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Title: Analysis of bovine viral diarrhoea virus: biobank and sequence database to support eradication in Scotland

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Abstract

Samples from bovine viral diarrhoea virus (BVDV)–positive cattle were gathered by Scottish diagnostic laboratories and used to produce a biobank of samples with associated location and identification data in support of the Scottish BVDV eradication scheme. The samples were subject to direct amplification and sequencing of the 5'-untranslated region (5'-UTR) to define the viral types and subtypes present. From 2693 samples collected prior to 2016, approximately 2300 sequences were obtained, representing 8 BVDV type 1 subtypes. No BVDV type 2 samples were detected. The samples came from all regions of the UK but 66% were from Scotland. Analysis of the sequences showed great diversity in the 5'-UTR, with 1206 different sequences. Many samples carried virus with identical 5'-UTR sequences; often from single locations, but there were also examples of the same sequence being obtained from samples at several different locations. This work provides a resource that can be used to analyse the movement of BVDV strains both within Scotland and between Scotland and other nations, particularly in the latter stages of the Scottish eradication programme, and so inform the advice available to both livestock keepers and policymakers.

Introduction

The pestivirus bovine viral diarrhoea virus (BVDV) is a major pathogen of cattle that causes important economic losses for cattle industries around the world (OIE, 2015).

Herds with active BVDV infection may have reduced conception and increased embryonic and foetal loss, and also experience more severe calfhood disease due to the immunosuppressive nature of the infection. Infection of naïve pregnant cattle with BVDV in the first 120 days of gestation can lead to the generation of persistently infected (PI) calves that are tolerant of the virus and which are the major source of infection within and between herds. The increased prevalence or severity of other endemic infections among BVDV-affected herds, and the potential for reductions in antimicrobial use if such secondary infections can be prevented through control of BVDV, provide additional incentives for eradication (Givens and Newcomer 2015; Lindberg and others 2006).

BVDV has a positive sense single-stranded RNA genome of about 12500 nucleotides that is replicated in an error-prone manner, generating a wide range of related virus strains. BVDV exists as two major types, of which type 1 prevails in Britain and type 2 is only rarely identified (Booth and Brownlie 2015; Courtenay and others 2007; Wakeley and others 2004). BVDV type 1 has about 15 recognized sub-types (named 1a to 1o) that are defined by sequence similarity, particularly in the 5' untranslated region (5'-UTR) (Booth and others 2013; Vilcek 2005; Vilcek and others 2001). Previous studies in the UK have shown that BVDV1a is the most prevalent BVDV sub-type in the UK and that the variety of BVDV sub-types may have increased in recent years (Booth and others 2013; Strong and others 2013). These publications identified BVDV sub-types 1a, 1b, 1d, 1e and 1i as being present in GB, with BVDV1a being most prevalent.

Due to the economic impact of this infection, several European nations, including Scotland, have ongoing control and/or eradication programmes. During such programmes, re-infection of previously BVDV-free herds may occur, depending on the degree of biosecurity that can be maintained and the number of PI animals remaining in the national population. In this situation, molecular epidemiology is a useful approach to finding sources of infections and routes of virus transmission (Rossmanith and others 2014; Ståhl and others 2005). For effective molecular tracing, a comprehensive collection of local BVDV sequences is required for comparison with 'new' virus samples. The usefulness of this approach has been demonstrated recently by the publication of work using a sample biobank and associated database to support the Swiss BVDV eradication programme (Stalder and others 2015).

To facilitate the establishment of a sample biobank and sequence database in support of BVDV eradication in Scotland, we developed a method for direct RT-PCR and sequencing of BVDV from serum without prior RNA isolation. This study is one of several research activities within the Scottish Government-funded EPIC Centre of Expertise on Animal Disease Outbreaks (www.epicscotland.org) that are intended to support the eradication of BVDV in Scotland. Here we summarise the status (to the end of 2015) of the collection and discuss the implications of the data for veterinarians, livestock keepers and policymakers.

Materials and Methods

Initially, a small batch of 33 RNA samples from 2010 and 2011, provided by the Moredun Research Institute Virus Surveillance Unit, was used to test PCR primers and sequencing protocols for sequence-based typing of BVDV samples. Subsequently, a panel of 93 samples was used to optimise a method for the direct RT-PCR and sequencing of BVDV from serum samples without purification of RNA (Bachofen and others 2013). This method was then employed to characterise BVDV-positive serum samples collected in the course of the Scottish eradication programme and the UK Cattle Health Certification Standards BVD control and accreditation programmes (www.checs.co.uk/) between 2012 and the end of 2015 (Table 1). Samples identified as positive by diagnostic real-time PCR were subjected to direct RT-PCR and sequencing in batches of 95 as follows. Briefly, 10µl of each serum sample was diluted 1:5 with phosphate buffered saline (PBS) and incubated at 95 °C for 3 min. After chilling on ice, 2.5µl of each serum sample was transferred to a 96-well PCR plate containing 22.5 µl of RT-PCR mix (SuperScript III one-step RT-PCR system with Platinum Taq polymerase, Invitrogen), and cDNA synthesis and amplification were performed according to the manufacturer's protocol. Briefly, samples were incubated at 50 °C for 30 minutes for cDNA synthesis, followed by 94 °C for two minutes for Taq polymerase activation and sample denaturation. Amplification conditions were 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 60 s, followed by a final extension at 68 °C for 5 minutes.The previously described primer pair 324/326 (Vilcek 1994) was used to amplify a 244–247 base pair (bp) fragment of the conserved 5'-UTR of BVDV. This fragment is widely used for phylogenetic analyses of pestiviruses. Sequencing of the N^{pro} region was done similarly, using a nested protocol due to the larger size of the amplified region. An initial RT-PCR was done as above from serum with primers 324 and 1400R (Becher and others 1997) to amplify a 1340 bp fragment which was then further amplified using KOD Hotstart polymerase (Merck Millipore, Watford, UK) and primers 17F and 1400R to produce a 1269 bp fragment containing the entire N^{pro} coding region. Amplicons were checked for product size and yield by capillary electrophoresis and submitted to commercial service providers (GATC Biotech, Eurofins MWG or Beckman Coulter Genomics) for sequencing with the PCR primers. Sequence trace data for each sample were assembled using

Lasergene software (DNASTAR Inc., Madison, USA) and a consensus sequence representing the region bounded by the sequencing primers was generated. For analysis of 5'-UTR sequences, duplicate sequences were first identified using the "ElimDupes" online comparison tool (https://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) and were removed from the phylogenetic analysis. The remaining sequences were aligned with the 5'-UTR sequences of 68 BVDV type 1 and type 2 reference strains (Table 2)(Booth and others 2013; Stalder and others 2015; Vilcek and others 2001) using MAFFT (Katoh and others 2002; Katoh and Standley 2013). Assignment of genotypes was performed following phylogenetic analysis of the aligned sequences using the Neighbour-Joining method within MEGA6 (Tamura and others 2013). Information on each sample (including biobank sample number, sample provider, provider sample number, sample type, date taken, inferred BVDV type and sequence) was stored in a spreadsheet. Animal identification and sample location data for most samples were obtained subsequently on the undertaking that no holding would be identified in outputs from the work. Sample sources in Figure 1 were assigned to regions of Great Britain on the basis of postcode information supplied with the samples. Using regionally-aggregated data ensured that there is no breach of confidentiality in areas where there are few cattle holdings.

Results

Approximately 2700 BVDV-positive samples, submitted prior to 2016, were analysed by RT-PCR and sequencing, yielding 2292 readable 5'-UTR sequences of length >200 bases (Table 1). Among these, 1206 different 5'-UTR sequences were identified. These were assigned to BVDV subtypes after phylogenetic analysis as described above. This showed that 1921 of the sequenced samples contained BVDV subtype 1a (84%), 322 contained BVDV1b (14%), 33 contained BVDV1i (1.4%), and the remaining 16 samples contained subtypes 1d, 1e, 1f, 1g and 1h (each <0.25 %). The diversity of the 5'-UTR sequences determined here is illustrated in Figure 2, showing a subset of the BVDV sequences and the reference sequences used for genotype assignment.

Location data (UK postcode) were obtained for 2015 of the sequenced samples after typing had been performed. These showed that while the majority of samples came from within Scotland (1322), the remainder of the samples (Figure 1) were spread across the UK.

Additional sequencing of the N^{pro} region of the first 93 samples analysed yielded only a small number of readable sequences (26). This was likely to be due to the larger size of the N^{pro} PCR product and no further samples were analysed by this method. These N^{pro} sequences confirmed the BVDV sub-types assigned from the 5'-UTR sequence. Three pairs and two sets of three samples analysed at both loci had identical 5'UTR sequences. All of the samples that were identical in 5'-UTR sequence

were also more than 99.5 % identical in their N^{pro} sequence, suggesting that for these samples at least, samples with identical 5'UTR appeared to represent the same virus strains. There was no evidence of recombination among the samples tested at these two loci.

Of the 2292 samples sequenced, 1405 contained 5'-UTR sequences that were found more than once, with 319 sequences shared among these samples, whereas the remaining 887 samples each contained a distinct BVDV sequence (i.e. unique to that sample). To determine the distribution of samples with shared sequences, those with location data (UK postcode) were analysed. Among 2015 samples with sequence and location data, there were 1060 different sequences at 721 different locations, of which 304 sequences were found in multiple samples. Fewer than half of the locations (328) had a single BVDV positive sample and a further 99 locations had multiple copies of the same BVDV sequence (average 2.8, range 2-10; Table 3). Multiple BVDV sequences were found at the remaining locations (Table 3). This shows, for example, that 16 locations submitted samples representing 5 different BVDV sequences, with the most frequently detected sequence identified in an average of 4 samples at each location (with a range of 1-18 identical samples).

Animal identification (full 12-digit UK number) was obtained for 1537 of the sequenced samples, representing 1285 different animals. The remaining samples had partial numbers, non-UK numbers or had identification that was withheld or unavailable. Among the 1537 samples with full UK numbers, 484 samples came from 232 animals that were represented more than once in the Biobank. Assuming a similar geographical distribution and degree of replication among the samples without complete identification data, the biobank probably represents about 1200 PI cattle from Scottish premises and a further 550 from the rest of the UK.

Discussion

In this work we have demonstrated that it is possible to generate sequence data for the 5'-UTR of BVDV rapidly and directly from hundreds of serum samples submitted for diagnostic testing. This is an improvement on previous approaches that depended on prior RNA extraction before amplification on the grounds of both costs (reagents, staff and time) and in the quantity of sample required. All of the samples collected were submitted for amplification and sequencing but only those that yielded readable sequence data for more than 200 nucleotides of the amplified 5'UTR fragment were typed. Thus while 2693 samples were analysed, only 2292 sequences were obtained due to PCR failures, sequencing failures and poor quality data (85 % success rate). The primers used are considered pan-pestivirus and have been used to amplify the 5'UTR from a broad range of BVDV strains (Vilcek 1994). Repeat testing of failed samples yielded sequence data in some but not all cases, suggesting technical failure is more likely than failure due to sequence variation in the primer

sites. This method cannot so far be applied to tissues but it will be tested on ear-tag tissue sample lysates in the near future.

The 5'-UTR sequence data allowed the virus in each sample to be assigned to one of eight BVDV type 1 subtypes. Although only 26 samples were also typed by sequencing of the N^{pro} locus, all of these genotype assignments agreed with the 5'-UTR data. This data also provided support for the view that samples with identical 5'UTR sequences were likely to represent the same strain, based on N^{pro} sequencing. Attempts to use our method to routinely sequence the N^{pro} locus directly from serum have been unsuccessful to date, with only a small number of samples producing usable sequence data. This may reflect the quality of the serum samples, which can vary (Bachofen and others 2013) or the larger size of the N^{pro} amplicon tested (1269 bp compared with 247 bp for the 5'-UTR amplicon). Efforts to amplify the Npro gene directly from serum samples are ongoing, using alternative primer sets.

This dataset provides a significant improvement in our understanding of the BVDV strains circulating in Scotland and more widely in UK. While the estimated 1200 Scottish PI cattle samples analysed are a relatively small fraction of the Scottish cattle population affected by BVDV, they represent a significant proportion of the Scottish PI population. As of September 2015, the total number of PI cattle identified in Scotland was 2876 (K. Davie, personal communication), so the EPIC BVDV biobank is estimated to represent almost half of the Scottish PI cattle identified since the eradication began in 2013.

Our data suggest that BVDV type 1a and 1b remain the most frequent strains of BVDV in the UK, with other types remaining at low frequency. We identified a larger range of BVDV sub-types than previous studies (Booth and others 2013; Strong and others 2013; Vilcek and others 1999a; Vilcek and others 2001), possibly because of the larger number of samples analysed, but an increase in BVDV strain diversity within the UK due to animal movements into UK cannot be excluded. The current study also demonstrates the high diversity of this virus with over 1200 different 5'-UTR sequences found among 2292 sequenced samples. This diversity is illustrated in Figure 2, which shows a subset of about 140 sequences that differed by more than 10 %.

Despite the large number of different 5'-UTR sequences identified, the use of the short 5'-UTR sequence in this analysis limited our ability to make strong inferences with respect to relationships between strains with highly similar 5'-UTR sequences. While it is likely that most samples with the same 5'-UTR sequence will represent the same BVDV strain, this will not be true in all cases and the analysis of other BVDV loci would help to differentiate between similar strains. In addition, previous studies (Becher and others 1997; Booth and others 2013) have highlighted difficulties in

differentiating between some subtypes using 5'-UTR sequences only. In the current study, only a small number of samples were typed by sequencing of N^{pro}; these samples included BVDV 1a, 1b and 1i subtypes (based on 5'-UTR sequence) and in each case the same subtype assignment was made by analysis of N^{pro}. Thus, the sequencing of additional virus genes, such as N^{pro} or E2, may improve the confidence of sub-type assignment for some samples and will add to our confidence in characterising highly similar BVDV strains. The sequencing of thousands of BVDV positive samples in this study was made feasible by the use of direct sequencing to reduce the time and expense required for the analysis. It is therefore a focus of current research to extend this approach to the analysis of other loci, specifically N^{pro} and E2, to facilitate further characterisation of BVDV strains with identical 5'-UTR sequences and to understand the evolutionary relationships between BVDV isolates.

Analysis of the sharing of sequences between samples suggests that most of the locations (302 of 393) that provided more than one sample had multiple copies of a 5'-UTR sequence. Similarly, among the 304 sequences with location data found more than once, almost half (133) had all copies at a single location. These observations provide some support for previous suggestions that BVDV strains may be holding-specific (Booth and others 2013; Rossmanith and others 2014; Stalder and others 2015). A report from the recent Swiss BVDV eradication has indicated that several BVDV sequences were found in more than 100 samples from locations across that country, possibly due to the use of communal alpine pastures (C. Bachofen, personal communication). Thus, the less widespread nature of BVDV strains in Britain may reflect better biosecurity associated with local farming practices.

We also found that a number of duplicated sequences were found in samples from multiple locations. In the most frequent case, 45 samples with the same sequence came from at least 17 different locations. This suggests that BVDV strains were being transferred between premises by either animal movements, over-the-fence contacts between contiguous premises (Flood and others 2013) or by transfer of virus in fomites (Rossmanith and others 2014; Ståhl and others 2005; Stalder and others 2015). Initial analysis of animal birth dates, sample dates and locations in a number of such cases in our dataset suggests that recorded animal movements alone may not explain the presence of these sequences at all of the locations found. Further analysis of these widely-disseminated samples with identical 5'-UTR sequences will focus initially on sequencing of the N^{pro} region to determine whether they represent identical strains or different but similar strains. While an extensive analysis of animal movements related to the samples within the database is now possible, it will also be important to consider all possible connections between premises in cases where the tracing of chains of infection is required. Recent publications suggest that additional

factors such as over-the-fence contacts between contiguous premises, animal haulage, farm visitors (family, staff, veterinarians, and others), and shared machinery may each contribute to spread in individual cases (Gethmann and others 2015; Rossmanith and others 2014; Stalder and others 2015). To provide livestock keepers and policy makers with appropriate evidence on which to base improvements to biosecurity measures and policies, we need to study chains of BVDV infection in greater depth and in a larger number of cases.

As the BVDV eradication in Scotland proceeds, the number of BVD-free premises will increase and the possibility of re-introduction of BVDV will be a significant risk for these livestock keepers. The usefulness of sequencing as a tool to track the introduction of novel BVDV strains has been demonstrated in England & Wales (Vilcek and others 1999b), in Northern Ireland and the Republic of Ireland (Guelbenzu-Gonzalo and others 2016;Graham and others 2001) and in Scotland (Nettleton and Gunn 2002). The resources developed and described here may provide a means to understand the contribution of animal movements and other factors to the spread of BVDV and thereby focus the efforts of livestock keepers on the relevant sources of risk.

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TABLE 1: summary of sample data obtained by year of sampling

Year	Samples analysed[a]	samples sequenced[b]	samples with sequence & location data[c]	samples with sequence & identification data[d]
2010	21	21	0	0
2011	12	12	0	1
2012	155	147	141	83
2013	427	388	359	243
2014	968	855	808	644
2015	1110	869	707	566
totals	**2693**	**2292**	**2015**	**1537**

[a] number of samples tested with submission date within the given year

[b] number of samples for which a consensus sequence >200 nucleotides could be derived

[c] number of samples with sequence as in b and with a UK postcode for the submitting location

[d] number of samples with sequence as in b and with a full UK number associated with the sample

TABLE 2: accession numbers of BVDV reference sequences

Sub-type	Accession numbers of reference sequences
BVDV1a	AF298061, M96751, EU180026, U94916, AF039181
BVDV1b	EF101530, AF298072, AF298060, JQ994197, M96687, U63479, AF298070
BVDV1c	JQ743607, JQ743606, JQ743605, AF049222, AF049221, AY763036, AY762998
BVDV1d	AF298056, JQ994205, EU224234, EU224230, AF298065
BVDV1e	AF298062, AF298054, JQ994203, JQ994204, EU180028, JQ994207, AF298058
BVDV1f	AF298071, AF298067, AF298073, EU224240, EU224221
BVDV1g	AF298069, EU224236, U97455, AF298064
BVDV1h	AF298066, JQ994208, JQ994196, AF298068
BVDV1i	AF298059, FJ493484, U97409
BVDV1j	U97429, AB078950, U97454, U97411
BVDV1k	AF299317, AF117699, JQ994206, EU224237
BVDV1L	EU180024, KF205294, KF205306
BVDV1m	AF526381, GU120245, GU120257
BVDV1n	AB359930, AB042661, GQ495676, DQ973181
BVDV2	U18059, EU224242, AF298055, AF298063

TABLE 3: number of BVDV sequences among samples from individual locations

Number of sequences[a]	number of samples (or range) with most frequent sequence[b]	Mean[c]	locations[d]
1	2-10	2.8	99
2	1-23	2.0	144
3	1-7	2.1	75
4	1-17	3.0	31
5	1-18	4.0	16
6	1-7	2.6	8
7	1-4	2.6	7
8	2-10	4.8	5
9	4	4	1
10	2-3	2.3	3
11	8	8	1
13	8	8	1
14	6-8	7	2

[a] number of different 5'UTR sequences within the samples from each postcode location

[b] number of samples (or range of sample numbers) carrying the most frequent BVDV 5'UTR sequence at each location

[c] average number of samples with the most frequent BVDV 5'UTR sequence at each location

[d] number of locations submitting the same number of different BVDV 5'UTR sequences

Figure Legends

Figure 1. Map of sample locations

Sample location details were available for most of the samples submitted for analysis. These were allocated to UK regions based on postcode and the number of samples submitted from each region is indicated.

Figure 2. Phylogenetic tree of BVDV 5'-UTR sequences.

An alignment of 139 different BVDV 5'-UTR sequences representing the overall diversity of the 1206 BVDV 5'-UTR sequences from the biobank was generated by excluding sequences more than 90% identical, as described in the text. A phylogenetic analysis was done by the Maximum-Likelihood method and the optimal tree is shown in circular format, where the closed circles represent sample sequences and open circles represent reference sequences, coloured according to BVDV sub-type. The BVDV sub-types are indicated outside the figure, underlined in the case of sub-types represented among the sample set. Branch lengths are proportional to genetic distance, measured as the number of base substitutions per site (scale bar vertical at top of circle). Phylogenetic analyses were conducted in MEGA6 (Tamura and others 2013).

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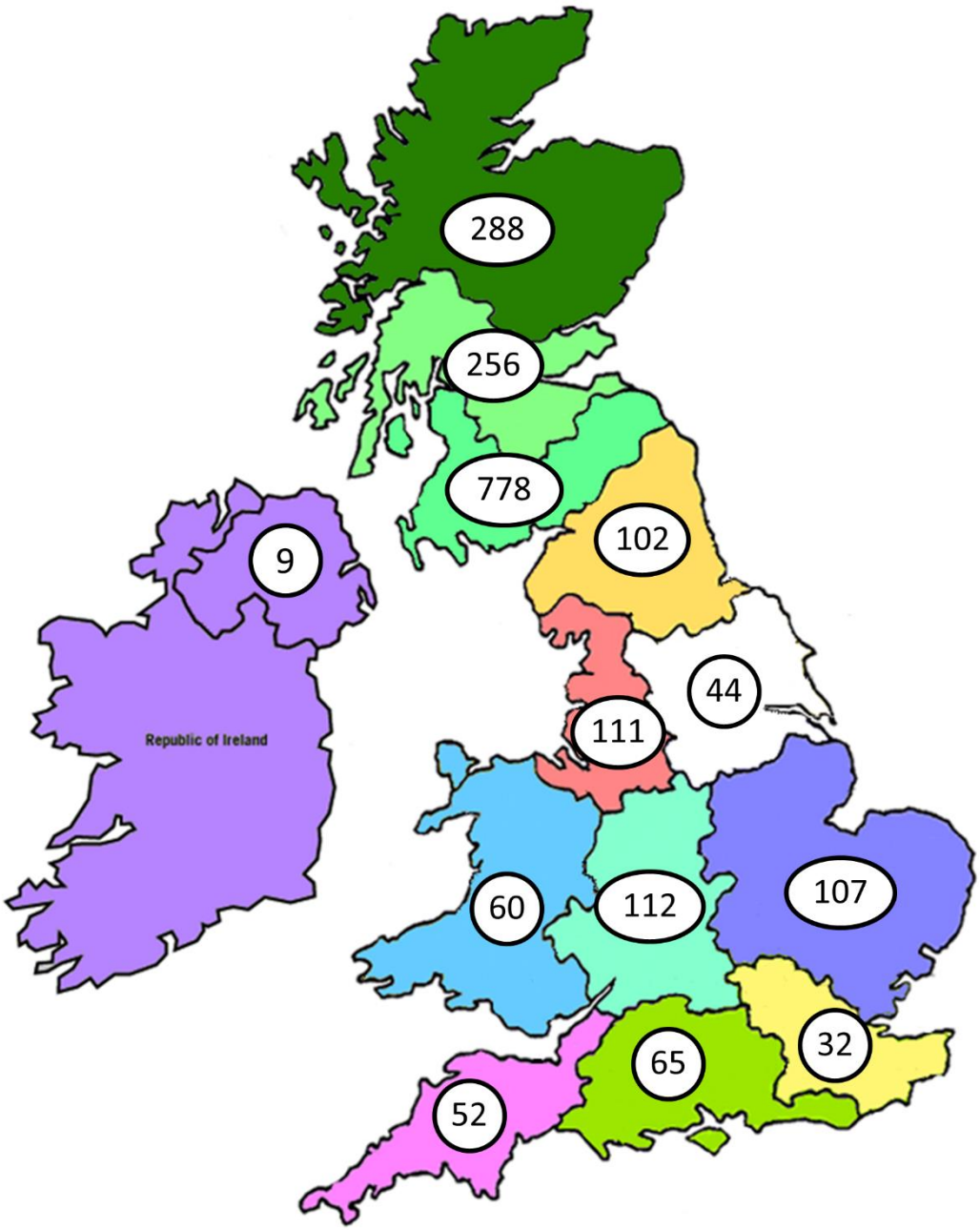
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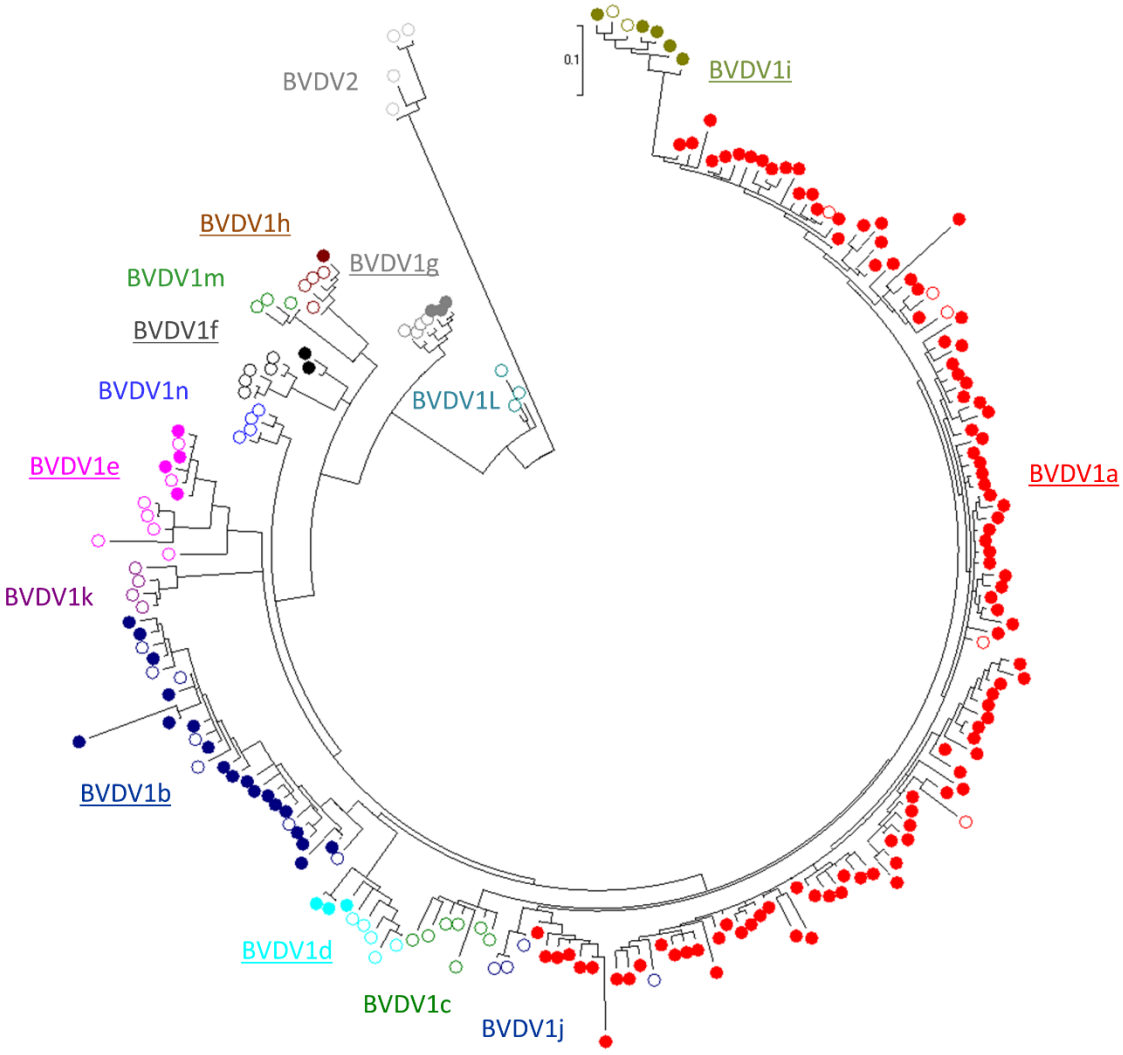
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BVDV1i
BVDV1h
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BVDV1n
BVDV1L
BVDV1e
BVDV1a
BVDV1k
BVDV1b
BVDV1d
BVDV1c
BVDV1j