sciendo

J Vet Res 67, 529-536, 2023
DOI: 10.2478/jvetres-2023-0063

The serological and genetic diversity of the *Leptospira interrogans* Icterohaemorrhagiae serogroup circulating in the UK

Zbigniew Arent[1]✉, Colm Gilmore[2], Laura Pardyak[1], Klaudia Dubniewicz[1], Barry McInerney[2], William Ellis[2]

[1]University Centre of Veterinary Medicine, Centre of Experimental and Innovative Medicine, University of Agriculture in Krakow, 30-248 Kraków, Poland
[2]Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stormont, Belfast BT4 3SD, UK
zbigniew.arent@urk.edu.pl

Received: June 12, 2023 *Accepted: November 6, 2023*

Abstract

Introduction: Strains of *Leptospira interrogans* belonging to two very closely related serovars, Icterohaemorrhagiae and Copenhageni, have been associated with disease in mammalian species and are the most frequently reported agents of human leptospirosis. They are considered the most pathogenic serovars and represent more than half of the leptospires encountered in severe human infections. **Material and Methods:** Nineteen such isolates from the United Kingdom – human, domestic and wildlife species – were typed using three monoclonal antibodies (F12 C3, F70 C14 and F70 C24) in an attempt to elucidate their epidemiology. They were further examined by restriction endonuclease analysis (REA), multiple-locus variable-number tandem repeat analysis (MLVA) and lic12008 gene sequence analysis. **Results:** Monoclonal antibody F12 C3, which is highly specific for Icterohaemorrhagiae and Copenhageni, confirmed that all the strains belonged to these two serovars. Sixteen strains were identified as Copenhageni and three as Icterohaemorrhagiae serovar. Only one restriction pattern type was identified, thus confirming that REA is not able to discriminate between the Icterohaemorrhagiae and Copenhageni serovars. Variable-number tandem-repeat analysis found three loci with differences in the repeat number, indicating genetic diversity between British isolates. Sequences of the lic12008 gene showed that all isolates identified as the Icterohaemorrhagiae serotype have a single base insertion, in contrast to the same sequences of the Copenhageni serotype. **Conclusion:** Copenhageni is the predominant serovar in the Icterohaemorrhagiae serogroup isolated in British Isles. There is a genetic diversity of MLVA patterns of the isolates but no genetic tool used in the study was able to determine serovars.

Keywords: *Leptospira*, leptospirosis, Icterohaemorrhagiae, Copenhageni, animal pathogens.

Introduction

Leptospirosis is an emerging major public health problem in many countries and is one of the most widespread zoonoses in the world. It is an excellent example of the rightness of the "One Health" approach, where the relationship between humans, animals and ecosystems needs be considered in order to better understand and manage a disease (20). The disease is caused by infection with pathogenic spirochetes of the *Leptospira* genus, which can chronically infect domestic and wild animal species and in particular small rodents – being harboured in their renal tubules before being shed into the environment *via* their urine.

The *Leptospira* genus is currently subdivided into 68 genomically distinct *Leptospira* species (5). Besides this taxonomical division, members of the genus are also divided according to the serological similarity of surface-exposed epitopes as determined by cross-agglutinin absorption tests (CAAT); over 300 pathogenic serovars have been identified. Of all the pathogenic genospecies, *Leptospira interrogans* is the most frequently reported, with the Icterohaemorrhagiae serogroup representing more than half of the leptospires encountered in human infections (33, 38).

In Europe, the terms "Weil's disease" and "Icterohaemorrhagiae infection" have become synonymous with the acute icteric form of leptospirosis

transmitted by the brown rat, *Rattus norvegicus*. This has arisen since Weil originally described the disease in humans and the first isolations of the causative organism from clinical cases were shown to be what is now recognised as *Leptospira interrogans* serovar Icterohaemorrhagiae. In 1938 Borg-Peterson (29) described two antigenic subtypes of this serovar represented by the RGA and M20 strains. Each of these subtypes has since been given the status of serovar: RGA has remained Icterohaemorrhagiae and M20 has been named Copenhageni. The majority of strains isolated from acute cases of leptospirosis in high risk areas such as the favelas of large South American cities have been shown to be serovar Copenhageni (21).

The Icterohaemorrhagiae serogroup is currently composed of at least 16 serovars. They are primarily found in rats, which act as a maintenance host for them (6, 7, 27). Serovar identification is rarely performed because the serological methods used to distinguish between serovars is highly time-consuming, and consequently the causative organism of rat-transmitted leptospirosis is loosely referred to as "Icterohaemorrhagiae infection" or "Copenhageni infection". Rat-borne strains also cause acute disease in animal species other than humans (11, 13).

Pathogenic *Leptospira* species, the aetiological agents of leptospirosis, infect a broad spectrum of hosts, annually causing 1 million human cases and 60,000 deaths globally (10). In the United Kingdom, leptospirosis and the particularly severe cases presenting as Weil's disease are uncommon, but almost half such cases are attributed to the Icterohaemorrhagiae serogroup (15) after serogroup-specific serological tests. The situation is similar throughout Europe. Little is known about the strains that are circulating. Isolation and identification of the causative organism are rarely performed because of the fastidious nature of the bacteria and the difficulty of its isolation from cultures. Modern bacterial taxonomy is based on genomic analysis of the organisms. However, mainly for epidemiological reasons, it is still necessary to identify the serological status of *Leptospira* strains. It helps in understanding the immune response and is important for routine diagnostic purposes and vaccine-specific immunisation. The adaptation of strains to animal species is also at the serovar or sub-serovar level. Therefore, both genetic and serological typing are still of great practical importance. Whole-genome sequencing (WGS) requires sophisticated tools – both to carry out the WGS itself and among the resources needed to link the WGS data to typing and taxonomy (16). For this reason, there is an ongoing requirement for methods that can be routinely used in research laboratories to identify strains and show phylogenetic differences between them. Therefore, this study aimed to characterise 19 *Leptospira* isolates identified as belonging to the Icterohaemorrhagiae serogroup. The studied bacteria were retrieved from humans and wild and domestic animals in the United Kingdom using both serological and molecular typing methods in order to identify the dominating serovar and show genetic differences between serovars.

Material and Methods

Strains and cultures. Nineteen UK isolates belonging to the Icterohaemorrhagiae serogroup were included in this study (Table 1). They were recovered from wildlife, domestic animals and humans. The reference strains of 16 serovars belonging to the Icterohaemorrhagiae serogroup used in the study are presented in Table 2. All of the strains were propagated in liquid Ellinghausen, McCullough, Johnson and Harris medium at 29°C and used after 7–10 days' growth.

Table 1. Source and origin characteristics of the Icterohaemorrhagiae serogroup *Leptospira interrogans* isolates used in this study

Strain identifier	Host	Tissue of origin	Clinical symptoms	Serovar	GenBank accession No.
23112	Human	Blood	Anorexia, fever	Copenhageni	
S72	Horse	Foetal kidney	Abortion	Copenhageni	GCA_017653945.1
X240	Dog	Kidney	Anorexia, fever, prostration, death	Icterohaemorrhagiae	GCA_017653925.1
R287	Cow	Foetal kidney	Abortion	Copenhageni	GCA_017653725.1
AB102	Cow	Foetal kidney	Abortion	Copenhageni	
S606	Cow	Foetal lung	Abortion	Copenhageni	GCA_017653855.1
W26/2	Hedgehog	Kidney	Asymptomatic	Copenhageni	GCA_017653845.1
B33	Badger	Kidney	Asymptomatic	Copenhageni	GCA_017653825.1
B42	Badger	Kidney	Asymptomatic	Copenhageni	GCA_017653785.1
B126	Badger	Kidney	Asymptomatic	Icterohaemorrhagiae	GCA_017654005.1
B167	Badger	Kidney	Asymptomatic	Copenhageni	GCA_017653985.1
S1006	Badger	Kidney	Asymptomatic	Copenhageni	GCA_017654105.1
S199/2	Brown rat	Kidney	Asymptomatic	Icterohaemorrhagiae	GCA_017653805.1
S199/3	Brown rat	Kidney	Asymptomatic	Copenhageni	GCA_017654025.1
S199/4	Brown rat	Kidney	Asymptomatic	Copenhageni	GCA_017653755.1
S199/5	Brown rat	Kidney	Asymptomatic	Copenhageni	GCA_017654055.1
S199/6	Brown rat	Kidney	Asymptomatic	Copenhageni	GCA_017653965.1
S199/7	Brown rat	Kidney	Asymptomatic	Copenhageni	
S199/12	Brown rat	Kidney	Asymptomatic	Copenhageni	GCA_017654085.1

Table 2. List of the Icterohaemorrhagiae serogroup *Leptospira* reference strains used in the restriction endonuclease analysis

Species	Serogroup	Serovar	Strain	Country	Source
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	Belgium	Human
L. interrogans	Icterohaemorrhagiae	Copenhageni	M20	Denmark	Human
L. interrogans	Icterohaemorrhagiae	Monymusk	LT 75-68	Jamaica	Rat
L. interrogans	Icterohaemorrhagiae	Budapest	PV-1	Hungary	Laboratory rat
L. interrogans	Icterohaemorrhagiae	Mankarso	Mankarso	Indonesia	Human
L. interrogans	Icterohaemorrhagiae	Naam	Naam	Indonesia	Human
L. interrogans	Icterohaemorrhagiae	Birkini	Birkin	Malaysia	Human
L. kirschneri	Icterohaemorrhagiae	Bogvere	LT 60-69	Jamaica	Rat
L. kirschneri	Icterohaemorrhagiae	Dakota	Grand River	USA	Water
L. interrogans	Icterohaemorrhagiae	Gem	Simon	Sri Lanka	Human
L. interrogans	Icterohaemorrhagiae	Lai	Lai	China	Human
L. kirschneri	Icterohaemorrhagiae	Mwogolo	Mwogolo	Zaire	Human
L. kirschneri	Icterohaemorrhagiae	Ndambari	Ndambari	Zaire	Human
L. kirschneri	Icterohaemorrhagiae	Ndahambukuje	Ndahambukuje	Zaire	Human
L. interrogans	Icterohaemorrhagiae	Smithi	Smith	Malaysia	Human
L. borgpetersenii	Icterohaemorrhagiae	Tonkini	LT 96-68	Vietnam	Human

Serological typing: microscopic agglutination test with group sera and monoclonal antibodies. Serological identification of the strains was initially performed by cross-agglutination. In this procedure a microscopic agglutination test was carried out using a panel of 17 *Leptospira* antisera against 15 major pathogenic serogroups (12, 37). The *Leptospira* serogroups tested included Australis (serovars Australis and Bratislava), Autumnalis, Ballum, Canicola, Cynopteri, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Mini, Pomona, Pyrogenes, Sejroe (serovars Saxkoebing and Hardjo) and Tarassovi. Rabbit sera were prepared as described by the World Health Organization (37). Monoclonal antibody (mAb) typing was carried out using the three mAbs F12C3, F70C14 and F70C24 (Royal Tropical Institute (KIT), Amsterdam, the Netherlands) in a panel that characteristically agglutinates serovars of the Icterohaemorrhagiae serogroup. The F12C3 antibody is specific for both serovars, while F70C14 is so only for Icterohaemorrhagiae and F70C24 only for Copenhageni (26).

Species-specific PCR. PCR analyses were performed with seven species-specific primer pairs as described by Reitstetter (32). The primer sets amplified the *ompL1* gene sequences for unique non-homologous DNA sequences representing *Leptospira* species: *L. borgpetersenii*, *L. kirschneri*, *L. santarosai*, *L. noguchii*, *L. weilii* and *L. interrogans*, which splits into two distinct groups (group A with *L. interrogans* serogroups Icterohaemorrhagia, Wolffi, Grippotyhosa, Autumnalis and group B with *L. interrogans* serogroups Australis, Canicola, Hebdomadis, Paidjan, Lai and Pyrogenes). Amplification was performed in a final volume of 25 µL containing 12.5 µL of Jumpstart REDTaq ReadyMix for High Throughput PCR (final concentrations: 20 mM of Tris-HCl, pH 8.3, 100 mM of KCl, 4 mM of $MgCl_2$, 0.002% gelatin, 0.4 mM of each dNTP, 0.03 U/µL *Taq* polymerase and Jumpstart Taq antibody) (Sigma-Aldrich, St. Louis, MO, USA), 120 nM of forward and reverse primers, 2.0 µL of DNA extract and nuclease-free water to make up the final volume. Thermal cycling was as follows: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 7 min. For negative controls, nuclease-free water was used instead of the DNA template.

Restriction endonuclease analysis. The reference strains and field isolates were further identified by restriction endonuclease analysis (REA). Preparation of DNA, restriction endonuclease digestion, electrophoresis and gel analysis was carried out as described by Ellis *et al.* (14). All restriction enzymes were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA), and the reaction conditions were performed as recommended by the manufacturer. Twenty-seven restriction enzymes were screened for cleavage of leptospiral DNA of the two serovars Icterohaemorrhagiae and Copenhageni (*Eco*RI, *Bgl*II, *Alu*I, *Hha*I, *Hind*III, *Eco*RV, *Bst*EII, *Xho*I, *Pst*I, *Sst*II, *Bcl*I, *Bgl*I, *Sma*I, *Tha*I, *Cla*I, *Taq*I, *Sph*I, *Sau*3AI, *Hpa*II, *Hae*III, *Dde*I, *Bgl*III, *Dra*I, *Pru*I, *Sal*I, *Sav*96I and *Xma*III). Complete digestion and the best results were obtained with *Eco*RI, *Hha*I, *Hpa*II, *Alu*I, *Hae*III and *Dde*I; consequently, all strains were examined by using *Eco*RI and *Hha*I.

Multiple-locus variable-number tandem repeat. Multiple-locus variable-number tandem repeat analysis was carried out using 13 sets of primers (V4, V7, V9, V10, V11, V19, V23, V27, V29, V30, V31, V36 and V50) (28, 35). One change was made to the V29 forward primer sequence as transcribed in the original paper (35) – a sequence of only four thymines near the 3′ end instead of five was used as this produced more significant alignments with *Leptospira interrogans* sequences logged in GenBank. A second change was necessary to the reported V11 reverse primer sequence – a thymine was substituted for the adenine at the 3′ end. All the PCRs to identify multiple loci of variable-number tandem repeats (VNTR) were

performed using the HotStarTaq DNA Polymerase Kit (Qiagen, Hilden, Germany). The PCR reaction mix consisted of 2.5 µL of Qiagen 10 × buffer, 5.0 µL of 5 × Q Solution, 1.5 µL of 25 mM $MgCl_2$, 120 nM of forward and reverse primers, 240 µM of Sigma dNTP mix, 1.0 µL of the template DNA and sterile water to make the volume up to 25 µL. The reactions were set up individually for each VNTR locus. The amplification protocol consisted of a hot start at 95°C for 15 min; 35 cycles of denaturing at 95°C for 30 s, annealing at 52°C for 40 s and extension at 72°C for 40 s; and a final extension at 72°C for 7 min. Negative controls (reagents only without DNA) were included each time the PCR reaction was performed. The positive control was the M20 strain of *Leptospira interrogans*, serovar Copenhageni. The products of the PCR were confirmed by electrophoresis on gels containing 2% (wt/vol) agarose (Invitrogen) in tris/boric acid/ethylenediaminetetraacetic acid buffer and stained with ethidium bromide (Invitrogen). A 100 bp DNA ladder (Invitrogen) was run as a size marker. Gels were visualised using UV transillumination and photographed and digitised using Kodak ID Image Analysis software version 3.6.1 (Eastman Kodak, Rochester, NY, USA). The software was used to accurately determine the size of the amplicons. Data was recorded in an Excel 2003 spreadsheet (Microsoft, Redmond, WA, USA). Distance trees were constructed using the unweighted pair group method with arithmetic means and plotted as dendrograms using Sequence Type Analysis and Recombinational Tests (START) software version 1.0.5 (http://pubmlst.org/software/analysis) (31).

Analysis of *lic12008* sequences. The genomes of the 16 field strains described in this study were sequenced as a part of another (paper in preparation) using next-generation sequencing (NGS), and whole-genome reads were deposited in GenBank. The accession numbers are listed in Table 1. The sequences of the *lic12008* gene were downloaded for comparative analysis using basic local alignment search tool searches against a reference strain.

Results

Serological characterisation. Serological characterisation carried out with polyclonal antibodies indicated that the field isolates belonged to the Icterohaemorrhagiae serogroup (data not shown). This was confirmed by monoclonal antibody testing: all field strains were agglutinated by the F12C3 mAb which specifically agglutinates both the Icterohaemorrhagiae and Copenhageni serovars. Only three strains (15.8%) gave positive reactions with the F70C14 mAb, which is specific for the Icterohaemorrhagiae serovar, but sixteen (84.2%) agglutinated with anti-Copenhageni F70C24 (Tables 1 and 3).

Species-specific PCR. The species-specific PCR gave a clear *ompL1*-derived DNA product with an electrophoretic mobility corresponding to a size of 396 bp with the Intergroup A primer set. No DNA products were generated using the primer pairs for specifically amplifying DNA from intergroup B, *L. borgpetersenii*, *L. santarosai*, *L weilii* or *L. noguchii* (data not shown). It may be concluded that all 19 strains belonged to the *L. interrogans* species.

Table 3. Reciprocals of agglutination titres of a panel of three monoclonal antibodies with reference strains of the Copenhageni (M20) and Icterohaemorrhagiae (RGA) serovars of *Leptospira interrogans* and two field strains of the bacteria. The rest of the field strains gave the same results as those two representatives of each serovar

Serovar	Strain	Monoclonal antibody		
		F12C3	F70C14	F70C24
Copenhageni	M20	10,000	-	10,000
Copenhageni	AB102	10,000		10,000
Icterohaemorrhagiae	RGA	30,000	10,000	-
Icterohaemorrhagiae	X240	30,000	10,000	-

Restriction endonuclease analysis. To choose the best restriction enzyme for REA analysis, chromosomal DNA from the M20 and RGA reference strains was digested with 27 endonucleases. Each enzyme gave the same DNA pattern for those two serovars (data not shown). *Eco*RI and *Hha*I gave complete digestion with good results and they were chosen to compare the DNA profiles for sixteen reference strains from the Icterohaemorrhagiae serogroup (Fig. 1) and 19 field strains used in the study (data not shown). Most of the serovars from the Icterohaemorrhagiae serogroup had a unique restriction fragment length polymorphism pattern, and only four serovars had patterns which were alike: Copenhageni, Icterohaemorrhagiae, Monymusk and Budapest (Fig. 1, lanes 1, 2, 3 and 4).

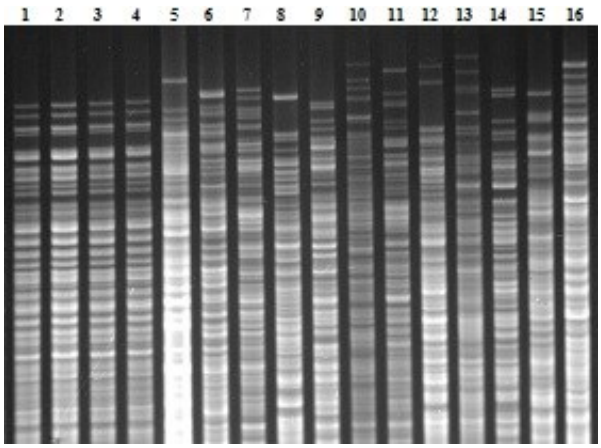

Fig. 1. Restriction endonuclease analysis patterns of chromosomal DNA from *Leptospira* strains digested with *Eco*RI. Lanes: 1 – Icterohaemorrhagiae (RGA strain); 2 – Copenhageni (M20 strain); 3 – Monymusk (LT 75-68 strain); 4 – Budapest (PV-1 strain); 5 – Mankarso (Mankarso strain); 6 – Naam (Naam strain); 7 – Birkini (Birkin strain); 8 – Bogvere (LT 60-69 strain); 9 – Dakota (Grand River strain); 10 – Gem (Simon strain); 11 – Lai (Lai strain); 12 – Mwogolo (Mwogolo strain); 13 – Ndambari (Ndambari strain); 14 – Ndahambukuje (Ndahambukuje strain); 15 – Smithi (Smith strain); 16 – Tonkini (LT 96-68 strain)

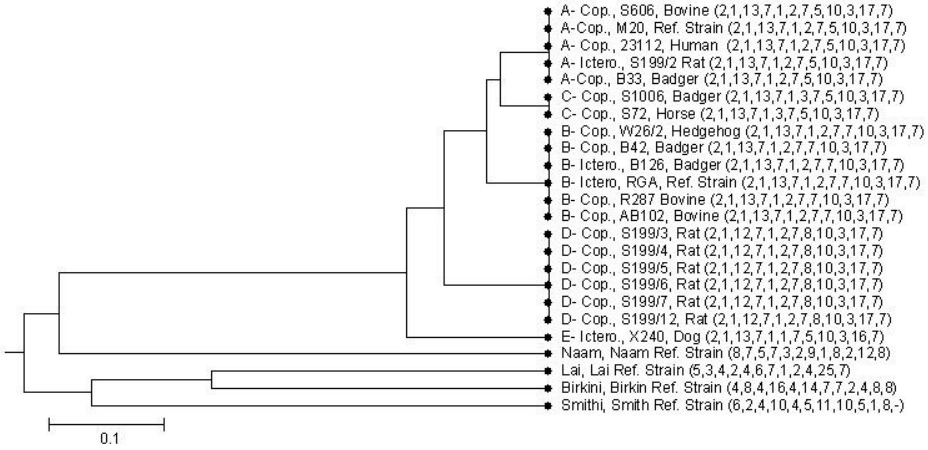

Fig. 2. Unweighted pair-group method with arithmetic mean (UPGMA) tree based on the 12 variable-number tandem-repeat (VNTR) loci (V4, V7, V9, V10, V11, V19, V27, V29, V30, V31, V36 and V50). As only a partial repeat was present in V23 for all studied isolates, this locus was omitted in the UPGMA cluster analysis. Different VNTR profiles of 19 isolates revealed 5 distinct genotypes (A to E)

The *Eco*RI and *Hha*I restriction patterns of the 19 isolates were compared with reference strains. All of them showed the same *Eco*RI and *Hha*I restriction patterns, which were compatible with the DNA profile of the four indistinguishable reference strains (Icterohaemorrhagiae, Copenhageni, Monymusk and Budapest).

Multiple-locus variable-number tandem repeat. Unweighted pair-group method with arithmetic mean cluster analysis of MLVA profiles divided the isolates into five clusters, which were assigned a letter designation from A to E (Fig. 2). Genotypes A, B and D were dominant, C was found twice, but E only once. Isolates from different hosts were distributed across several genotypes.

Sequences of *lic12008*. A comparison of *lic12008* gene sequences showed that all isolates identified as the Icterohaemorrhagiae serovar had a single base insertion of a thiamine nucleotide, resulting in a completely different translation from Copenhageni strains. The mutation caused the codon reading after the mutation to encode other amino acids and caused the first stop codon encountered in the sequence to appear in the additional sequence. The resulting polypeptide may have been abnormally short and was unlikely to be functional.

Discussion

This study described serological and molecular characterisation of strains of the Icterohaemorrhagiae serogroup isolated in the United Kingdom. Classification to serogroup level based on cross-agglutination tests using group specific hyperimmune sera showed strong cross-reactivity between the isolates and the Icterohaemorrhagiae serovar but little cross-reactivity with the other 15 serogroups tested, indicating that all 19 strains belonged to the Icterohaemorrhagiae serogroup. It is known that Icterohaemorrhagiae serogroup strains can belong to different *Leptospira* species (38), but in this study species identification by PCR analysis of the *ompL1* gene confirmed that all UK isolates belonged to *Leptospira interrogans.*

In *Leptospira* classification, the serovar is a basic taxon, and identification of isolates to serovar level is essential for epidemiological studies and the development of appropriate preventive measures. The classical method for identifying *Leptospira* serovars is the CAAT (12); however, it is prohibitively time-consuming and costly for the rapid examination of large numbers of isolates and is used mainly on new strains representing new serovars or problematic isolates (30). Since the 1980s, REA has been widely used as a supplementary or alternative method to classify *Leptospira* cultures. Although there is a poor correlation between the serological and most genotypic classification systems, REA was proved to be a sensitive, reproducible technique for the identification of isolates to serovar or even subserovar level (1–4, 9, 36). In our study 16 Icterohaemorrhagiae serogroup reference strains were used to identify the digesting enzyme and discriminate between serovars. The results revealed that most of the serovars had a unique restriction fragment length polymorphism pattern when digested with the *Eco*RI and *Hha*I enzymes, and only four serovars, namely Copenhageni, Icterohaemorrhagiae, Monymusk and Budapest had patterns with strong mutual resemblance. Further REA typing of the field strains used in the study showed the same *Eco*RI and *Hha*I restriction patterns for all tested isolates, and they were compatible with the DNA profiles of those four very closely related serovars. These results correlated with those of some previous studies, revealing difficulties in genetic discrimination between those strains (8, 17–19). Moreover, using monoclonal antibody typing, Kobayashi *et al.* (22, 23) also showed a very close antigenic similarity of those strains, which led the authors to put those four serovars

in one subgroup. Studies using a panel of monoclonal antibodies prepared by Korver *et al.* (26) found that the F12C3 mAb was able to reduce the group of highly similar serovars from four to two, namely Icterohaemorrhagiae and Copenhageni. Further typing using F70C24 and F70C14, each specific only for one serovar, revealed that 16 of the isolates were identified as serovar Copenhageni and only 3 as serovar Icterohaemorrhagiae. No association between serovar and specific animal species was observed.

Recent whole-genome sequencing analysis of 67 isolates belonging to the *L. interrogans* Copenhageni and Icterohaemorrhagiae serovars identified 1,072 SNPs and 258 indels (34). The phylogenetic analysis during this study revealed that both serovars are closely related but showed distinct spatial clustering. In the genetic era of bacterial identification, many molecular tools have been employed as alternatives to classical methods for the identification and classification of *Leptospira* or to evaluate genetic diversity among strains, such as multiple-locus variable-number tandem repeat analysis and multilocus sequence typing (MLST). In our studies we employed MLVA, which was shown to have a higher discriminatory power and was more concordant with serotyping than MLST for *L. interrogans* (24, 25). Analysis of 13 MLVA loci revealed genetic diversity between the studied isolates and their clustering into five different types. Strains belonging to the dominant groups A and B corresponded to the profile of both reference strains. Group E was constituted only by rat isolates, which were typed as serovar Copenhageni and had a unique VNTR profile consistent at loci V9 and V29. One of the rat isolates (S199/2) belonged to the Icterohaemorrhagiae serovar; however, its genetic profile was the same as that of strain M20, which is the reference strain for the Copenhageni serovar. When MLVA results were compared with those of the monoclonal antibody typing, this diversity did not correspond to the serovar status of the isolates. This is in agreement with a recent study conducted in China, where 128 serogroup Icterohaemorrhagiae strains were analysed using another genetic technique – MLST – and it was found that some isolates belonged to the same sequence type (ST) but different serovars. Conversely, some serovars were usually associated with more than one ST (38). This further substantiates the non-correlation of such genetic diversity to serological status and the unsuitability of serovars as indicators of genetic relatedness. The diversity of serovars is most likely to be due to horizontal gene transfer, even within these closely related examples. The studies referred to earlier of 67 different strains of *L. interrogans* of the Copenhageni and Icterohaemorrhagiae serovars, which showed 1,072 SNPs and 258 indels, only found one insertion of the *lic12008* gene within the Icterohaemorrhagiae serotype, which may be important in differentiating these two serovars (34). This was also confirmed in our study.

However, the biological significance of the other genetic variations found in our studies is unclear. They could be useful in epidemiological tracking analysis and have potential application in the understanding of leptospiral molecular epidemiology. Clonality studies in outbreaks and rapid determination of the source of infection is critical in limiting the spread of infection and important to the development of appropriate prevention strategies. Zhang *et al.* (38) showed some clustering of serogroup Icterohaemorrhagiae strains from specific geographical regions in China and that the genetic diversity of Chinese strains was generally different to that of other countries. When disease outcome is considered rather than geography, there is no apparent difference between the Icterohaemorrhagiae and Copenhageni serovars. The genetic diversity revealed between strains nevertheless undoubtedly affords a better understanding of the diversity of those which are pathogenic and could also help identify the genotypes responsible for severe, often lethal infections (11).

In summary, the results demonstrate that monoclonal antibody typing is a useful method for discriminating between these two closely related serovars and is more cost effective than other methods used. Restriction endonuclease analysis which is based on the whole *Leptospira* genome does not reveal any differences in the DNA profiles of either reference strains or studied field isolates. In contrast to REA, MLVA revealed the diversity of VNTR loci among the strains, but this was not concordant with the serotyping results. Additionally, differences were brought to light in the *lic12008* gene sequence which may be helpful in serovar identification. The results of the study indicate that the Icterohaemorrhagiae and Copenhageni serovars have both been isolated in UK. Most of those tested in the study (84.2%) were identified as the Copenhageni serovar. These data may help identify better options for infection prevention measures and contribute to the epidemiological characterisation of common strains circulating among infected animals and humans.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was funded by the financial resources of the Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast, UK and was also funded and supported by the National Science Centre in Poland under grant number 2019/33/B/NZ9/02159.

Animal Rights Statement: None required.

References

1. Arent Z.J., Frizzell C., Gilmore C., Allen A., Ellis W.A.: *Leptospira interrogans* serovars Bratislava and Muenchen animal infections: Implications for epidemiology and control. Vet Microbiol 2016, 190, 19–26, doi: 10.1016/j.vetmic.2016.05.004.
2. Arent Z.J., Gilmore C., Ayanz J.M.S., Neyra L.Q., Garcia-Pena F.J.: Molecular Epidemiology of *Leptospira* Serogroup Pomona Infections Among Wild and Domestic Animals in Spain. Ecohealth 2017, 14, 48–57, doi: 10.1007/s10393-017-1210-8.
3. Arent Z.J., Gilmore C., Barlow A.M., Smith L., Ellis W.A.: *Leptospira interrogans* serogroup Pomona infections in the UK: is there a real threat for farm animals? Vet Rec 2017, 180, 424, doi: 10.1136/vr.103891.
4. Arent Z.J., Gilmore C., Brem S., Ellis W.A.: Molecular studies on European equine isolates of *Leptospira interrogans* serovars Bratislava and Muenchen. Infect Genet Evol 2015, 34, 26–31, doi: 10.1016/j.meegid.2015.07.009.
5. Arent Z.J., Pardyak L., Dubniewicz K., Płachno B., Kotula-Balak M.: Leptospira taxonomy: then and now. Med Weter 2022, 78, 489–496, doi: 10.21521/mw.6694.
6. Boey K., Shiokawa K., Rajeev S.: *Leptospira* infection in rats: A literature review of global prevalence and distribution. PLoS Negl Trop Dis 2019, 13, doi: 10.1371/journal.pntd.0007499.
7. Brem S., Radu O., Bauer T., Schönberg A., Reisshauer K., Waidmann R., Kopp H., Meyer P.: Leptospira infected rat population as probable cause of a case of fatal Weil's disease (in German). Berl Munch Tierarzt Wochenschr 1995, 108, 405–407.
8. Collares Pereira M., Korver H., Thi B.V.C., Santos-Reis M., Bellenger E., Baranton G., Terpstra W.J.: Analysis of *Leptospira* isolates from mainland Portugal and the Azores islands. FEMS Microbiol Lett 2000, 185, 181–187.
9. Corn J.L., Swiderek P.K., Blackburn B.O., Erickson G.A., Thiermann A.B., Nettles V.F.: Survey of selected diseases in wild swine in Texas. J Am Vet Med Assoc 1986, 189, 1029–1032.
10. Costa F., Hagan J.E., Calcagno J., Kane M., Torgerson P., Martinez-Silveira M.S., Stein C., Abela-Ridder B., Ko A.I.: Global Morbidity and Mortality of Leptospirosis: A Systematic Review. Plos Negl Trop Dis 2015, 9, doi: 10.1371/journal.pntd.0003898.
11. de Oliveira N.R., Kremer F.S., de Brito R.S.A., Zamboni R., Dellagostin O.A., Jorge S.: Pathogenesis and Genomic Analysis of a Virulent *Leptospira interrogans* Serovar Copenhageni Isolated from a Dog with Lethal Infection. Trop Med Infect Dis 2022, 7, doi: 10.3390/tropicalmed7110333.
12. Dikken H., Kmety E.: Chapter VIII: Serological Typing Methods of Leptospires, in: *Methods in Microbiology* Volume II, Academic Press, London, 1978, pp. 259–307.
13. Ellis W.A.: Animal Leptospirosis. In: *Current Topics in Microbiology and Immunology, Volume 387, Leptospira and Leptospirosis*, edited by B. Adler, Springer, Berlin, 2015, pp. 99–137, doi: 10.1007/978-3-662-45059-8_6.
14. Ellis W.A., Montgomery J.M., Thiermann A.B.: Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of *Leptospira interrogans*. J Clin Microbiol 1991, 29, 957–961, doi: 10.1128/jcm.29.5.957-961.1991.
15. Forbes A.E., Zochowski W.J., Dubrey S.W., Sivaprakasam V.: Leptospirosis and Weil's disease in the UK. QJM 2012, 105, 1151–1162, doi: 10.1093/qjmed/hcs145.
16. Guglielmini J., Bourhy P., Schiettekatte O., Zinini F., Brisse S., Picardeau M.: Genus-wide Leptospira core genome multilocus sequence typing for strain taxonomy and global surveillance. PLoS Negl Trop Dis 2019, 13, doi: 10.1371/journal.pntd.0007374.
17. Herrmann J.L., Baril C., Bellenger E., Perolat P., Baranton G., Saint Girons I.: Genome conservation in isolates of *Leptospira interrogans*. J Bacteriol 1991, 173, 7582–7588, doi: 10.1128/jb.173.23.7582-7588.1991.
18. Herrmann J.L., Bellenger E., Perolat P., Baranton G., Saint Girons I.: Pulsed-Field Gel Electrophoresis of NotI Digests of Leptospiral DNA - A New Rapid Method of Serovar Identification. J Clin Microbiol 1992, 30, 1696–1702, doi: 10.1128/jcm.30.7.1696-1702.1992.
19. Hookey J.V., Palmer M.F.: A comparative investigation and identification of *Leptospira interrogans* serogroup icterohaemorrhagiae strains by monoclonal antibody and DNA fingerprint analyses. Zentralbl Bakteriol 1991, 275, 185–199, doi: 10.1016/s0934-8840(11)80065-4.
20. Jancloes M., Bertherat E., Schneider C., Belmain S., Munoz-Zanzi C., Hartskeerl R., Costa F., Denis J., Benschop J.: Towards a "One Health" Strategy against Leptospirosis. In: *Planet@Risk 2(3), Special Issue on One Health (Part I/II),* Global Risk Forum, Davos, 2014, pp. 204–206.
21. Ko A.I., Reis M.G., Dourado C.M.R., Johnson W.D.Jr., Riley L.W.: Urban epidemic of severe leptospirosis in Brazil. Lancet 1999, 354, 820–825, doi: 10.1016/s0140-6736(99)80012-9.
22. Kobayashi Y., Tamai T., Sada E.: Serological analysis of serogroup Icterohaemorrhagiae using monoclonal antibodies. Microbiol Immunol 1985, 29, 1229–1235, doi: 10.1111/j.1348-0421.1985.tb00912.x.
23. Kobayashi Y., Tamai T., Sada E., Imoto A.: Antigenic analysis of serogroup Icterohaemorrhagiae using monoclonal antibodies. Isr J Vet Med 1987, 43, 277–287.
24. Koizumi N., Izumiya H., Mu J.J., Arent Z.J., Okano S., Nakajima C., Suzuki Y., Muto M.M., Tanikawa T., Taylor K.R., Komatsu N., Yoshimatsu K., Ha H.T.T., Ohnishi M.: Multiple-locus variable-number tandem repeat analysis of *Leptospira interrogans* and *Leptospira borgpetersenii* isolated from small feral and wild mammals in East Asia. Infect Genet Evol 2015, 36, 434–440, doi: 10.1016/j.meegid.2015.08.013.
25. Koizumi N., Morita M., Nuradji H., Susanti, Noor S.M., Dharmayanti N., Randusari P., Mu J.J., Solante R.M., Saito N., Ariyoshi K., Ha H.T.T., Wada T., Akeda Y., Miura K.: Comparative genomic analysis of *Leptospira* spp. isolated from *Rattus norvegicus* in Indonesia. Infect Genet Evol 2022, 102, doi: 10.1016/j.meegid.2022.105306.
26. Korver H., Kolk A.H.J., Vingerhoed J., van Leeuwen J., Terpstra W.J.: Classification of serovars of the Icterhaemorrhagiae serogroup by monoclonal antibodies. Isr J Vet Med 1988, 44, 15–18.
27. Krojgaard L.H., Villumsen S., Markussen M.D.K., Jensen J.S., Leirs H., Heiberg A.C.: High prevalence of *Leptospira* spp. in sewer rats (*Rattus norvegicus*). Epidemiol Infect 2009, 137, 1586–1592, doi: 10.1017/s0950268809002647.
28. Majed Z., Bellenger E., Postic D., Pourcel C., Baranton G., Picardeau M.: Identification of Variable-Number Tandem-Repeat Loci in *Leptospira interrogans* Sensu Stricto. J Clin Microbiol 2005, 43, 539–545, doi: 10.1128/JCM.43.2.539-545.2005.
29. Marshall R.B., Winter P.J., Yanagawa R.: Restriction Endonuclease DNA Analysis of *Leptospira interrogans* Serovars *icterohaemorrhagiae* and *hebdomadis*. J Clin Microbiol 1984, 20, 808–810.
30. Paiva-Cardoso M.D., Arent Z., Gilmore C., Hartskeerl R., Ellis W.A.: Altodouro, a new *Leptospira* serovar of the Pomona serogroup isolated from rodents in northern Portugal. Infect Genet Evol 2013, 13, 211–217, doi: 10.1016/j.meegid.2012.09.013.
31. Pavan M.E., Cairo F., Pettinari M.J., Samartino L., Brihuega B.: Genotyping of *Leptospira interrogans* strains from Argentina by Multiple-Locus Variable-number tandem repeat Analysis (MLVA). Comp Immunol Microbiol Infect Dis 2011, 34, 135–141, doi: 10.1016/j.cimid.2010.06.002.
32. Reitstetter R.E.: Development of species-specific PCR primer sets for the detection of *Leptospira*. FEMS Microbiol Lett 2006, 264, 31–39, doi: 10.1111/j.1574-6968.2006.00431.x.

33. Salaün L., Merien F., Gurianova S., Baranton G., Picardeau M.: Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. J Clin Microbiol 2006, 44, 3954–3962, doi: 10.1128/JCM.00336-06.
34. Santos L.A., Adhikarla H., Yan X., Wang Z., Fouts D.E., Vinetz J.M., Alcantara L.C.J., Hartskeerl R.A., Goris M.G.A., Picardeau M., Reis M.G., Townsend J.P., Zhao H., Ko A.I., Wunder E.A.Jr.: Genomic Comparison Among Global Isolates of *L. interrogans* Serovars Copenhageni and Icterohaemorrhagiae Identified Natural Genetic Variation Caused by an Indel. Front Cell Infect Microbiol 2018, 8, 193, doi: 10.3389/fcimb. 2018.00193.
35. Slack A.T., Dohnt M.F., Symonds M.L., Smythe L.D.: Development of a Multiple-Locus Variable number of tandem repeat Analysis (MLVA) for *Leptospira interrogans* and its application to *Leptospira interrogans* serovar Australis isolates from Far North Queensland, Australia. Ann Clin Microbiol Antimicrob 2005, 4, 10, doi: 10.1186/1476-0711-4-10.
36. Thiermann A.B., Ellis W.A.: Identification of leptospires of veterinary importance by restriction endonuclease analysis. In: *Current Topics in Veterinary Medicine and Animal Science, Volume 36, The present state of leptospirosis diagnosis and control,* edited by W.A. Ellis and T.W.A. Little, Martinus Nijhoff, Dordrecht, 1986, pp. 91–104.
37. World Health Organization: *Human Leptospirosis: Guidance for Diagnosis Surveillance and Control*, WHO, Geneva, 2003.
38. Zhang C., Yang H., Li X., Cao Z., Zhou H., Zeng L., Xu J., Xu Y., Chang Y.-F., Guo X., Zhu Y., Jiang X.: Molecular Typing of Pathogenic *Leptospira* Serogroup Icterohaemorrhagiae Strains Circulating in China during the Past 50 Years. PLoS Negl Trop Dis 2015, 9, doi: 10.1371/journal.pntd.0003762.