9% in the frequency of the ARR allele from 43.3% to 52.3% (Ortiz-Pelaez
306 et al., 2014). This value reaches an absolute increase of 15.7% in 2015 in comparison with 2002-2003 and
307 indicates, that despite the termination of the NSP six years ago, the trends have continued. A possible
308 explanation would be that many flock owners in GB are still replacing their sheep from farms where the ARR
309 allele is known to be present in order to prevent future classical scrapie cases.

310 It has been described that while the occurrence of the ARR allele decreases significantly the number of CS
311 cases, the presence of VRQ alleles increases significantly among the CS cases (Ortiz-Pelaez and Bianchini,
312 2011; Ortiz-Pelaez et al., 2014). In this context, over this 14-years study period, only 0.1% of positive CS was
313 detected in animals with ARR allele. The allele ARR is known to confer resistance to all strains of CS, although
314 the genetic resistance of homozygous ARR genotype is not absolute (Groschup et al., 2007). The results of
315 this study, together with the culling of scrapie flocks, would explain the low scrapie incidence trend in the
316 last four years in GB, as it has been suggested for other national breeding programs (Hagenaars et al., 2010).
317 In GB, a 90% reduction in the prevalence of CS between 2005 and 2012 has been reported (Ortiz-Pelaez et
318 al., 2014), and only 2 cases have been detected through active surveillance in 2013-2015 despite 77,510
319 tested (EFSA 2016). The fact that changes in genotype was targeted towards infected farm in the NSP might
320 explain, to some extent, the large decrease in incidence of CS compare to the moderate change in frequency
321 of PRNP genotypes in the population.
322 Several studies have examined risk factors associated with an increased risk of occurrence of CS. These relates
323 to PRNP genotype frequency, flock size, seasonality, soil drainage, breed and geographical region (Baylis et
324 al., 2000; Hoinville et al., 2000; Sivam et al., 2003, Del Rio Vilas et al., 2006; McIntyre et al., 2006:2008;
325 Tongue et al., 2006; Green et al., 2007; Gubbins, 2008; Stevens et al., 2009) (McLean 1999; Gubbins, 2003;
326 Del Rio Vilas et al., 2005; McIntyre et al., 2006:2010; Dobly et al., 2013); the purchase of female from scrapie-
327 infected flocks, purchase of replacement sheep from markets and the spreading of sheep compost on land
328 (Healy et al., 2004); the use of concentrates and milk replacements (Philippe et al., 2005), and the ratio of
329 iron-to-manganese in forage grown on scrapie-affected farms (Gudmundsdottir et al., 2006). Our model
330 showed that breed and country were associated with the most resistant and most susceptible alleles. It is
331 has been reported that there is considerable variation in the distribution of alleles between breeds and that
332 clearly some breeds have allele frequency associated with a higher or lower risk for CS (Eglin et al., 2005; del
333 Rio Vilas et al., 2006; McIntyre et al., 2006; Melchior et al., 2010; Hautaniemi et al., 2012; Dobly et al., 2013).
334 Our model findings on breed as risk factor are in line with findings from a previous study (Eglin et al., 2005).
335 Of particular interest are the breeds such as Shetland, Herdwick and Swaledale identified in this study as
336 having high odds of having the susceptible VRQ allele, and at the same time presenting very low odds of

337 having the resistant ARR allele. Surprisingly, according to the GB farm animal genetic resources breed
338 inventory, the population of Swaledale and Herdwick breeding females has fallen steeply between 2002 to
339 2017 (78% and 82%, respectively), with an actual male population estimated at approximately 2,585 and 480
340 for Swaledale and Herdwick, respectively (Defra, 2017). It is unknown to what extent this reduction can be
341 attributed to the NSP breeding programme. At the present time, the largest female breeding population is
342 the Swaledale breed. These results suggest that future scrapie control programs (surveillance and breeding
343 strategies) could be breed-specific. However, the large number of British sheep breeds and production types
344 compared with other European countries could make these breed-specific scrapie control programs more
345 difficult to implement.
346 There have been previous attempts to compare the surveillance performance of the two active surveillance
347 sources among EU countries (Bird et al., 2003; Del Rio Vilas et al., 2007). The present study shows, in the
348 univariable analysis, that active survey detects higher frequency of the VRQ allele relative to the Fallen stock
349 survey, while this route is able to detect higher number of sheep with the ARR allele than the abattoir survey.
350 Despite fallen stock survey being a higher risk-based source of classical scrapie (SSC, 2001; Del Rio Vilas et al.,
351 2005:2008), the current results suggest that the abattoir survey is potentially covering areas with farms with
352 increased presence of susceptible genotype. Similarly, breeding farms and those farms located in less
353 favoured areas were found to have lower odds of having the ARR allele. Given that the better predictions
354 where obtained from the multivariable models without breed, the risk factors identified in those models
355 could be prioritized for improving the sensitivity of the scrapie surveillance system. Therefore, the
356 surveillance could be modified to target the breeding farms, those in less favored areas and those specialized
357 in wool production; and reduce surveillance intensity in Scotland. Nonetheless, breed remains an important
358 factor to consider given the fact that some breeds have at the same time high odds of having the VRQ allele
359 and very low odds of having the ARR allele, and viceversa.
360 It is know that in GB some geographical regions have been associated with an increased risk of occurrence of
361 CS (Tongue et al., 2006). A more recent study, based on data from 2001 to 2005, shows that the distribution
362 of cases of CS in GB exhibits a definite spatial pattern (Stevens et al., 2009). Specifically, South and central
363 Wales were identified as areas with a generally higher occurrence of the disease than the rest of GB.

Although our multivariate model associates Wales with higher odds of the most resistant ARR allele and Scotland with lower odds of the most susceptible VRQ alleles, the spatial scan statistic identified one significant cluster in Wales, in line with a previous study based on the risk of being confirmed positive for scrapie (Stevens et al., 2009).

This study contains some important biases and limitations. Sheep geographical source is determined in the fallen stock survey by the farm address where the sheep were collected and in the abattoir survey by the flock mark, which indicates farm where animal was born. Hence, if animals are moved to different farms, this information is not captured. Coverage of the current surveillance system is dependent on the location and through-put of participating abattoirs and fallen stock sampling sites. Therefore, some counties in GB may have a lower representation than others. In addition, large numbers of samples were genotyped in 2002-2003 and 2012 with a specific project in order to determine the PRNP genotype distribution of the sheep population and to assess the impact of the NSP (Ortiz-Pelaez et al., 2014). These large number of animals tested would have an important influence on the farm-level risk factors observed. Nevertheless, the aggregation of year data allowed the analysis to have sufficient power to detect trends and risk factors. Taken together, this study provides further knowledge about the prevalence and geographic distribution of ARR and VRQ alleles over the last 14 years, specifically during and after the ending of the NSP. The increase in the frequency of the ARR allele was evident, but most importantly is that the trend continues, with a frequency of 77.2% estimated in 2016. Taken together, these results indicate that the selective breeding programme that promotes the resistant ARR allele is still maintained despite the ending of the programme in 2009.

This study shows spatial differences in GB, with a higher density of the ARR allele in the southern regions of England and Wales, but a higher density of VRQ alleles in Wales. Nevertheless, breed seems to be the most important factor affecting allele distribution. Although GB surveillance is already balanced to try and ensure geographical coverage, this study suggests that sensitivity of scrapie surveillance could be further improved by developing a risk-based approach focussing on genotype with more samples submitted from those areas and farms identified to have higher frequency of VRQ genotypes and less frequency of ARR genotypes. Of particular concern is Swaledale sheep, which despite a significant reduction in the total population between

2002 and 2017, are still the largest female breeding population with a high frequency of VRQ allele together with a low frequency of ARR allele.

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Table 1. Summary of the number of samples tested and the frequency distribution of active scrapie surveillance program and genotyping between 2002 and 2015 in sheep population in Great Britain.

Summary	Testing route	Year													
		2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Surveillance	Data numbers	33,657	80,988	16,941	21,506	75,370	45,472	24,175	22,107	18,228	19,040	19,538	19,482	19,287	19,368
	Abattoir (%)	97.0	93.9	67.8	54.1	65.4	57.3	45.2	49.6	43.7	36.3	32.9	35.4	35.3	25.7
	Fallen stock (%)	3.0	5.4	29.4	42.8	34.2	40.7	51.2	47.4	56.3	62.9	66.1	62.9	63.9	73.4
	Dead in transit (%)	0.0	0.7	2.8	3.1	1.2	1.9	3.6	0.0	0.8	1.0	1.7	0.9	0.9	1.0
Genotyping	Data numbers	30,095	20,333	558	85	515	402	734	599	587	699	8,639	1,192	607	626
	Abattoir (%)	97.70	93.83	44.95	38.46	55.93	17.38	0.96	52.51	55.29	48.64	67.37	81.48	50.00	50.32
	Fallen stock (%)	2.27	5.81	51.38	57.69	40.32	69.77	97.27	35.45	49.10	51.20	31.80	13.19	49.83	49.68
	Dead in transit (%)	0.03	0.36	3.67	3.85	3.75	12.85	1.78	12.04	0.00	0.16	0.83	3.70	0.17	0.00

Table 4. Multivariate mix-effect logistic regression model showing risk factors associated with "most resistant" (homozygous or heterozygous combinations of ARR allele and excluding VRQ allele) and "most susceptible" (homozygous or heterozygous combinations of VRQ allele) to classical scrapie including breed as exposure variable. Year has been included as fixed effect and farm as random effect.

Risk factor		Most resistant n = 5,552			Most susceptible n = 5,852		
		Odds ratio	**95% CI**	**p-value**	**Odds ratio**	**95% CI**	**p-value**
Year		1.04	1.01-1.06	0.002	0.97	0.94-1.01	0.170
Country	England	Ref.			Ref.		
	Scotland	1.10	0.91-1.32	0.326	0.71	0.53-0.95	0.021
	Wales	1.28	1.04-1.59	0.022	0.97	0.72-1.33	0.867
Breed	Mule	Ref.			Ref.		
	Beulah speckled face	1.73	1.14-2.61	0.009	0.63	0.33-1.21	0.170
	Bluefaced Leicester	1.53	0.90-2.60	0.116	0.81	0.35-1.88	0.627
	Border Leicester	1.12	0.55-2.32	0.750	2.47	1.02-5.97	0.045
	Charollais	1.65	0.86-3.18	0.132	0.74	0.30-1.81	0.503
	Cheviot	0.77	0.57-1.04	0.088	1.47	0.94-2.31	0.093
	Dorset Horn & Poll	1.17	0.83-1.64	0.368	1.59	1.03-2.44	0.036
	English Leicester	0.93	0.55-1.58	0.798	1.79	0.85-3.75	0.125
	Herdwick	0.27	0.15-0.48	0.000	2.05	1.02-4.14	0.045
	Jacob	2.98	1.36-6.53	0.006	1.00*		
	Lleyn	2.91	1.27-6.66	0.012	0.15	0.02-1.12	0.064
	Scottish Blackface	0.53	0.41-0.70	0.000	0.79	0.50-1.25	0.310
	Shetland	0.21	0.12-0.37	0.000	4.29	2.31-7.98	0.000
	Suffolk	2.15	1.66-2.78	0.000	0.43	0.27-0.66	0.000
	Suffolk cross	1.54	1.25-1.91	0.000	0.72	0.52-1.00	0.051
	Swaledale	0.56	0.45-0.71	0.000	1.53	1.11-2.10	0.009
	Texel	0.79	0.62-1.01	0.057	0.68	0.45-1.04	0.073
	Texel cross	0.95	0.74-1.24	0.738	1.22	0.84-1.79	0.300
	Welsh Mountain	0.61	0.45-0.81	0.001	1.37	0.90-2.08	0.143
Animal purpose	Breeding	Ref.					
	Dealer	1.04	0.82-1.31	0.756			
	Finishing	1.03	0.85-1.25	0.746			
	Grazing	1.24	0.97-1.59	0.084			
	Pet	2.25	1.12-4.52	0.022			
	Producer/processor	0.81	0.25-2.59	0.725			
	Stores	1.04	0.83-1.21	0.729			
	Wool	0.67	0.48-0.93	0.018			
Random effect – Farm cons		0.10	0.03-0.41		0.53	0.25-1.09	
Model prediction (prob>=0.5%)		79.2% of non-ARR sheep 95.0% of ARR sheep Chi-square p<0.001				100% of non-VRQ sheep 0% of VRQ sheep	

Ref.:Reference ; **CI**: Confidence interval; * lack of convergence

Table 5. Multivariate mix-effect logistic regression model showing risk factor associated with "most resistant" genotypes (homozygous or heterozygous combinations of ARR allele and excluding VRQ allele) and "most susceptible" (homozygous or heterozygous combinations of VRQ allele) genotypes to classical scrapie excluding breed as an exposure variable. Year has been included as fixed effect and farm was include as a random effect.

Model including country and excluding farm purpose

Risk factor		Most resistant n = 46,766			Most susceptible n = 48,264		
		Odds ratio	**95% CI**	**p-value**	**Odds ratio**	**95% CI**	**p-value**
Year		1.06	1.05-1.06	0.000	0.93	0.92-0.94	0.001
Country	England	Ref.			Ref.		
	Scotland	0.78	0.74-0.84	0.000	0.85	0.77-0.93	0.001
	Wales	1.00	0.95-1.06	0.976	0.98	0.91-1.06	0.678
Animal purpose	Breeding	Ref.			Ref.		
	Dealer	1.11	1.01-1.23	0.029	0.95	0.83-1.10	0.510
	Finishing	1.15	1.07-1.25	0.000	0.99	0.89-1.11	0.892
	Grazing	1.16	1.05-1.27	0.002	0.93	0.82-1.06	0.280
	Pet	1.39	1.08-1.78	0.010	0.87	0.61-1.24	0.438
	Producer/processor	0.77	0.46-1.27	0.298	1.33	0.66-2.65	0.417
	Stores	1.13	1.02-1.24	0.014	0.89	0.77-1.02	0.090
	Wool	1.33	1.18-1.49	0.000	0.79	0.67-0.95	0.010
Herd size	<250	Ref.					
	250-1000	0.94	0.89-0.99	0.028			
	>1000	0.87	0.81-0.93	0.000			
Random effect – Farm cons		0.29	0.25-0.34		0.37	0.30-0.46	
Model prediction (prob>=0.5%)		91.5% of non-ARR sheep 98.5% of ARR sheep Chi-square p<0.001				100% of non-VRQ sheep 0% of VRQ sheep	

Model excluding country and including farm purpose

		Most resistant n = 17,766			Most susceptible[1]		
		Odds ratio	**95% CI**	**p-value**	**Odds ratio**	**95% CI**	**p-value**
Year		1.07	1.06-1.08	0.000			
Animal purpose	Breeding	Ref.					
	Dealer	1.09	0.96-1.24	0.163			
	Finishing	1.18	1.06-1.32	0.003			
	Grazing	1.38	1.10-1.72	0.004			
	Pet	1.29	0.83-2.00	0.252			
	Producer/processor	0.52	0.29-0.92	0.025			
	Stores	1.11	0.98-1.27	0.105			
	Wool	1.47	1.18-1.83	0.001			
Farm purpose	LFA grazing	Ref.					
	Lowland grazing	1.52	1.39-1.67	0.000			
	Dairy	1.29	1.07-1.55	0.008			
	Other	1.51	1.37-1.68	0.000			
Random effect – Farm cons		0.33	0.26-0.42				
Model prediction (prob>=0.5%)		88.1% of non-ARR sheep 98.5% of ARR sheep Chi-square p<0.001					

Ref.:Reference ; **CI**: Confidence interval; * lack of convergence, [1]Farm purpose non-significant, hence model not considered

Figure 1. Description of the structure of the scrapie surveillance program in Great Britain (Abbreviations: IMC = Immunochemistry; MWB = Modified Western Blot; CSFS = Classical Scrapie Flock Scheme; ASM = Atypical Scrapie Monitoring scheme)

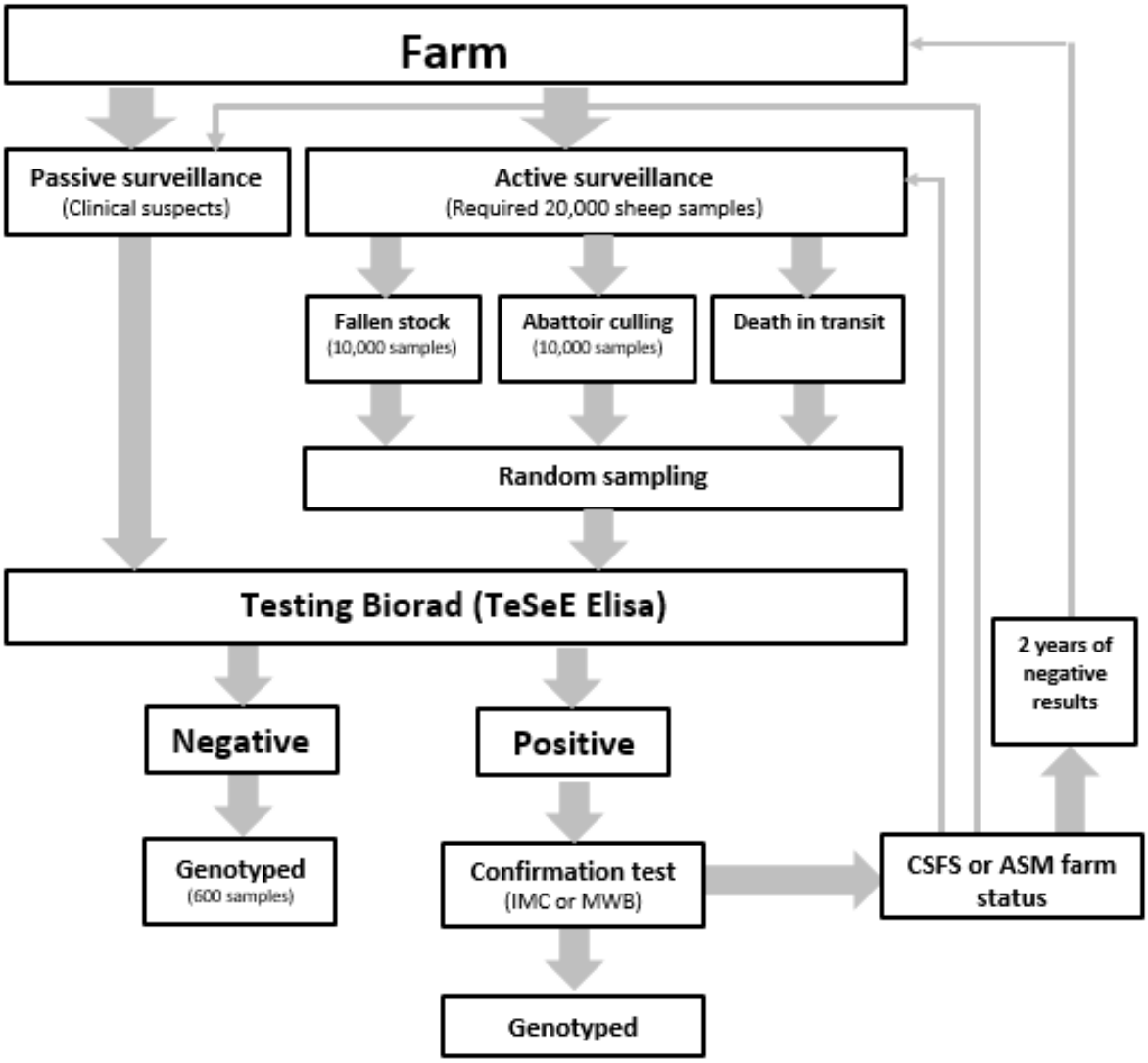


Figure 2. Annual frequency of genotypes of sheep in the Great Britain from 2002 to 2015. The prion protein gene (PrP) genotypes were defined as: type 1 (ARR/ARR), type 2 (ARR/AHQ, ARR/ARH, ARR/ARQ), type 3 (AHQ/AHQ, AHQ/ARH, AHQ/ARQ, ARH/ARH, ARH/ARQ, ARQ/ARQ), type 4 (ARR/VRQ) and type 5 (AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ). They establish decreasing levels of resistant to classical scrapie with type 1 or genotype ARR/ARR being the most resistant and type 5 or genotypes with VRQ alleles and non-ARR alleles being the most susceptible.

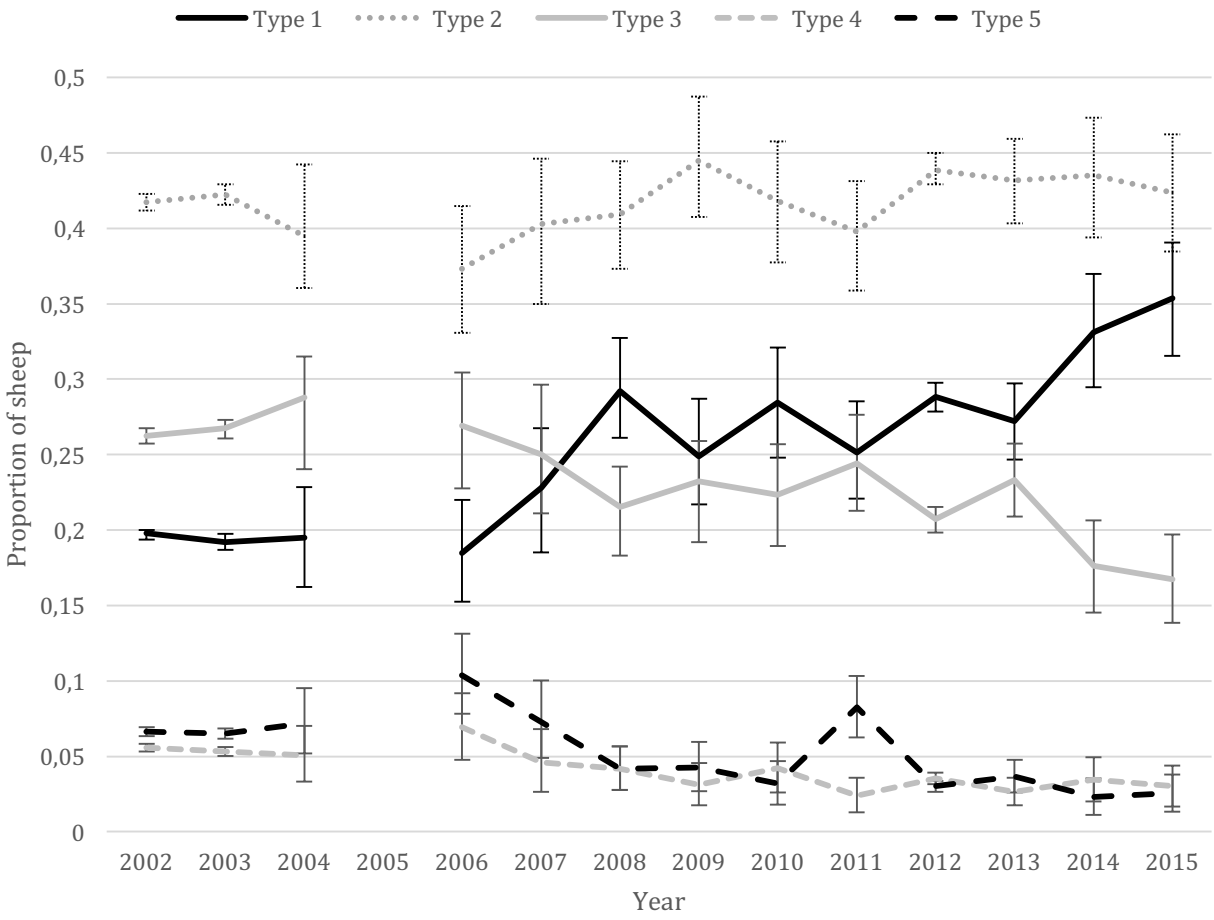
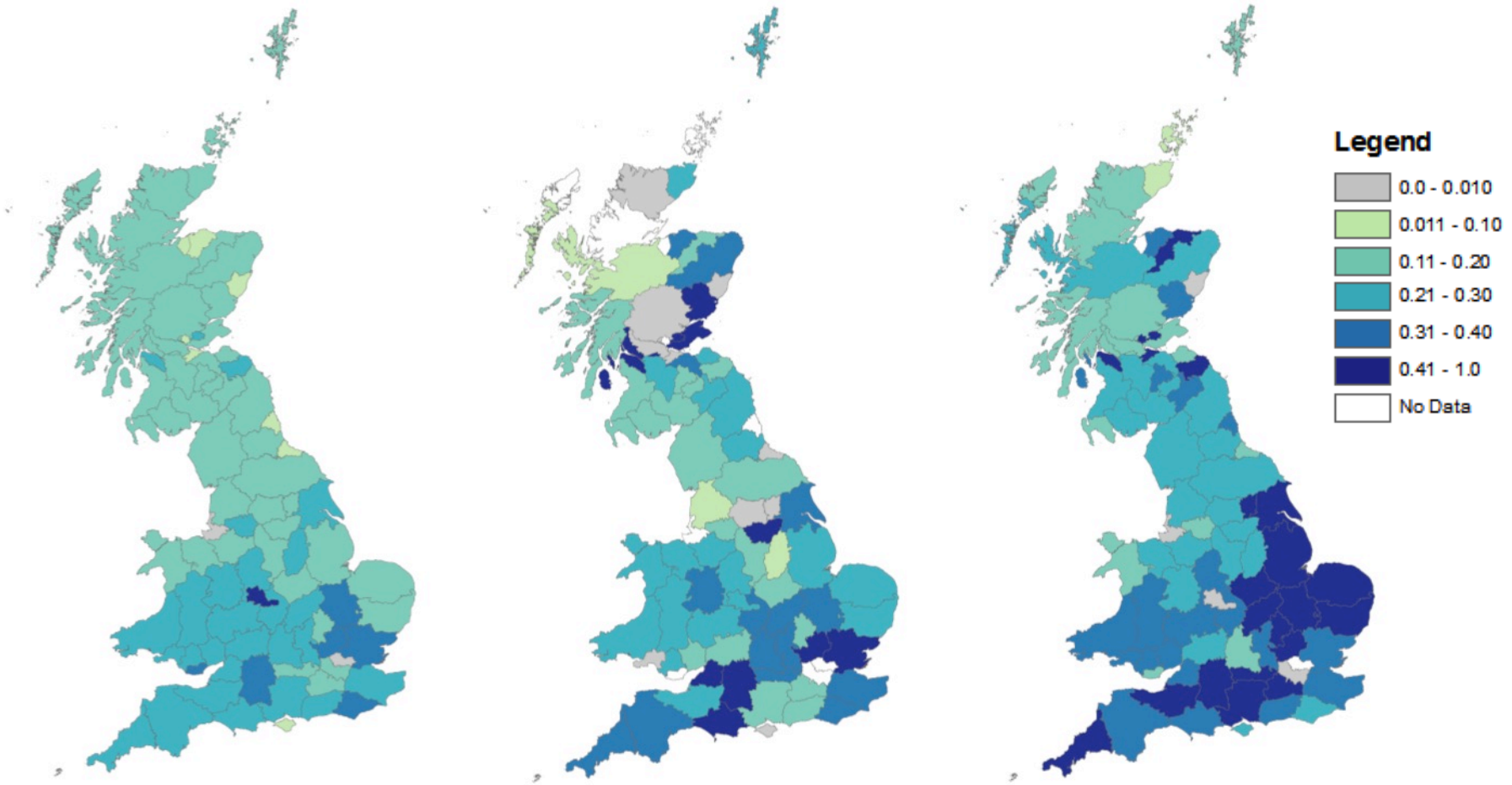


Figure 3. Geographic distribution of sheep with “most resistant” (ARR) genotype to classical scrapie by administrative area from 2002 to 2015 in Great Britain.

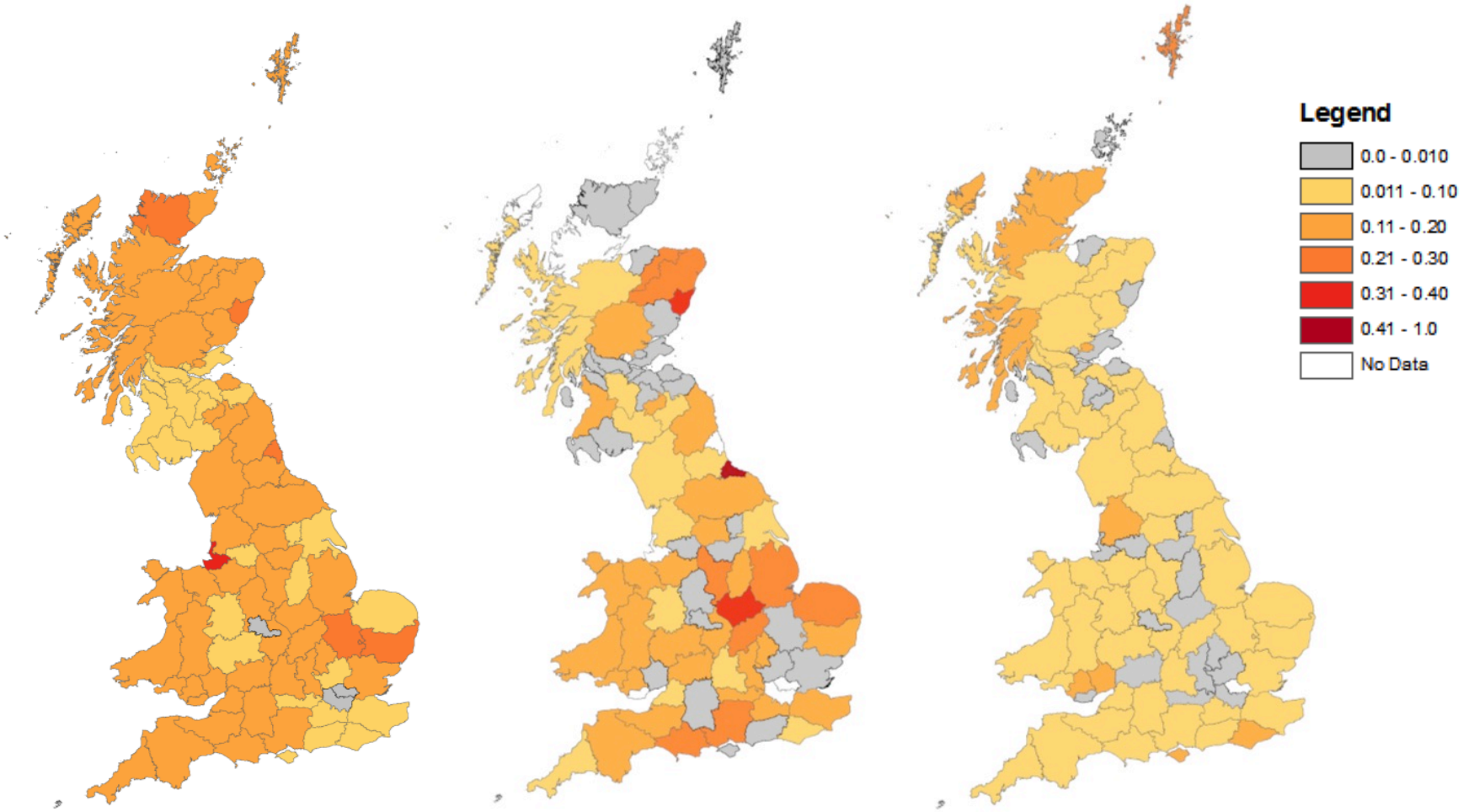


2002 - 2003
(n = 35,457)

2004 - 2009
(n = 2,323)

2010 - 2015
(n = 10.369)

Figure 4. Geographic distribution of sheep "most susceptible" including homozygous or heterozygous combinations of VRQ allele and excluding ARR allele to classical scrapie by administrative area from 2002 to 2015 in Great Britain.

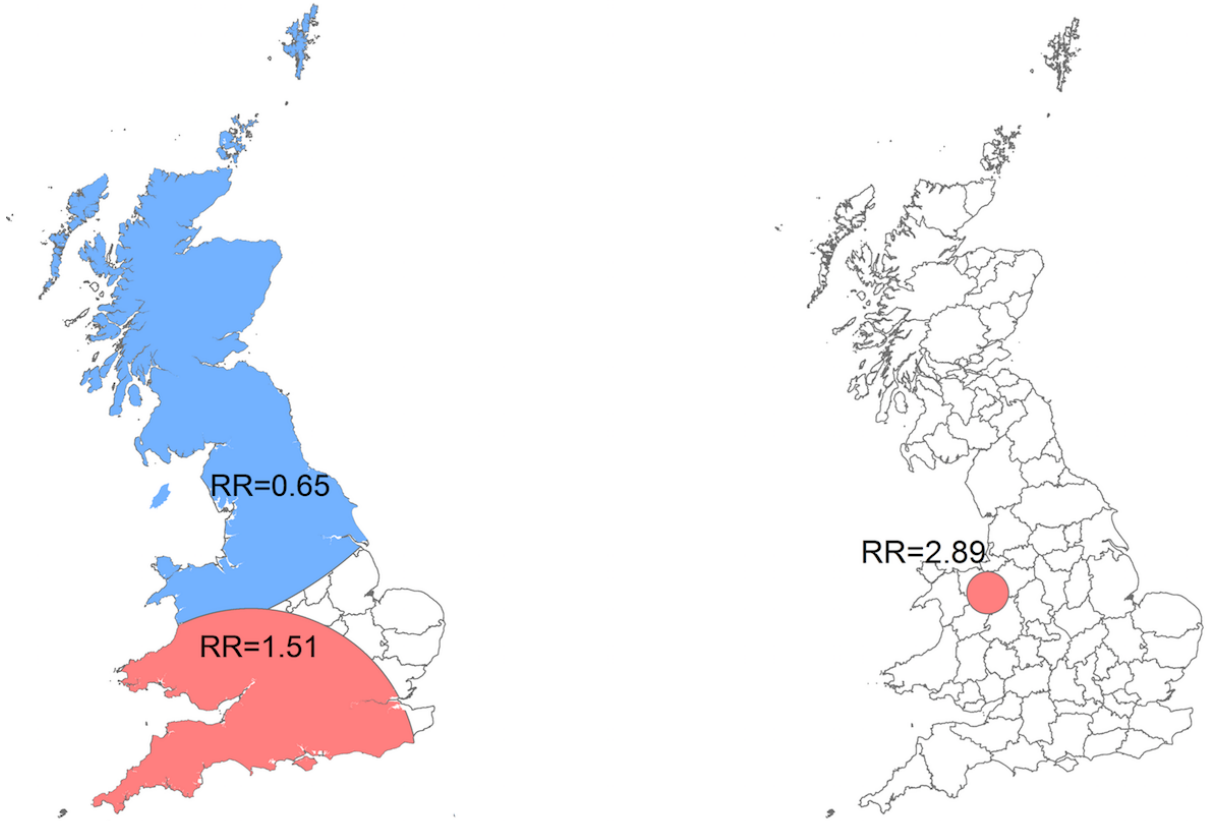


2002 - 2003
(n = 35,457)

2004 - 2009
(n = 2,323)

2010 - 2015
(n = 10.369)

Figure 5. Spatial clusters of sheep with ARR/ARR genotype (left) and of the VRQ allele (right) using sheep samples from 2012 to 2015 in Great Britain. Relative risks (RR) of genotype or allele presence are show in red for RR>1 and low for RR<1.



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