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Undiagnosed leptospirosis cases in naïve and vaccinated dogs: Properties of a serological test based on a synthetic peptide derived from Hap1/LipL32 (residues 154–178)

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ABSTRACT

Leptospirosis is a common disease in dogs, despite having current vaccinations. However, leptospirosis diagnosis based on the routine Microscopic Agglutination Test (MAT) leads to confusing conclusions, especially for infected vaccinated dogs. Indeed, both bacterin and natural infection stimulate the production of agglutinating antibodies.

In experimentally infected dogs, antibodies against the peptide PP derived from Hap1/Lipl32 were raised earlier than agglutinating antibodies. The background level of these antibodies was determined in a group of 109 healthy dogs, either vaccinated or not against leptospirosis, with a specificity for IgM of 96.4% and for IgG of 95.5%.

PP ELISA was subsequently performed with 118 sera from dogs with suspected leptospirosis that was not confirmed by MAT. New leptospirosis cases based on the PP ELISA results were suspected in 14 out of 102 vaccinated dogs and in two out of 16 non-vaccinated dogs. These results highlight the importance of serological diagnosis corresponding to an interesting window when it is too late for PCR detection and too early to be confirmed by MAT.

1. Introduction

Leptospirosis is one of the most common infectious diseases of canine species worldwide. Vaccination is justified by the mortality rate associated with acute forms of this disease. When infection starts, the host reacts with an immune response for which the typical agglutinating antibodies are specific to the antigenic pattern shared by the infecting strain. The lipopolyosidic determinants triggering this response are particularly numerous in the pathogenic species formerly known as *Leptospira interrogans* "sensu lato". Detection of these agglutinating antibodies *in vitro* by the gold-standard microscopic agglutination test (MAT) has to be performed in specialized laboratories. Therefore, the resulting serological diagnosis is not very convenient in practice.

Dogs are typically vaccinated against only two of the major serogroups (out of approximately 20) – Icterohaemorrhagiae (IH) and Canicola (Can) – and produce agglutinating antibodies. A dog can therefore be affected by leptospirosis even if it has been vaccinated [1,2]. To make an early and appropriate therapeutic decision, veterinarians have

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to discriminate leptospirosis from other diseases (piroplasmosis or parvoviral enteritis, for example), which exhibit similar clinical signs but have very different specific treatments. Veterinarians often try to confirm their clinical diagnosis by MAT. However, post-infection MAT results are confused by post-vaccination agglutination [3].

Due to the recent development of genotypic classification, diagnosis has been feasible by PCR at the beginning of the disease, when the bacteria spread through the blood stream during the first ten days of infection or later when they are eliminated in the urine [4]; however, this has not been helpful in the serological diagnosis of human or animal leptospirosis. A quick and early diagnosis is required to differentiate between vaccinated and/or infected animals [5,6].

Apart from the lipopolyosidic antigens, *Leptospira* spp share many other proteinaceous [7,8] or lipoproteinaceous antigens [9–12]. Some of them are common to the whole *Leptospira* genus (pathogenic and saprophytic), but others are specific to pathogenic *Leptospira* [13,14], such as OMPL1 [15] and Hap1/LipL32 [10,16]. Several of these have been used as coated antigen as an alternative to MAT [17].

Based on bioinformatic analysis and epitope mapping, we selected a particular sequence of Hap1/LipL32 (GenBank: AF366366), remarkable by its poly-aspartic-acid sequence (residues 154–178). The peptide sequence of 25 amino acids, hereafter designated "PP," was chemically synthesized (WO 2004/04855).

Our aim was to study the feasibility of using this synthetic peptide as coated antigen in an ELISA (PP ELISA) similar to the use of the C6 peptide in borreliosis diagnosis [18]. Our goal was to use this antigen for canine sera, but we previously checked the serological response to the typical bacterins in the rodent model (*Meriones unguiculatus*) used in our laboratory. We then studied three groups of dogs for which sera had been previously tested by MAT. Group 1 consisted of laboratory dogs subjected to an experimental challenge to check the humoral response to this peptide after infection (positive controls). Group 2 consisted of healthy dogs obtained from the typically vaccinated canine population and used as a negative (no disease) reference population. Finally, group 3 consisted of clinically suspect pets presenting symptoms suggestive of leptospirosis onset, either vaccinated or not, but for which leptospirosis remained doubtful or had been ruled out by practitioners on the basis of MAT results [3].

2. Methods

2.1. Animals

2.1.1. Gerbils

Three groups of adult gerbils (breeding JANVIER, France) were vaccinated three weeks apart by the subcutaneous route. The first group ($N = 6$) was injected with a canine bivalent vaccine (Icterohaemorrhagiae (IH) and Canicola (Can)). Each animal received a dilution of the canine dose (1:40, as suggested by the European Pharmacopea). The second group ($N = 6$, gerbil positive controls) received by the same route a preparation of the peptide PP linked to a carrier. The third group (gerbil negative controls) received the same volume of PBS. Sera were collected on D0 (time of the first injection), D10, D20, D31, D41 and D66.

2.1.2. Dogs

2.1.2.1. Positive controls (experimentally infected specific-pathogen free (SPF) dogs). Six SPF dogs were subjected to a Canicola challenge infection with a fresh culture of 2×10^8/mL of the virulent Canicola serovar. They received 3 mL of the *Leptospira* culture by the intra-peritoneal route and 0.5 mL by conjunctival instillation [19]. Blood was sampled on the day of infection (D0) and on D3, D7, D10, D14 and D18 post-infection. The sera were then analyzed by MAT and PP ELISA.

All animals were treated according to the Ethical Guidelines of the Veterinary School of Nantes.

2.1.2.2. Reference population (healthy dogs) [3]. In 2006, blood was sampled from 109 healthy dogs without any clinical signs of disease regardless of their vaccination status (the time since their primary vaccination or their last booster ranged from less than three months to more than three years). All animals included in the study were six months to nine years old.

2.1.2.3. Sick and clinically suspect dogs. Leptospirosis was clinically suspected in dogs by vets who identified relevant hematological and biochemical changes. Most of the dogs (101 out of 132) exhibited kidney and liver failures, while the remaining dogs displayed less common signs of leptospirosis, such as reproductive disorders. The veterinarians sent sera from these dogs to the laboratory for serological diagnosis, along with complete clinical data (age, breed, date of last vaccination, date of the first clinical sign, date of sample collection). MAT was performed on these 132 well-documented sera. Leptospirosis was confirmed in 14 cases according to the diagnosis algorithm previously defined in the same laboratory [3]. This algorithm took into account several factors:

(1) Vaccination status (vaccinated for leptospirosis or not).
(2) Time lapse between the last booster and sampling (< or >6 months).
(3) Delay between the onset of the first clinical sign and the sampling time (< or $\geq$10 days).
(4) MAT titers against serogroups included in the bacterins, which in France included IH and/or Can (if vaccinated, < or $\geq$320; if not vaccinated, < or $\geq$40).
(5) MAT titers against other serogroups not included in the bacterins (< or $\geq$40).

PP ELISA was then performed with the 118 sera (102 vaccinated and 16 not vaccinated) for which leptospirosis etiology remained doubtful or was rejected.

2.2. Leptospira

2.2.1. Strains used for the challenge

The dogs were challenged with the pathogenic serovar Canicola strain Moulton as previouslydescribed [20]. Strain