Multiple-locus variable-number tandem repeat analysis and clinical characterization of *Leptospira interrogans* canine isolates

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Canine leptospirosis occurs worldwide; however, information on the relationship between *Leptospira* serotypes/genotypes and virulence in dogs remains limited. We investigated the molecular characteristics of *Leptospira interrogans* canine isolates belonging to three serogroups using multiple-locus variable-number tandem repeat analysis (MLVA) and the effects of each serotype/genotype on the clinical characteristics of leptospirosis in dogs. MLVA using 11 loci of the three major *L. interrogans* serogroups in Japan, Australis (32 strains from 21 dogs), Autumnalis (12; 7) and Hebdomadis (66; 39), revealed more divergent genetic heterogeneity within each serogroup than multilocus sequence typing (MLST), and they formed two, three and five clusters (CLs), respectively. Lethal infections were caused by all *Leptospira* serogroup isolates (70.3 % with Hebdomadis, 83.3 % with Australis and 100 % with Autumnalis) or *Leptospira* isolates belonging to all the CLs (57.1–100 %) without any significant differences. A significant difference in hyperaemia and haemorrhage of mucus membrane was observed between serogroups Australis and Autumnalis (*P* < 0.03). *Leptospira* isolates of Australis CL2 caused no hyperaemia and haemorrhage from mucus membrane, whereas those of Australis CL1, Autumnalis CL3 and Hebdomadis CL1 and CL3 did (*P* < 0.05). Significant differences in creatinine (Cre) levels were observed between serogroups Australis and Hebdomadis (*P* = 0.02). In addition, significant differences in blood urea nitrogen levels were observed between serogroups Australis and Hebdomadis (*P* = 0.004) and Australis and Autumnalis (*P* = 0.02). Based on MLVA types, a significant difference in Cre levels was observed between Hebdomadis CL1 and CL4 (*P* = 0.0018). Our results indicated that MLVA had a higher discriminatory power and was more concordant with serotyping than MLST. Although all *Leptospira* serotypes and genotypes caused lethal infections in dogs, the *L. interrogans* serogroup Australis strains were more likely to cause severe kidney damage than Autumnalis and Hebdomadis, which may be more critical to the outcome of infected dogs than haemorrhage. Our results also suggest that the virulence mechanisms and target organs in dogs may differ by *Leptospira* genotype.

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by infection with pathogenic spirochaetes, *Leptospira* spp., which affects almost all mammals (Bharti et al., 2003; Faine et al., 1999). Canine leptospirosis is widespread throughout the world and dogs serve as incidental hosts for various leptospiral serovar strains as well as maintenance hosts for *Leptospira interrogans* serovar Canicola (Sykes et al., 2011). In incidental infections, dogs exhibit acute or subacute hepatic and renal failure, which historically has been associated with the serovars Canicola and Icterohaemorrhagiae (Goldstein,
2010). However, because of the widespread use of bivalent vaccines comprising these serovars and the increased contact between dogs and wildlife reservoirs in expanding suburban environments, new L. interrogans serovars such as Grippotyphosa and Pomona have become common in North America (Gautam et al., 2010; Stokes et al., 2007).

Serovars Icterohaemorrhagiae and Pomona have been most commonly associated with severe hepatic dysfunction, whereas serovar Canicola is more likely to cause acute interstitial nephritis with less hepatic involvement (Wohl, 1996; Langston & Heuter, 2003). An infection with serovar Grippotyphosa is typically associated with minimal liver involvement (Langston & Heuter, 2003). A serological study indicated that serogroup Pomona caused more severe renal disease and was associated with worse outcomes in dogs than diseases caused by Autumnalis or Grippotyphosa (Goldstein et al., 2006). However, except for these four serovars, characterizations of the Leptospira isolates causing clinical disease in dogs are limited. In addition, the differences in virulence between Leptospira genotypes in dogs remain unknown.

We recently characterized L. interrogans isolated from dogs with acute leptospirosis in Japan by conventional serology and multilocus sequence typing (MLST) (Koizumi et al., 2013). MLST revealed that there was genetic heterogeneity in the major serogroups in Japan, i.e. Australis, Autumnalis and Hebdomadis, and suggested that the virulence of serogroup Hebdomadis in dogs may be associated with its genotypes. On the other hand, the same sequence types determined by MLST were found in multiple serogroups, indicating that MLST was not suitable for serovar (or serogroup) identification. Recently, multiple-locus variable-number tandem repeat analysis (MLVA) of L. interrogans was demonstrated as a useful tool for identifying serovars or discriminating strains within the same serovar (Majed et al., 2005; Slack et al., 2006; Zuerner & Alt, 2009).

In this study, we characterized L. interrogans isolates obtained from blood samples of dogs with acute leptospirosis by MLVA using 11 loci and assessed the associations of Leptospira serotypes and genotypes with the outcomes, clinical manifestations and blood test results of infected dogs.

METHODS

Leptospira isolates. In total, we characterized 112 Leptospira strains, which were isolated from 69 dogs with acute leptospirosis by blood culture using liquid Korthof’s medium containing 10% rabbit serum and/or Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Faine et al., 1999). Leptospira isolates were obtained from nine prefectures (Chiba, Shizuoka, Mie, Fukuoka, Saga, Kumamoto, Miyazaki, Kagoshima and Okinawa) of Japan during the period from August 2007 to March 2013; 73 strains were derived from our previous study (Koizumi et al., 2013) and 39 strains were isolated in this study. We also used 25 Leptospira serovar strains from five serogroups as references for MLVA (Table S1, available in the online supplementary material). The serogroups of the isolates obtained in this study were identified by the microscopic agglutination test using a panel of anti-Leptospira rabbit sera for 18 serovars (Koizumi et al., 2013). Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). All isolates were identified as the species L. interrogans by DNA sequencing of the flaB gene (data not shown).

MLST for the isolates was performed as described by Thaipadungsanan et al. (2007), and sequence types were assigned based on the MLST database (http://leptospira.mlst.net/).

Fourteen dogs had a vaccination history with either a trivalent vaccine (serovars Canicola, Copenhageni and Hebdomadis) or a bivalent vaccine (serovars Canicola and Icterohaemorrhagiae).

MLVA. MLVA was performed using 11 loci: variable number tandem repeat (VNTR)-4, -7, -10 (Salatin et al., 2006), -19 (Majed et al., 2005), -23 (Zuerner & Alt, 2009), -31 (Majed et al., 2005), -27, -29, -30, -36 and -51 (Slack et al., 2006) for the isolates. The 25 Leptospira serovar strains were also subjected to MLVA as reference. The PCR conditions used were as follows: after an initial 1 min denaturation step at 95 °C, the reaction mixture was subjected to 32 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 1.5 min. The amplified products were analysed by 2% agarose gel electrophoresis and their sizes were estimated by comparisons with a 100 bp ladder DNA marker. Some of the amplified products were directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the PCR primers. The sizes of PCR products were converted to repeat copy numbers for analysis using a categorical multi-coefficient and unweighted pair group method using arithmetic averages clustering algorithms with BioNumerics software, version 6.6 (Applied Maths). Simpson’s index of diversity was calculated as previously described (Grundmann et al., 2001).

Statistical methods. Statistical comparisons of mortality rates between males and females and vaccination rates between dead and recovered animals were made by the Chi-squared test or Fisher’s exact test. Clinical characteristics and laboratory data were compared between serogroups or MLVA types using the Chi-squared test or Kruskal–Wallis test. Post-hoc pairwise comparisons were made using Fisher’s exact test or Wilcoxon rank-sum test. A P value of <0.05 was considered significant; Bonferroni-corrected significance levels were used for post-hoc comparisons.

RESULTS AND DISCUSSION

Genotyping and cluster analysis of L. interrogans isolates by MLVA

Leptospira isolate typing is essential for epidemiological studies, identifying reservoir animals and developing appropriate preventive measures. Serotyping remains important because each serovar is usually associated with a particular host (Bharti et al., 2003). However, determining serovars by the cross-agglutination absorption test (CAAT) is cumbersome and time-consuming, and thus the usage of molecular methods has been explored. PFGE is useful for identifying leptospiral serovars (Herrmann et al., 1992), but the resulting complex patterns may be challenging for inter-laboratory pattern comparisons. MLST has also been used for Leptospira species genotyping (Boonslip et al., 2013). However, our previous study demonstrated that MLST was unsuitable for serovar (or serogroup) identification and had less discriminatory power than PFGE (Koizumi et al., 2013). Thus, for this study, we used MLVA to type our canine L. interrogans isolates.
In total, we analysed 112 Leptospira-positive cultures, which were obtained from 69 dogs using EMJH and/or Korthof’s media and were included in MLVA using 11 loci. Positive cultures from both EMJH and Korthof's media were obtained from 43 dogs; the strains from only two dogs showed differences in MLVA types from each other (FO11-5E, FO11-5K, MZ08-20E and MZ08-20K in Fig. 1). Thus, clustering analysis was performed for 71 isolates. With this method, the 71 L. interrogans isolates were clustered into 48 different MLVA types with 12 clusters (identity for at least four of 11 loci was considered as the same cluster) and 37 singleton MLVA types (Fig. 1). Although the same sequence types (STs) defined by MLST were found in multiple serogroups previously (Koizumi et al., 2013) and this study, different serogroup strains were clustered into different clusters on the basis of MLVA (Fig. 1). For serogroup Australis, there were 17 MLVA types, which formed two clusters, whereas two STs were detected by MLST (Fig. 1). For serogroups Autumnalis and Hebdomadis, there were six and 23 MLVA types, which were clustered into three and five clusters, respectively, which were more divergent than MLST (Fig. 1). Simpson’s index of diversity with MLVA was 0.972 [95% confidence interval (CI): 0.951–0.993], which was greater than that with MLST (0.695, 95% CI: 0.601–0.789).

All these results indicated that MLVA had a higher discriminatory power and was more concordant with serotyping than MLST. In this study, we characterized the isolates at the serogroup level and were unaware if each cluster represented a serovar. A previous study demonstrated that MLVA for L. interrogans was useful for identifying serovars (Majed et al., 2005), whereas other studies indicated considerable diversity of genotypes defined by MLVA within the same serovar (Slack et al., 2006; Zuerner & Alt, 2009). We performed MLVA on 25 serovar strains and compared their profiles with those of the isolates (Table S1). Serovar Icterohaemorrhagiae strain Icter No.1 formed the same cluster with serogroup Icterohaemorrhagiae isolate CB09-8E (identity: 10/11) and serovar Australis strain Akiyami C was clustered into Australis CL1 (identity: 4/11), which may indicate the isolates’ serovar. The other 23 serovar strains showed unique MLVA types and did not form the same cluster with the isolates obtained in this study. On the other hand, some serovar strains in the same serogroups showed their identity 4/11. Since the VNTRs employed in this study are designed based on the genome sequences of serogroup Icterohaemorrhagiae strains, some of them may be inadequate for the discrimination of other serogroups. Therefore, identification of VNTR loci from other Leptospira serogroups (serovars) is required to improve the discriminatory power of MLVA. Only a few laboratories are capable of performing CAAT to identify serovars, and molecular methods to replace this complex test have been explored (Cerqueira & Picardeau, 2009). More investigations on serovar strains using newly identified VNTRs would verify the availability of MLVA as an alternative to the classic serovar identification method for L. interrogans.

Compared to serogroups Hebdomadis and Australis isolates, Autumnalis isolates appear not to form close clusters. This observation may suggest that this serogroup is polyphyletic, though a larger sample size is needed to evaluate the nature of serogroup Autumnalis.

**Associations of Leptospira serotypes and genotypes with fatality rates, clinical signs and blood test results of infected dogs**

Canine leptospirosis occurs worldwide; however, information on the causative Leptospira serotypes and genotypes and their effects on virulence in dogs remains limited. Associations of some serotypes and the clinical involvement of infected dogs have been described. Infection with serovar Icterohaemorrhagiae or Pomona is commonly associated with severe hepatic dysfunction, whereas serovar Canicola tends to cause acute interstitial nephritis (Wohl, 1996; Langston & Heuter, 2003). Although an association of specific genotypes and enhanced virulence has been suggested in human leptospirosis (Gouveia et al., 2008; Thaipadungpanit et al., 2007), differences in the virulence of Leptospira genotypes in canine leptospirosis remain unknown.

In this study, we investigated the associations of Leptospira serotypes [three major serogroups: Australis (n = 21), Autumnalis (n = 7) and Hebdomadis (n = 37)] and MLVA types (genotypes) in these serogroups; CL1 (n = 11) and CL2 (n = 10) in serogroup Australis; CL1 (n = 2), CL2 (n = 1) and CL3 (n = 4) in serogroup Autumnalis; and CL1 (n = 14), CL2 (n = 1), CL3 (n = 12) and CL4 (n = 10) in serogroup Hebdomadis (no information was obtained for the dog in CL5 and one case of mixed-infection with CL1 and CL3 was excluded) with the outcomes, clinical manifestations and blood test results of infected dogs.

Information on the outcomes of 61 of the 65 dogs, which were included in this analysis, was obtained at 3 months after the first visit (Table 1). Their mortality rate was 77%. There was no significant difference in the mortality rates between males (80%, 32/40) and females (68.4%, 13/19). Furthermore, there was no significant difference in the vaccination rates of serovar Hebdomadis-containing vaccine between dead and recovered animals (12.8%, 6/47 and 23.1%, 2/13, respectively). This indicated that sex and previous vaccination did not affect the outcomes of the infected dogs.

Lethal infections were caused by all three Leptospira serogroup isolates (83.3% with Australis, 100% with Autumnalis and 69.4% with Hebdomadis) or Leptospira isolates belonging to all CLs (57.1–100%), but there were no significant differences in the mortality rates among serogroups or MLVA types (Table 1).

Among four clinical signs recorded, there was a significant difference in hyperaemia and haemorrhage of the mucous membrane between serogroups Australis and Autumnalis (P = 0.03). A multigroup comparison between nine CLs...
Fig. 1. Dendrogram based on MLVA using 11 loci showing relationships between the 71 *L. interrogans* isolates. Strain name contains the place, year, sample number and positive culture medium: for example, strain KM11-3EK indicates that the strain was collected in Kumamoto in 2011, sample no. 3 and isolated from both EMJH and Korthof's media. CB, Chiba; FO, Fukuoka; KM, Kumamoto; KS, Kagoshima; ME, Mie; MZ, Miyazaki; OW, Okinawa; SA, Saga; SO, Shizuoka.
revealed a significant difference in hyperaemia and haemorrhage of mucus membrane ($P=0.002$). Australis CL2 isolates did not cause hyperaemia and haemorrhage from mucus membrane, which was significantly different from the results with Australis CL1, Autumnalis CL3 and Hebdomadis CL1 and CL3 ($P<0.05$, Table 1). However, these significant differences were negated after making Bonferroni corrections.

Among the blood tests used, only data for creatinine (Cre), blood urea nitrogen (BUN), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were obtained from more than half of the infected dogs; these were used for statistical comparisons (Table 2). Significant differences in Cre levels were observed between serogroups Australis and Autumnalis ($P=0.003$), Australis CL2 and Hebdomadis CL4 ($P=0.004$) and Australis and Autumnalis ($P=0.004$). A multigroup comparison between nine CLs showed a significant difference in Cre levels ($P=0.05$). There were significant differences in Cre levels between Australis CL1 and Hebdomadis CL3 ($P=0.03$), Australis CL1 and Hebdomadis CL4 ($P=0.003$), Australis CL2 and Hebdomadis CL4 ($P=0.04$) and Hebdomadis CL1 and CL4 ($P=0.002$). The significant difference between Hebdomadis CL1 and CL4 was the only one that remained after making Bonferroni corrections.

Abnormalities in ALT and ALP values and jaundice (71.4–100%) were observed in all *Leptospira* CLs though there were no significant differences; therefore, there was no clear correlation between hepatic dysfunction and *Leptospira* genotypes. On the other hand, significant elevations in Cre and BUN levels among dogs infected with the *L. interrogans* serogroup Australis indicated that this serogroup was more likely to cause severe renal damage in dogs than Autumnalis or Hebdomadis. Regarding differences in virulence among genotypes, only serum Cre levels between dogs infected with Hebdomadis CL1 and CL4 were significantly different. Significant differences were found between serogroup Australis CLs and those with infections caused by Australis CLs and Hebdomadis CLs in terms of hyperaemia and haemorrhage in mucous membrane and serum Cre levels, although Bonferroni corrections negated these significant differences. In addition, Wilcoxon rank-sum tests indicated some differences in BUN levels among Australis CLs and Autumnalis or Hebdomadis CLs (data not shown), although a multigroup comparison showed no significant differences among the nine CLs. These results suggested that virulence mechanisms may be different among these genotypes. In addition, these results also suggested that renal damage was more critical to the outcome of infected dogs than haemorrhage. These statistical results may have been attributable to the small sample numbers used in this study. Thus, larger sample sizes may be required to verify the association between *Leptospira* genotypes and virulence. Furthermore, analysis on other blood markers as well as metabolome would identify a host marker specific to each *Leptospira* genotype infection.

### Conclusions

The results of this study indicated that MLVA had a higher discriminatory power and was more concordant with serotyping than MLST. Although all *Leptospira* serotypes...
and genotypes caused lethal infections in dogs, *L. interrogans* serogroup Australis strains were more likely to cause severe kidney damage than Autumnalis and Hebdomadis, which may be more critical to the outcomes of infected dogs than haemorrhage. Furthermore, our study results suggest that virulence mechanisms and target organs in dogs may be different based on *Leptospira* genotypes.

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