1 ***Leptospira interrogans* serovars Bratislava and Muenchen animal**
2 **infections: implications for epidemiology and control.**

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26 **Abstract**

27 Strains of *Leptospira interrogans* belonging to two very closely related
28 serovars – Bratislava and Muenchen - have been associated with disease in
29 domestic animals, in particular pigs, but also in horses and dogs. Similar
30 strains have also been recovered from various wildlife species. Their
31 epidemiology is poorly understood. Two hundred and forty seven such
32 isolates, from UK domestic animal and wildlife species, were examined by
33 restriction endonuclease analysis in an attempt to elucidate their
34 epidemiology. A representative sub-sample of 65 of these isolates was
35 further examined by multiple-locus variable-number tandem repeat analysis
36 and 22 by *secY* sequencing. Ten restriction pattern types were identified.
37 The majority of isolates fell into one of three restriction endonuclease analysis
38 pattern types designated B2a, B2b and M2a. B2a was ubiquitous and was
39 isolated from 10 species and represented the majority of the horse and all dog
40 isolates. B2b was very different, being isolated only from pigs, indicating that
41 this type was maintained by pigs. The pattern M2a was reported for the
42 majority of isolates from pigs but also was common in small rodents isolates.
43 Five restriction pattern types were found only in wildlife suggesting that they
44 are unlikely to pose a disease threat to domestic animals. Multiple-locus
45 variable-number tandem repeat analysis identified six clusters. The REA
46 types B2a and B2b were all found in one MLVA cluster while the majority of
47 the M2a strains examined occurred in another cluster. The *secY* sequencing
48 detected only one sequence type, clustered with other serovars of *Leptospira*
49 *interrogans*.

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51 **1. Introduction**

52 Widespread infection, by what serological evidence suggests is *Leptospira*
53 *interrogans* serovar Bratislava infection, is found in pig and horse populations
54 worldwide (Arent et al., 2015; Arent and Kedzierska-Mieszkowska 2012; Ellis,
55 1999a; Ellis, 2012; Ellis, 2015; Milas et al., 2013). Such infections have been
56 associated with abortion, reduced litter size and infertility in pigs (Bolin and
57 Cassells, 1990; Ellis et al., 1986c; Frantz et al., 1989) and abortion and uveitis
58 in horses (Ellis, 2015). There is also evidence linking Bratislava infection with
59 clinical disease in dogs (Adin and Cowgill, 2000; Nielsen et al., 1991)
60 Conventional serological identification of a limited number of United Kingdom
61 (UK) isolates has indicated that such titres are due to two closely related
62 serovars – Bratislava and Muenchen – belonging to the Australis serogroup
63 (Hathaway et al., 1983a; Hathaway et al., 1983c; Hathaway et al., 1983e).
64 Genetic typing of UK pig isolates concurred with the serological studies (Ellis
65 et al., 1991).

66 The epidemiology of these Bratislava/Muenchen infections is unclear and
67 diverging views have been expressed as to the relative roles of wildlife and
68 domestic animal hosts. This is particularly true in the UK, where widespread
69 infection of free-living animals has been identified (Hathaway et al., 1983a;
70 Hathaway et al., 1983c; Hathaway et al., 1983e). Hathaway and others
71 (1983a) suggested that free-living species constitute primary sources of
72 infection for domestic species. In contrast, serological evidence, supported by
73 some isolation studies, indicates that there is endemic infection in pigs and
74 possibly horse populations, and it has been suggested that pigs and possibly

75 horses (Arent et al., 2015; Ellis, 1999a; Ellis et al., 1986a, c; Ellis et al.,
76 1986e) and dogs (Arent et al., 2013; Ellis, 2010; Van Den Broek et al., 1991)
77 maintain strains of serovar Bratislava/Muenchen and that wildlife may not be
78 an important source of infection for pigs.

79 Conventional serological typing of isolates has been unable to differentiate
80 between wildlife and domestic animal isolates of serovars Bratislava and
81 Muenchen and so has been unable to provide further insights into this
82 conundrum. Molecular tools have been employed as alternatives to
83 serological methods for the identification and classification of *Leptospira*.
84 These include restriction endonuclease assay (REA) (Ellis et al., 1991), REA
85 and polymerase chain reaction (PCR) combined (Brown and Levett, 1997;
86 Savio et al., 1994), pulsed field gel electrophoresis (PFGE)(Herrmann et al.,
87 1991; Herrmann et al., 1992), restriction fragment length polymorphism
88 (RFLP) (Zuerner et al., 1993) arbitrarily primed PCR (Perolat et al., 1994),
89 fluorescent amplified fragment length polymorphism (FAFLP)(Vijayachari et
90 al., 2004), and multilocus sequence typing (MLS) (Ahmed et al., 2011; Ahmed
91 et al., 2006) Some of these techniques however, suffer from certain
92 disadvantages that include a requirement of large quantity of pure and high
93 quality DNA, variable discriminatory power, variable reproducibility and
94 ambiguous interpretation of data. Recently multiple-locus variable-number
95 tandem repeat analysis (MLVA) which involves the analysis of multiple loci of
96 variable-number tandem repeats (VNTR) (Majed et al., 2005; Salaun et al.,
97 2006; Slack et al., 2005) has been applied to the speciation and molecular
98 epidemiology of leptospires and promising results have been reported in the
99 study of the strains belonging to the serogroup Australis (includes Bratislava

and Muenchen) found in the *L. interrogans* genomospecies (Koizumi et al., 2015a, 2015b; Slack et al., 2006, 2005)

Using restriction endonuclease analysis, Ellis et al., (1991) were able to differentiate the serovars in the serogroup Australis, including two closely related serovars - Bratislava and Muenchen – found in the UK. They were also able to show differences at the sub serovar - genotype - level. This provided some possible insights into the clinical significance of different strains – e.g. strains which they designated as Bratislava genotype 2b (B2b) were found only in material with a history of reproductive wastage i.e., they were isolated only from aborted or stillborn pigs, sows which had aborted, or boars from farms from which the organisms had also been recovered from aborted piglets. Only Bratislava genotype 2a (B2a) strains were recovered from piglets with meningitis.

The purpose of this study was to examine isolates from UK wildlife and domestic animal species by REA in an attempt to elucidate the epidemiology of infection by the serogroup Australis in the United Kingdom and, in particular, to assess the relative importance of domestic and wildlife species as sources of infection for domestic animals species. VNTR analysis and *secY* sequencing were then applied to selected isolates to examine whether this methodology would provide additional information.

2. Materials and Methods

2.1. Cultures

Two hundred and forty seven UK isolates of *Leptospires* belonging to the Australis serogroup were included in this study (Table 1). They were recovered from 13 wild and domestic animal species. REA findings for the 157 pig strains included in the study have been described previously (Ellis et al., 1991). The majority of pig isolates were obtained from animals with clinical problems such as aborted fetuses (51), sows that had aborted fetuses (50), piglets with meningitides (11). The rest of the swine isolates were derived from boars which came from farms where culture-positive fetuses had been obtained (39) or recovered from pigs collected at an abattoir (6). Similarly most of the equine isolates (9) were obtained from aborted fetuses or neonate after stillbirth but the rest (5) came from asymptomatic animals. Most of canine isolates (14) came from animals showing clinical problems. All ovine isolates were obtained from aborted fetuses but bovine were recovered from cattle at an abattoir. All isolates derived from wild species were obtained from animals without known clinical status. Also included in the study were the type reference strains of serovars Bratislava (strain Jez Bratislava), Muenchen (strain München C90), Australis (strain Ballico) and two other closely related strains from the Australis serogroup - Lora (strain Lora) and Jalna (strain Jalna). Sixty five selected isolates and the five type strains noted above were included in the VNTR study (Tables 4 & 5). *Leptospira interrogans* serovar Copenhageni strain M20 was included as a control.

2.2. Preparation of whole-cell DNA.

Leptospire were harvested by centrifugation, from 300 ml of an exponentially growing culture in liquid E medium (Ellis, 1986) containing no rabbit serum, 5-fluorouracil or nalidixic acid. DNA was extracted from the pellet as described previously (Ellis et al., 1991) and held at -70°C until required.

2.3. Restriction endonuclease digestion of DNA.

Restriction endonuclease digestion and analysis was carried out as described by (Ellis et al., 1991). A variety of electrophoresis conditions were used depending on which restriction enzyme had been used: run times varied between 18 and 24 hours and the voltages used varied between 60 and 75 volts. Similarly different gel concentrations were used; these ranged from 0.5 to 0.7% agarose (A-9539, Sigma)(Figures 1 to 4) in Tris borate buffer (10.8 g of Tris, 0.99g of Na₄-EDTA and 5.5 g of boric acid per litre of distilled water). The gels were stained in ethidium bromide (0.5 µl/ml) for 45 min and photographed under short-wave UV light through a Kodak 23A red filter. All strains were examined using those enzymes which had previously been identified as appropriate for the typing of Bratislava and Muenchen strains (Ellis et al., 1991) - *Clal*, *Hhal*, *HpaII* and a combined digestion with *BglII* and *Hhal*

2.4. Multiple-locus variable-number tandem repeat analysis

Analysis of variable-number of tandem repeats was carried out using 13 sets of primers designed by (Majed et al., 2005) and (Slack et al., 2005) with some modification (Supplementary File 1, Supplementary Table 1).

2.5. Sequencing

Sequencing of 245 bp fragment of *secY* gene (G1-G2 domain), was performed using SecYII/SecYIV primer pair as described previously (Victoria et al., 2008). Amplicon were purified for sequencing using a QIAquick gel extraction kit (Qiagen) The nucleotide sequence was determined by gel capillary electrophoresis on an Applied Biosystems ABI3130xl Genetic Analyser. Eleven Bratislava strains and eleven Muenchen strains representing different REA types and derived from different hosts were selected for the sequencing.

2.6. Phylogenetic analysis

MLVA data were recorded in a Microsoft Excel 2003 spreadsheet. Distance trees were constructed using the Unweighted Pair Group Method with Arithmetic Means (UPGMA) and plotted as dendrograms using the Sequence Type Analysis and Recombinational Tests (START) v1.0.5 software (<http://pubmlst.org/software/analysis>) (Jolley et al., 2001). *SecY* sequences from tested strains and other sequences representing the genus *Leptospira*, retrieved from GenBank, were aligned with CLUSTAL W (Thompson et al., 1994). Phylogenetic analysis was conducted with MEGA4 (Tamura et al.,

2007) and the neighbor-joining method (Saitou and Nei, 1987) were used for estimation of distance of aligned sequences.

3. Results

3.1. REA

A total of ten restriction patterns were identified by the enzyme panel used – 3 within serovar Bratislava (B1, B2a and B2b) and 7 within serovar Muenchen (M1, M2a through M2f) (Tables 2 & 3, Figures 1 to 4). The M1 pattern was restricted to the serovar Muenchen type strain (Muenchen C90). All the UK isolates had the M2 pattern. Four patterns were identified in Northern Ireland (NI) isolates (Table 2) and seven patterns in the Great Britain (GB) isolates (Table 3, Figures 1, 2, 3 & 4).

Bratislava strain B2a was an ubiquitous strain being originally isolated from 10 of the 13 species from which isolates were studied and was common amongst both the NI and GB isolates. It was however the only strain isolated from dogs (26 of 26 strains) and accounted for the majority of the horse isolates (11 of 14 strains).

Bratislava B2b was only found in pigs with an association with clinical disease (Ellis et al., 1991). B2b was not detected in the GB strains but that sample contained no isolates from pig clinical material.

The pattern M2a was common in pig isolates from both GB and NI and accounted for the pattern seen with 16 of 21 small rodent isolates from GB.

215 Muenchen types M2b, M2c, M2d, M2e, M2f were found only in the GB wildlife
216 isolates (Table 3, Figure 4)

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218 3.2. MLVA

219 The PCR products amplified from the samples had sizes which corresponded
220 to the insertion or deletion of tandem repeat units. Length polymorphisms
221 were easily identified on agarose gels. The PCR product amplified from
222 *Leptospira interrogans* serovar *Copenhageni* strain M20 was the predicted
223 size. Serovars Muenchen and Bratislava demonstrated substantial length
224 polymorphisms from the other type strains across a number of the repeats
225 (Table 4). Serovars Muenchen and Bratislava could be separated by the
226 number of repeats at the V23, V29 and V30 loci, as could the isolates. No
227 differences were observed in the Bratislava and Muenchen genomes with
228 repeats V7, V9, V10, V11 and V31.

229 VNTR allele profiles of some strains were very similar to each other, differing
230 in 1 or 2 copies of a repeat at a few loci.

231 UPGMA cluster analysis (Figure 5) divided the isolates into two large clusters
232 (M and B): M contained all the strains with a Muenchen REA profile and those
233 strains that had been typed as serovar Muenchen by conventional serological
234 methods previously. Similarly B contained all the Bratislava strains. These two
235 large clusters were found in a wide variety of domestic and animal species
236 and were found across the UK.

Within the Bratislava cluster, MLVA discriminated between REA types B1 and B2 (clusters B1 and B2 – Figure 5) but did not distinguish between REA subtypes B2a and B2b.

Within the Muenchen cluster, MLVA distinguished between the type strain – REA type M1 (cluster M1) - and the isolates – REA type M2 (cluster M2). Subclusters within the REA M2 strains did not correlate with the REA findings (Figure 5), however all six of the NI M2 isolates were found within the same subcluster (M2(iii) – Figure 5), while GB isolates were found in all four M2 subclusters.

3.3. *secY* sequencing

In the next step, 245 bp of the *secY* gene sequence was used. The *secY* gene codes for preprotein translocase, a gene that has proven value for phylogenetic relationships between *Leptospira* (Victoria et al., 2008). In the present study *secY* sequencing detected only one sequence type, clustered with others serovars of *Leptospira interrogans* (data not shown).

4. Discussion

In this study we used three genotyping methods for identification and discrimination of Bratislava and Muenchen strains. For sequencing we employed 245bp fragment of *secY* gene whose discriminatory ability was shown to be high making it useful for a quick *Leptospira* typing (Victoria et al., 2008) However, there were no differences in the sequences of tested strains representing these two serovars, different REA types and those derived from

260 different animal hosts. All strains were put into the same cluster with other
261 strains of *L. interrogans*. It confirms that sequence based on *secY* gene is
262 suitable for rapid identification of *Leptospira* species and does not exhibit any
263 differences within serovar and sub-serovar level in the Bratislava / Muenchen
264 complex (Koizumi et al., 2013; Koizumi et al., 2015)
265 REA and MLVA had a higher discriminatory power than *secY* sequencing..
266 (Figure 5). There was a noticeable correlation between REA and VNTR
267 genotyping methods for the identification of Bratislava and Muenchen isolates
268 to serovar and further to two subserovars (M1/M2 and B1/B2) levels(Figure
269 5). REA was proved to be a sensitive, reproducible technique used in the
270 identification of isolates to serovar or even subserovar level (Marshall et al.,
271 1981; Thiermann and Ellis, 1986). Moreover, it has been useful technique to
272 connect the fingerprint patterns of the Bratislava/Muenchen isolates with
273 different clinical significance or animal origin (Ellis, 1988; Ellis et al., 1991). In
274 REA, genomic DNA is cut by enzymes which recognise specific base
275 sequences and the resulting fragments are separated by electrophoresis. Its
276 discriminatory value is limited by the fact that analysis is based on the
277 observation of a relatively small number of the largest fragments and
278 potentially significant differences in the smaller fragments cannot be
279 visualised. It also suffers from the problem of requiring relatively large
280 amounts of highly purified DNA. MLVA has the advantage of requiring much
281 smaller amounts of DNA and can utilise much simpler extraction methods.
282 Unfortunately MLVA did not reveal potentially significant differences in the B2
283 subtype which were detected by REA. The clusters in the M2 subtype also
284 did not correspond to REA subtypes and they show no links to animal origin.

Both REA and MLVA methods demonstrated differences at the subserovar level which may have important implications for our understanding of the epidemiology of infection by the Australis serogroup of *Leptospira* in the UK and consequently on control strategies present and future.

While there was insufficient balance, both in the number of isolates from each species and in the geographical spread of isolates to draw firm conclusions on some of the issues, there are strong indications of regional and species variations. The spectrum of variation found in the NI isolates was much smaller – 4 REA subtypes (Table 2) - than that found in GB – 7 REA subtypes (Table 3). This could have been anticipated as there is a greater diversity and number of wildlife present in the latter and the isolates came from a much greater geographical area – from Scotland to the south of England. Analysed isolates designated as B1 by REA and as cluster B1 by MLVA were only found in NI. Also isolates designated as B2b by REA have only been found in NI, whereas B2a isolates were distributed across the UK. Within REA type M2 and MLVA cluster M2 there were subtypes which are found throughout the UK – REA subtype M2a and MLVA cluster M2(5), however there were subtypes which were only found in GB – REA types M2b to M2f and MLVA clusters M2(3), M2(4) and M2(6).

As well as regional differences, there were important differences in the distribution among animal species. REA type B2b was only found in pigs (Table 2). In the absence of other identified hosts it is reasonable to conclude that these strains are maintained by pigs. These strains have been associated with reproductive wastage (Ellis et al., 1991) and therefore control

309 measures for such strains should be directed towards control in the pig
310 population.

311 There are other isolates of Bratislava designated B2a, which have been
312 associated with clinical disease in pigs, but which this study has shown to be
313 present in horses, dogs and a variety of wildlife. It is impossible to draw firm
314 conclusions as to the role of each species in the epidemiology of B2a
315 infections. It is possible that this is a fairly ubiquitous infection, which is
316 maintained by a variety of domestic and wildlife hosts. It is also possible that
317 within this grouping there are clusters of isolates, which show particular host
318 adaptation, but which the methodologies used were unable to delineate.

319 Isolates of serovar Muenchen – REA and MLVA type M2 – were found across
320 a range of wild and domestic animal species. Hathaway et al. (1983c)
321 believed that wood mice and voles were the maintenance host for such
322 strains and while the information from the REA study of GB isolates would
323 support the view of a rodent maintenance host or hosts (Table 3), the NI
324 situation is clearly different. Voles are absent from NI and culture studies of
325 wood mice in NI have failed to detect the presence of Bratislava/Muenchen
326 infection (Ellis, unpublished data). It is reasonable to conclude that in NI,
327 Muenchen (REA type M2a) is pig maintained.

328 Strains, which so far, have only been isolated from wildlife, designated M2b to
329 M2f (Table 3, Figure 4), may not be important as potential domestic animal
330 pathogens.

331 There is a need to control Bratislava/Muenchen infections in pig herds
332 (Hayden, 2000). Ideally control measures should be effective against pig

333 maintained strains (Bratislava REA types B2a and B2b and Muenchen type
334 M2a) plus strains of B2a and M2a maintained by other hosts, particularly as
335 16 pigs included in the study had mixed serovar infections or mixed genotype
336 infections of serovar Bratislava. The recovery of both serovars or two
337 genotypes from one animal indicates that natural infection by one serovar or
338 genotype may not always confer immunity to infection by the heterologous
339 strain. Options for controlling infection in pigs include: 1) Eliminating infection
340 from herds and subsequently preventing its reintroduction and; 2) Reducing or
341 preventing clinical disease.

342 Evidence so far would indicate that option 1 is impossible with the tools
343 currently at the industry's disposal. Attempts to eliminate infection and
344 establish disease free nucleus populations through the use of snatch
345 farrowing or hysterectomy have failed, presumably due to the fact that *in utero*
346 transmission may be an important feature of infection. Similarly attempts to
347 use antibiotic therapy with or without one of the above procedures have failed
348 (Ellis, unpublished data). Antibiotics have been used to affect a
349 microbiological cure for other leptospiral infections in pigs with variable results
350 (Ellis, 2012). Even if it was possible to eliminate infection from a herd,
351 preventing its re-introduction would be very unlikely to succeed, since it would
352 depend on the preventing direct or indirect contact with other infected animals
353 or establishing a solid microbiological immunity in the herd.

354 Preventing contact with horses and dogs are attainable objectives but
355 preventing contact with rodents in the context of a large piggery is
356 impracticable. Stopping the introduction of infected pigs is also impossible

357 since as already described medication of an animal will not give a
358 microbiological cure and there are no tests available to accurately determine
359 whether a pig is infected. In many infections, serological testing gives a good
360 indication of an animal's infectivity status but in Bratislava/Muenchen
361 infection, serological status gives no indication as to whether a pig is infected.
362 This is particularly so for boars which are the animals most likely to be
363 introduced to a breeding herd (Ellis et al., 1986a).

364 Option 2 is a more practical solution. Tetracyclines have been widely used as
365 in-feed medication, sometimes in combination with systemic penicillin and
366 streptomycin therapy. This improves both fertility and fecundity but has led to
367 problems with inadequate observation of withdrawal times ,development of
368 resistant strains of the animal and human pathogens and cause failure of
369 antibiotic therapy in clinical situations

370 Vaccines could be an important means of delivering option 2 and have the
371 potential to contribute to delivering part of option 1, namely the reintroduction
372 of infection. Bratislava vaccines are available in a number of countries in
373 North America and Europe but are not licensed for use in the UK. These are
374 believed to be produced against serovar Bratislava type strain (Jez Bratislava,
375 genotype B1), originally isolated in eastern Europe from a hedgehog. Only
376 three of the isolates in the present study had the B1 profile and none were
377 from pigs (Table 2). There is little published information on the efficacy of
378 such vaccines to prevent infection. Ellis et al. (Ellis et al., 1989) found that
379 pigs challenged with a B2a strain 6 weeks after vaccination with a B1 vaccine
380 did not become infected although they did show a transient bacteraemia.

381 Disappointingly, pigs challenged six months after vaccination were not
382 protected and became infected. The development of a vaccine which would
383 prevent infection should be considered. Vaccines which produce up to 12
384 months protection against Bratislava infection have been developed for dogs
385 (Klaasen et al., 2014; Wilson et al., 2013). Also effective vaccines have been
386 developed against heterologous species, namely *L.interrogans* and *L.*
387 *borgpetersenii* of serovar Hardjo in cattle (Ellis, 1999b) so it should be
388 possible to do this for strains of Bratislava and Muenchen.

389 While the high seroprevalences reported in horses from around the world
390 would suggest that horses maintain Bratislava infection, no clear-cut horse
391 maintained genotype has been found in this study (Tables 2, 3 and 5). Recent
392 studies on *Leptospira* Bratislava / Muenchen isolates from European horses,
393 led Arent et al. (2015) to the conclusion that genotypes B1 and M1 may be
394 adaptated to this species.

395

396 The majority of UK strains belonged to the ubiquitous REA type B2a (11 of
397 14) and MLVA Cluster 2 per Figure 5 (9 of 11 tested) suggesting that the
398 horse is particularly susceptible to Bratislava/Muenchen strains maintained by
399 other hosts which share its environment but is not necessarily a maintenance
400 host. It would appear to be much more susceptible to Bratislava infection
401 than other domestic herbivores (cattle and sheep) who are also exposed to
402 wildlife infections (Arent et al., 2015).

403 Only one genotype has been recovered from dogs supporting a possible
404 maintenance host role for dogs however this genotype (B2a) was ubiquitous,

being recovered from a range of hosts. This could possibly be a reflection of the inability of the REA techniques used to differentiate strains within the B2a genotype. As more sensitive methods become available it may be possible to distinguish dog specific strains within this group. There is some circumstantial evidence to suggest that the dog strains could be unique in that they have proved to be more difficult to isolate and adapt in the laboratory than other B2a strains (Ellis unpublished observation).

The application of other molecular tools might resolve the issues surrounding the role of pigs, dogs and horses in the epidemiology of REA type B2a. For example, a study of Restriction Fragment Length Polymorphisms (RFLP) has shown the value of that method in identifying clonal populations within *Leptospira borgpetersenii* serovar Hardjo (Zuerner et al., 1993). The whole-genome sequencing, which is now available for some *Leptospira* strains, can reveal some sequence differences between serovars Bratislava and Muenchen genotypes, which could have potential value for routine strain typing. However the overriding consideration is to get a meaningful number of pig, dog and horse isolates from GB and other geographical regions to include in such a study.

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Figures

Figure 1. Differentiation of serovar Bratislava genotypes 1 & 2 and serovar Muenchen following digestion with a combination of the restriction enzymes *BglII/HhaI* and electrophoresis in a 0.6% agarose gel for 19.5 hours at 65 volts.

Lane 1. Molecular weight marker (Kb); Lane 2. 82/1532 ex pig (B2); Lane 3. 78/886 ex hedgehog (B1); Lane 4. Bratislava type strain – Jez Bratislava (B1); Lane 5. Muenchen type strain – München C90 (M1). Differentiation is in the bands between arrows a and b.

Figure 2. Differentiation of serovar Bratislava genotypes B1, B2a, B2b and serovar Muenchen 2a with restriction enzyme *ClaI* and subsequent electrophoresis in an 0.5% agarose gel for 23.5 hours at 70 volts.

Lane 1. MW marker (Kb); Lane 2. 82/960 ex horse (M2a); Lane 3. 80/304 ex horse (B1); Lane 4. 82/1056 ex dog (B2a); Lane 5. 82/1532 ex pig (B2b). Differentiation is in the region between arrows a and b and between c and d.

Figure 3. Differentiation of serovar Muenchen genotypes 1 and 2 using restriction enzyme *HhaI* with subsequent electrophoresis in a 0.6% agarose gel for 24 hours at 70 volts.

Lane 1. MW markers; Lane 2. 82/764 ex pig (M2); Lane 3. Muenchen type strain – München C90. Differentiation is in the area between arrows a and b.

Figure 4. Differentiation of serovar Muenchen genotype 2 following cutting with *HpaII* and electrophoresis in a 0.7% agarose gel for 23 hours at 70 volts.

Lane 1. MW marker (Kb); Lane 2. OW303/28 ex wood mouse (M2a); Lane 3. M116 ex badger (M2b); Lane 4. M331 ex hedgehog (M2b); Lane 5. M767 ex squirrel (M2c); Lane 6. M229/3 ex mink (M2d); Lane 7. DM63 ex wood mouse (M2e); Lane 8. LC85/24 wood mouse (M2f); Lane 9. 82/960 ex horse (M2a). Differentiation is in the area between arrows a and b.

Figure 5. UPGMA Cluster Analysis based on MLVA profile. The number of isolates with the same profile in brackets.

TABLES

Table 1. Animal species from which original isolates were made and the region from which they came.

Animal species	Northern Ireland	Great Britain	Total –UK
Pig	155	2	157
Cattle	2		2
Horse	13	1	14
Sheep	2	2	4
Dog	20	6	26
Badger	1	6	7
Fox		1	1
Mink		4	4
Hedgehog	2	7	9
Wood mouse		11	11
Rat	1		1
Squirrel		1	1
Vole		10	10
TOTAL	196	51	247

Table 2. REA types of N. Ireland isolates

Animal species	B1	B2a	B2b	M2a
Pig		36	64	55
Cattle		2		
Sheep		2		
Dog		20		
Horse	2	10		1
Hedgehog	1	1		
Rat		1		
Badger		1		
TOTAL	3	73	64	56

Table 3. REA types of GB isolates

Species	Geno-type									
	B1	B2a	B2b	M1	M2a	M2b	M2c	M2d	M2e	M2f
Badger						3		2	1	
Fox		1								
Hedgehog		5				1				1
Mink		1						3		
Wood mouse					7				3	1
Vole					9				1	
Squirrel							1			
Horse		1								
Dog		6								
Sheep		2								
Pig					2					
TOTAL		16			18	4	1	5	5	2

Table 4. MLVA profiles of the type strains.

REA type	Sample ID	V4	V7	V9	V10	V11	V19	V23	V27	V29	V30	V31	V36	V50
Australis	Type strain	1	6	10	13	3	1	7	4	9	7	6	15	8
Lora	Type strain	1	11	3	9	2	3	4	9	1	4	1	11	4
Jalna	Type strain	4	11	3	9	2	10	6	5	3	4	1	13	4
Bratislava	Type strain	1	10	3	9	2	11	11	7	8	4	1	9	1
Muenchen	Type strain	2	10	3	9	2	11	6	8	15	2	1	10	4

Table 5. MLVA profiles of UK Bratislava and Muenchen

Cluster	Number of strains	REA	Animal (numbers)	V4	V19	V23	V27	V29	V30	V36	V50
1	2	B1	Hedgehog(1), horse (1)	1	11	11	7	8	4	9	1
2	35	B2a	Dog(7), fox(1), hedgehog(4), horse(9), pig(4), rat(1), sheep(4)	2	11	9	8	10	4	9	4
		B2a	Hedgehogs(2)	3	11	9	8	10	4	9	4
		B2b	Pig(3)	2	11	9	8	10	4	9	4
3	7	M2a	Pig(1)	1	13	6	8	15	2	12	4
		M2d	Badger(1)	2	13	6	8	15	2	11	4
		M2d	Badger(1), mink(3)	1	13	6	8	15	2	11	4
		M2e	Badger(1)	1	13	6	8	15	2	11	4
4	3	M2b	Badger(2)	1	13	6	4	15	2	11	4
		M2c	Squirrel(1)	1	13	6	4	15	2	11	4
5	11	M2a	Horse(1), pig(5), vole(1),	1	13	5	8	15	2	11	4
		M2a	Pig(1)	1	13	5	8	15	2	13	4
		M2e	Vole(1), wood mouse(2)	1	13	5	8	15	2	11	4
6	7	M2a	Wood mouse(5)	1	13	5	8	15	2	11	1
		M2f	Hedgehog(1)	1	13	6	8	15	2	11	1
		M2a	Vole(1)	1	13	5	1	15	2	11	1

*All had identical profiles for V7(10), V9(3), V10(9), V11(2) and V31(1)

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4

Figure 5