

Recent advances in canine leptospirosis: focus on vaccine development

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Abstract: Leptospirosis is a global infection of humans and animals caused by pathogenic *Leptospira* spp. Leptospirosis is a major zoonosis, with infection acquired from wild and domestic animals. It is also a significant cause of morbidity, mortality, and economic loss in production and companion animals. Leptospirosis in dogs is prevalent worldwide and as well as a cause of canine disease, it presents a zoonotic risk to human contacts. Canine leptospirosis does not differ greatly from the syndromes seen in other animal species, with hepatic, renal, and pulmonary involvement being the main manifestations. While the pathogenesis of disease is well documented at the whole animal level, the cellular and molecular basis remains obscure. Killed, whole-cell bacterin vaccines are licensed worldwide and have not changed greatly over the past several decades. Vaccine-induced immunity is restricted to serologically related serovars and is generally short-lived, necessitating annual revaccination. The appearance of new serovars as causes of canine leptospirosis requires constant epidemiological surveillance and tailoring of vaccines to cover emerging serovars. At the present time, there is no realistic prospect of alternative, non-bacterin vaccines in the foreseeable future.

Keywords: canine leptospirosis, vaccines, diagnosis, epidemiology, pathogenesis

Introduction to leptospirosis

Leptospirosis is almost certainly the most widespread global zoonosis. It has been reported on all continents and in virtually all mammalian species examined.¹ Leptospirosis arises from infection with one of the more than 230 serovars belonging to one of at least ten pathogenic species of the *Leptospira* genus.² As well being a serious human infection with upward of one million severe cases annually, leptospirosis is a major cause of disease in production and companion animals such as dogs, cattle, swine, horses, deer, and probably sheep.³ Leptospirosis is a systemic disease, characterized by fever, renal and hepatic insufficiency, pulmonary manifestations, and reproductive failure. In cattle and pigs, signs of leptospirosis include reproductive failure, abortion, weak piglets or calves, and agalactia. A commonly seen chronic manifestation of leptospirosis in horses presents as recurrent uveitis.⁴ Typical signs of leptospirosis in dogs may include fever, jaundice, vomiting, diarrhea, intravascular disseminated coagulation, uremia caused by renal failure, hemorrhages, and death.^{3,5} Animals that have either recovered from acute infection or have acquired inapparent infection may then go on to become asymptomatic renal carriers for extended periods and shed infectious leptospires into the environment. Susceptible animals can then acquire the infection by either direct or indirect contact with the urine or tissues of infected animals. Other species such as rats, mice, and other rodents may serve as

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reservoirs for their host-adapted serovars, such as Copenhageni in rats and Arborea in mice. They usually do not show signs of infection, but can harbor leptospires in their kidneys for the life of the animal, thereby constituting an important source of infection for other animals or humans. While most human cases of leptospirosis are contracted from farm animals or rodents, the potential of infection from dogs should always be borne in mind;⁵⁻⁹ in particular, vaccinated and/or treated dogs may continue to excrete leptospires in their urine, constituting a human infection risk. For more detailed descriptions of the biology and taxonomy of *Leptospira*, the reader is referred to the appropriate chapter(s) in *Leptospira and leptospirosis*.¹⁰

Pathogenesis of leptospirosis: the basics

At the level of the whole animal, leptospirosis in dogs does not differ from syndromes seen in other animal species. Leptospire enter via mucosal membranes or damaged skin and spread hematogenously throughout the body during a febrile, bacteremic phase, which may last up to several days. When numbers of leptospire in various tissues reach a critical level, characteristic signs appear, such as pulmonary hemorrhage, jaundice due to liver damage, and nephritis due to kidney damage. In general, the appearance of circulating antibodies results in the clearance of leptospire by opsonophagocytosis and/or complement-mediated killing; recovery is usually complete as long as irreversible organ damage has not occurred. The exception to this clearance occurs in the proximal renal tubules, in which leptospire may persist for extended periods of time and can be shed, continuously or intermittently, sometimes for the life of the animal.³

At the molecular and cellular level, the picture is far less clear.¹¹ It would appear to be a sine qua non that leptospire must adhere to host tissues in order to initiate infection. Indeed, a large range of leptospiral proteins has been shown to interact with a large number of host components.¹² Some proteins appear to associate with multiple host proteins; for example, the lipoprotein LipL32 was shown to interact with laminin, fibronectin, and several collagens.¹³ The reverse is also true, with some host proteins apparently binding a multitude of leptospiral proteins; at least 20 leptospiral proteins have been reported to interact with laminin.¹¹ In almost all cases, the studies involved recombinant leptospiral proteins, usually expressed in *Escherichia coli*.¹² While these in vitro studies are clearly real, a caveat should be considered when assessing the biological significance of these reported interactions. Even taking into account the high functional

redundancy within pathogenic *Leptospira* spp.,¹⁴ is it really the case that leptospire possess more than 20 specific, laminin-binding proteins? Genetic studies confirming the absolute requirement or even relevance of these putative adhesins are not yet available. In fact, defined *lipL32* and *ligB* mutants retained virulence and host component binding capacity.^{15,16}

The availability of genome sequences and the development of transposon and directed mutagenesis systems have allowed the identification of a small number of defined virulence factors; these include lipopolysaccharide (LPS), motility, heme oxygenase, catalase, collagenase, the stress proteins ClpB and HtpG, the cell entry protein Mce, and the ApoA1-interacting protein LruA. For a detailed description of these factors the reader is referred to Murray¹¹ and Adler,¹² where the original references may also be found. Comparative genomic analysis has indicated that genes of unknown function are highly overrepresented in the subset of genes unique to pathogenic *Leptospira* spp. Likewise, transcriptomic analyses have found that the majority of genes differentially regulated when leptospire were grown under simulated in vivo conditions were genes of unknown, or poorly defined, function.^{14,17} These findings are consistent with the notion that the *Leptospira* genus possesses unique virulence factors not found in other bacterial species.

Canine leptospirosis: epidemiology and clinical characteristics

The clinical signs of leptospirosis in humans and animals, including dogs, are mostly vague and always non-specific. Therefore, in practice, laboratory tests are needed to achieve a definitive diagnosis. Since isolation of the *Leptospira* spp. bacteria requires specific growth media and skills, and because PCR testing is not yet a routine test, the definitive diagnosis is mostly based on the microscopic agglutination test (MAT; see the “Laboratory diagnosis” section), which is the reference method for serological diagnosis of leptospirosis.¹⁸ As a consequence, most of the information relating to canine leptospirosis worldwide is based on sero-epidemiological studies performed using the MAT. However, direct comparisons between different studies are complicated by the variability in cut-off MAT titers used, ranging from 10 to 800. As is generally applied to large human populations, a low titer would be appropriate in a population in which exposure to leptospirosis is uncommon, but if exposure is frequent, as in most tropical countries, a higher cut-off titer is necessary.¹⁹ Therefore, part of the observed variation between studies in cut-off MAT titers may be attributed to

the presumed level of exposure in the geographic region of the dogs studied. In addition, within their studies, some investigators used two different cut-off titers: a higher titer (eg, 800) for vaccine serovars and a lower titer (eg, 100) for non-vaccine serovars. As explained by Ellis et al,²⁰ the leptospirosis literature has been bedeviled by the question of usage for arbitrary, “significant titers”. They emphasized the high risk of errors in interpretation of results against arbitrary cut-off MAT titers in sero-epidemiological studies, most commonly greater than or equal to 100, when conclusions based solely on MAT titers are drawn with regard to (sub)acute versus chronic infections, infection titers versus vaccine-induced titers, and the identity of the infecting serogroup. The identity of the infecting serogroup may be erroneous in the case of paradoxical reactions in acute phase sera. Provided that the caveats of this assay and the interpretation thereof are taken into account, sero-epidemiological studies can roughly indicate the prevalence of leptospirosis and which serogroups (not serovars²¹) of *Leptospira* spp. are predominant in dogs in a certain geographical area.

Table 1 gives an overview of data on seroprevalence and predominating serogroups from peer-reviewed scientific papers of sero-epidemiological studies in dogs worldwide published in the period 1973–2014. Apart from the caveats described above, the large variety between the published studies in sample size, type of dog population studied, and the number of MAT antigens (serovars) used makes it difficult to draw conclusions. The seroprevalence in randomly sampled groups of privately owned or stray dogs is an indicator of the spread of, and exposure to, *Leptospira* spp. bacteria in these dog populations. The variation in seroprevalence in this category of dogs, even after removal of “low MAT titers possibly induced by vaccine serovars”, appears to be strikingly large: Europe, 13%–57%; North America, 6%–38%; South and Central America and the Caribbean, 7%–62%; Asia and the South Pacific, 7%–46%; and Africa and the Middle East, 5%–27%. Seroprevalence in ten studies was lower than or equal to 15% (Table 1). In seven of these ten studies, at least eleven serovars representing eleven serogroups were used for the MAT, suggesting that most of the low seroprevalences were not due to missing serovars in the MAT. However, the use of only single-serum samples in most studies and the large variation in MAT cut-off titers (20–400 in healthy dogs and 100–3,200 in dogs with acute leptospirosis) are two factors that undoubtedly contribute to the large variation in seropositivity. Despite these limitations and confounding factors, it can be concluded from the reviewed studies that canine leptospirosis is an important

infectious and zoonotic disease in dogs worldwide, with an increasing prevalence of seropositive dogs in time as reported in publications from various countries, including Canada,²² USA,²³ Switzerland,²⁴ and the Netherlands (EM Broens, personal communication, 2015).

As explained earlier in this section, assessment of the predominant infecting serogroups based on only serological tests is unreliable. In the reviewed studies (Table 1), very few serological data were generated with paired serum samples, and high MAT titers in acute sera from patients are unreliable indicators of the infecting serogroup, therefore, the serological results on serogroups in these studies must be interpreted with caution. However, when in a certain region the same serogroup patterns based on representative numbers of dogs are observed repeatedly and are complemented with results of identification of the infecting serovar(s) isolated from clinical cases, assessment of the predominant serovars or serogroups is more reliable.

Table 2 provides an overview of the large variety of clinical pictures and courses of leptospiral infections in dogs. The clinical presentation varies from subclinical disease (probably the majority of cases) and minimal clinical signs to severe renal, hepatic, or pulmonary disease, sometimes with intestinal complications. Whereas formerly it was considered that distinct clinical syndromes were associated with specific serogroups, more intense study over the past 30 years has refuted this hypothesis.¹⁸ Apart from the poor ability of the MAT to predict the infecting serogroup, variations in the host’s immune response and possibly lateral transfer of virulence factors between serovars are other main determinants for the type and severity of clinical disease. Lateral gene transfer has not been demonstrated experimentally in *Leptospira* spp., but appears likely given the high degree of plasticity in leptospiral genomes. In the early years, there was a reported predominance of the classical syndromes: severe hepatic failure and acute renal insufficiency, which were thought to be caused mainly by strains of serogroups Icterohaemorrhagiae and Canicola, respectively. It is unclear whether the introduction in the 1960s in USA and Europe of bivalent vaccines directed against these two serogroups is the main cause of the observed shift in serogroup prevalence in USA and Europe, with serogroups Grippotyphosa, Pomona, and Australis emerging in dogs. In addition, increased awareness by veterinarians and more adequate tests, and subsequently, more frequent recognition of less apparent forms of the disease in dogs may well have contributed to a higher frequency of reported cases of other serogroups and other clinical presentations.

Table 1 Overview of most common serogroups in dogs in different geographical regions and periods based on serological data published between 1973 and 2014

Geographic region	Country	Reference	Year(s) of investigations	Number of sera, % positive ^a	Predominant serogroups, expressed in % of seropositive dogs ^b	Remarks
Europe	Scotland	91	≤1973	120, 39%	Can, 79%; lct, 11%	Vaccinated and non-vaccinated urban dogs
	Germany	92	≤1974	347, 13%	Grip, 56%; lct, 15%; Sej, 15%	Different service and working breeds
	Ireland	93	≤1974	446, 34%	Can, 50%; lct, 46%	Urban and suburban dogs
	the Netherlands	38	1969–1982	1,289, 10%	lct, 76%; Can, 24%	Serum samples submitted for MAT
	Germany	94	1985–1988	993, 31%	Grip + Aus, 28%; Sej, 3%	South Bavaria
				408, 33%	Grip + Aus, 18%; Sej, 6%	North Bavaria and other regions
	Italy	95	≤2002	245, 29%	Aus, 36%; Gri, 19%; Can, 11%; lct, 8%	Privately owned dogs and kennel dogs (most stray dogs)
	Greece	96	≤2003	254, 12%	lct	Randomly selected serum samples
	Germany	97	1999–2002	3,671, 42%	lct; Gri; Can; Aus; Sej ^c	Serum samples submitted for MAT by veterinary practitioners
	France	98	≤2006 (5-year period)	2,457, N/A%	lct, Aus, Sej, Can, Gri ^c	N/A
	Germany	99	1990–2003	42, 100%	Gri, 31%; Sej, 24%; lct, 17%; Can, 12%; Aus, 7%	Dogs diagnosed with leptospirosis
	Germany	100	2003–2006	39, 100%	lct, 26%; Aus, 26%; Sej, 8%; Gri, 5%; Pom, 5%	Dogs diagnosed with leptospirosis
	France	101	2003–2008	37, 100%	lct ^c	Dogs suspected of leptospirosis
	the Netherlands	102	2003	86, 74%	lct, Can ^c	Healthy dogs
North America			2003	26, 85%	lct, Aus ^c	Non-vaccinated dogs in hunting pack
			2009	100, 37%	lct ^c	Dog sera submitted to commercial labs
	Switzerland	103	2007–2008	52, 100%	Aus, Gri, Pom, Aut, lct ^c	Dogs diagnosed with leptospirosis
	Germany	104	2006–2011	329, N/A%	Aus, 24%; Gri, 20%; Pom, 9%	Dogs suspected of leptospirosis
	Switzerland	24	2003–2012	298, 44%	Aus, Gri, Pom, Aut ^c	Dogs suspected of leptospirosis
	France	105	2008–2011	232, 100%	Aus, Gri ^c	Dogs diagnosed with leptospirosis
	Canada	106	≤1976	619, 6%	Aut, 40%; Can, 30%	600 stray dogs; 19 patients
	USA	107	≤1980	433, 38%	lct	Urban stray dogs
				123, 19%	lct	Suburban stray dogs
	Canada	108	≤1991	474, 39%	Can + lct, 14%	Dog sera submitted for MAT:
					Can, 10%; lct, 10%; Aus, 3%	Likely post-vaccination titers
						Post-infection titers
	Canada	22	1998–2001	462, 26%	Aut (paradoxical)	Dog sera submitted for MAT
	Canada	109	1996–2001	1,260, 29%	(≥1:800) Gri; Aus ^c	Dog sera submitted for MAT
North America				15, 100%	(≥1:800) Gri, 100%	Dogs diagnosed with leptospirosis
	USA	110	2001	500, 21%	Gri; lct; Can ^c	Privately owned dogs and patients
	USA	111	1997–2002	470, 11%	Gri, Aus, lct, Pom ^c	Dog sera submitted for MAT
	USA	112	1999–2001	34, 100%	Gri, 14%; Aus, 6%	Dogs diagnosed with leptospirosis
				950, 23%	Gri, 13%; Can, 8%; Aus, 4%	Healthy vacc dogs
				291, 30%	Pom, 40%; Can, 9%; Aus, 5%; Gri, 5%	Healthy non-vacc dogs
	USA	113	1998–2000	43, 100%	Gri, Pom, Aus, Aut ^c	Dogs diagnosed with leptospirosis
	USA	114	2000–2007	33, 119, 8%		Dog sera submitted for MAT

South, Central America; Caribbean	Argentina	115	≤1980	143, 51%	Can, 37%; Pyr, 5%; Aut, 2%	Stray dogs
	Chile	116	≤1996	25, 28% 12, 17% 13, 61%	All dogs: Can, 36%; Pyr, 27%	Dogs diagnosed with leptospirosis Dogs with other diagnosis Dogs without diagnosis
	Barbados	117	≤1997	48, 62% 46, 75%	Aut, 22%; lct, 8%; Aus, 8% lct, 36%; Aus, 13%; Aut, 8%	Healthy control dogs Acutely ill dogs
Asia, South Pacific	Eight countries in this region	118	2002–2014	Per reviewed study: <100 to >400, N/A%	Depending on country: lct, Can, Aut, Tar, Aus, Gri, Pom, Sej, Lou ^c	Different categories of dogs
	Australia	119	1971–1972	600, 7%	lct, 73%; Pom, 15%	Dogs from Sydney
	Fiji	120	≤1984	100, 57%	Bal, 12%; Aus, 11%; lct, 5%	N/A
	Micronesia	121	1995–1996	Unclear	Aus (serovars Aus and Bra)	Majority dogs: farm dogs
	New Zealand	122	≤2002	466, 14%	lct, 62%	Healthy dogs (lower North Island, New Zealand)
	Taiwan	123	2004	241, 46%	She, Can, Aus, lct ^c	Stray dogs
	Thailand	124	2004	210, 11%	Bat, Can, Aus, lct ^c	Randomly selected dog sera (university hospital)
	Australia	125	≤2008	956, 1.9%	Bal, 39%; lct, 22% (percentages of 18 seropositive dogs)	Dogs from different regions in Australia
	Japan	126	2006–2007	801, 27%	lct; Aut ^c	Patients from vet clinics from all 47 prefectures
	Japan	127	2007–2011	58, 52%	Heb, Aus, Aut ^c	Dogs suspected of leptospirosis
Africa; Middle East	India	128	2006–2008	40, 18%	Pyr, 100%	Dogs suspected of leptospirosis
	Egypt	129	≤1973	50, 12%	Can, 83%; lct, 17%	Stray dogs
	Turkey	130	≤2005	116, 44%	Aus, Can ^c	Healthy stray dogs
	Nigeria	131	2005–2006	52, 100%	Gri, Aus, Pom ^c	Privately owned dogs suspected of leptospirosis
	South Africa	132	2008–2009	530, 5%	Can, Pyr ^c	Privately owned dogs and stray dogs
	Zimbabwe	133	≤2012	250, 16%	Not tested (ELISA used instead of MAT)	Randomly selected urban dogs
	Uganda	134	2011	105, 27%	lct, Can, Pyr ^c	Privately owned rural dogs

Notes: ^aPercentage of seropositive dogs, with criterion for seropositivity (MAT cut-off) varying between studies from 1:20 to 1:400 in healthy dogs, and from 1:100 to 1:3,200 in dogs with acute leptospirosis; ^bmixed reactions with same titers against different serogroups not included; ^cpercentages with predominant serogroups not given.

Abbreviations: MAT, microscopic agglutination test; ELISA, enzyme-linked immunosorbent assay; Can, Canicola; lct, Icterohaemorrhagiae; Gri, Grippityphosa; Sej, Sejroe; Aus, Australis; Bal, Ballum; Pom, Pomona; Aut, Autumnalis; Pyr, Pyrogenes; Tar, Tarassovi; Heb, Hebdomadis; Bat, Bataviae; She, Shermani; Bra, Bratislava; Lou, Louisiana; pos, positive; N/A, not available; vacc, vaccinated; non-vacc, not vaccinated.

Table 2 Clinical characteristics of canine leptospirosis

Clinical presentation	Reported frequency of clinical signs	Clinical signs	References
Peracute (<24 hours; rarely diagnosed) ^a	Most frequent	Either sudden death or death preceded by few clinical signs, eg, lethargy/depression, anorexia	9,32,38,135–137
	Less frequent	Lethargy/depression, anorexia, vomiting, diarrhea	
Acute (1–2 days; not often diagnosed)	Most frequent	Lethargy/depression, anorexia, vomiting, muscle weakness, myalgia, death	9,32,33,38,39,48,135–140
	Less frequent	Fever, hypothermia, diarrhea, dehydration, PUPD, jaundice, red eyes, intestinal invaginations	
Subacute (2–7 days; most common form of diagnosed cases)	Most frequent	Lethargy/depression, anorexia, vomiting, fever, hypothermia, dehydration, polyuria and polydipsia, abdominal pain on palpation, diarrhea	6,9,32,33,36,38,39,48,135–140
	Less frequent	Intestinal invaginations, weight loss, muscle weakness, myalgia, coughing, dyspnea, jaundice, pale mucous membranes, petechiae, abortion, death	
Chronic (>1 week; not often diagnosed)	Most frequent	polyuria and polydipsia, jaundice, weight loss, emaciation, anterior uveitis	6,28,36,137
	Less frequent	Petechiae, hemoptysis, ascites, abortion, death	
None = subclinical leptospirosis (probably majority of infected dogs)	Not applicable	Not applicable	25,137

Note: ^aTime after commencement of clinical signs.

Abbreviation: PUPD, polyuria/polydipsia.

In two different studies^{25,26} in a group of clinically healthy laboratory beagle dogs used for research or quality control purposes, an association between subclinical infection with a serovar from serogroup Sejroe and interstitial nephritis were demonstrated. In two other studies with laboratory beagle dogs,^{27,28} it was strongly suggested that a leptospiral infection was the cause of the observed chronic hepatitis. These studies underline the broad spectrum of clinical symptoms of canine leptospirosis and in particular, the contrast between the reported and true prevalence of the chronic as well as subclinical forms of the disease. One of the clinical forms that has been reported more frequently in the last decade is the pulmonary form of canine leptospirosis. These investigations might partly have been triggered by the increasing number of reports of the leptospiral pulmonary hemorrhagic syndrome (LPHS) in humans.^{29–31} In the 1990s, there were two publications of cases of confirmed canine leptospirosis in which a small minority of the dogs showed pulmonary lesions in addition to other pathology.^{32,33} In other studies,^{34,35} pulmonary lesions were observed by radiographic examination in dogs with leptospirosis. In the study of Baumann and Fluckiger³⁵ it was concluded that the pulmonary findings might be misinterpreted as other lung diseases. In recent studies^{36,37} into LPHS-like pulmonary lesions in dogs with leptospirosis, the most important necropsy finding and cause of death was severe, acute, pulmonary hemorrhages. Severe lung involvement in canine leptospirosis is more prevalent than expected based

on clinical examination and causes increased case fatality rates. For a description of pathology findings in animal leptospirosis, the reader is referred to Ellis.³

Clinical diagnosis

Achieving a definitive diagnosis of leptospirosis in dogs should be of special importance to veterinary practitioners because of the zoonotic potential of the disease.^{5–8} In order to prevent misdiagnosis, a complete anamnesis should be carried out, particularly an assessment of potential previous exposure of the dog to sources of *Leptospira* spp. infection. These sources, mostly being urine from chronically infected rodents or other wild maintenance hosts, can vary greatly between geographic regions and seasons. In the past, within the group of privately owned dogs, mainly dogs in rural environments and sporting dogs were considered to be at risk. However, reports from the last decades showed that the risk of exposure to sources of *Leptospira* spp. infection can also be present in urban areas, particularly when dogs live in the vicinity of forests or parks or have free access to standing water. The first difficulty in making the correct diagnosis is that the clinical signs are vague and typically non-specific.⁹ Anorexia, lethargy, and depression are the most predominant, or the only signs. In addition, reluctance to move, abdominal pain on palpation, vomiting, polyuria/polydipsia, and diarrhea are frequently observed in cases of subacute disease. When ileus is suspected based on findings of abdominal palpation, intestinal intussusception as the underlying cause

may be revealed using abdominal radiography. Particularly in young dogs with gastrointestinal complaints and intestinal intussusception, leptospirosis should be included in the differential diagnosis.^{38–40} On initial presentation, overt icterus and fever (associated with the classical icteric form of leptospirosis) are less common. Peracute or acute death (rare and uncommon, respectively) probably occur more often in young dogs.⁴¹ A definitive diagnosis of canine leptospirosis thus requires laboratory confirmation.

Laboratory diagnosis

The laboratory diagnosis of canine leptospirosis does not differ from that used in other animal species or in humans; it is beyond the scope of this review to cover in detail what is readily available elsewhere.^{3,5} As with most bacterial infections, culture of the infecting organism provides a definitive diagnosis. However, in the case of *Leptospira* spp., there are several problems with culture. Pathogenic *Leptospira* spp. require specialized media and are slow growing; cultures therefore require long-term incubation (up to 3 months) with weekly checking by darkfield microscopy for the presence of leptospires. Contamination may also be a problem when culturing from urine or tissues at necropsy. For details of media and culture techniques, see Cameron.⁴² Culture is therefore of very limited use in individual diagnosis. However, for a proper understanding of local epizootiology, it is very important that prevalent serovars are isolated and identified in order to inform the selection of serovars for inclusion in vaccine formulations.

In regions where appropriate equipment and facilities are available, polymerase chain reaction (PCR) offers an alternative to culture. There exists now an extensive literature on the use of PCR to detect leptospiral deoxyribonucleic acid (DNA);^{3,43} procedures are equally applicable to animal and human tissues. A range of qualitative and quantitative PCR protocols and primers has been described, mostly designed to detect all leptospires (eg, *rRNA*, *secY*) or genes unique to pathogenic species (eg, *lipL32*). Sequencing of PCR products can be used to identify the leptospiral species and to drill down further to identify so-called “genotypes”.⁴⁴ There is currently no PCR protocol available that will identify the infecting serovar, although with the availability of genome sequences for all serovars, this may be possible in the near future. The large number of serovars, and therefore LPS biosynthesis loci, suggests that any serovar-specific PCR would need to be tailored for local serovars, again reinforcing the necessity of sound, regional epidemiological data. As with all PCR protocols, the potential presence of inhibitors in animal

tissues must be borne in mind. It is therefore very important to remember that, as for any diagnostic test, a negative PCR does not necessarily exclude the presence of leptospires, nor does a diagnosis of leptospirosis.

Antigen detection or staining methods such as Warthin–Starry and other silver deposition stains, fluorescent antibodies, or immunohistochemistry techniques in general lack sensitivity, but are useful for detecting leptospires in histological sections taken by biopsy or at necropsy (Figure 1). The use of darkfield microscopy on clinical specimens (blood, urine, or other tissues) lacks sensitivity and may give rise to false positives; it is not recommended.

Serological diagnosis remains the most commonly used method, with the MAT still considered the gold standard. Because of its serovar (or at best, serogroup) specificity, good epidemiological knowledge of locally prevalent serovars is critical so that they can be included in the battery of MAT test strains. As with all serological tests, a rise in paired sera is diagnostic. A single high titer (≥ 400) together with appropriate clinical signs (see Clinical Diagnosis section) is highly suggestive. As with other animal species or humans, so-called paradoxical reactions may occur in which the highest titer is not necessarily against the infecting serovar. Serological surveys are therefore unreliable indicators of locally circulating serovars, highlighting the importance of obtaining local isolates for diagnosis and vaccine usage and development. The MAT is problematic for the detection of renal carrier animals, which may have titers below the usually accepted minimal significant value, or may even be sero-negative. The presence of antibodies in the fetal circulation is an indicator of in utero infection.³

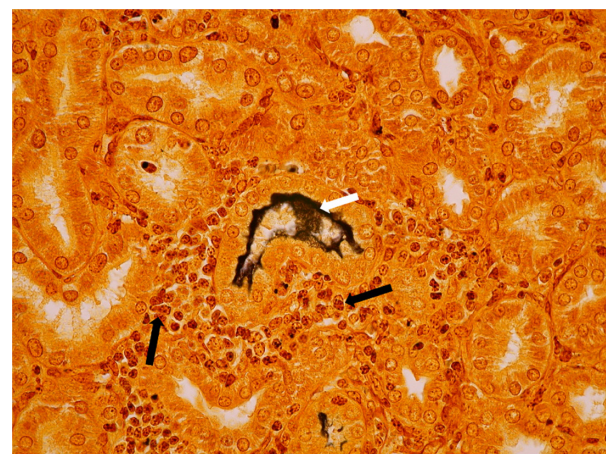


Figure 1 Kidney cortex, transverse section through convoluted tubules, Warthin–Starry staining (magnification 400x).

Notes: Dog from non-vaccinated control group challenged with serovar Canicola. White arrow indicates massive presence of leptospires in lumen of convoluted tubule; black arrows indicate mononuclear infiltration.

The most commonly used alternative to the MAT is the enzyme-linked immunosorbent assay (ELISA). Most published ELISA protocols use either whole cell lysates or conserved recombinant proteins (eg, LipL32, LigA) as antigens. They are therefore genus-specific and provide no information on the infecting serovar. An immunoglobulin M (IgM) ELISA may detect acute infection.⁴⁵ Several commercial ELISA kits for canine diagnosis are available, although proper validation presents a problem because of the imperfect nature of the MAT in situations where paired sera are not available. Other tests such as lateral flow tests⁴⁶ do not seem to have gained widespread use. Older tests such as those using complement fixation, macroscopic slide agglutination, or passive hemagglutination are not recommended.

Treatment strategies

Table 3 provides an overview of the most important treatments of peracute, acute, subacute, and chronic leptospirosis in dogs. Wohl⁴⁷ described the therapeutic management of acute renal failure, which is the most prevalent clinical syndrome in canine leptospirosis, although in some European countries an increased prevalence of severe pulmonary signs has been reported.⁶ In (sub)acute leptospirosis, dogs have to be treated immediately with adequate antibiotics (Table 3) to eliminate the leptospires from the bloodstream. Aggressive supportive therapy can be life-saving for many dogs with severe anuric leptospirosis or other severe conditions. If facilities are available, hemodialysis can be life-saving in cases of severe uremia (also known as azotemia⁴⁸). Ideally, serum biochemistry panels of dogs with acute leptospirosis should be performed every 24 hours during hospitalization to monitor renal function, liver enzyme activities, serum protein concentrations, and electrolyte and acid–base derangements. In chronic leptospirosis, antibiotics (particularly to clear leptospires from the kidneys) and supportive therapy may be needed as well. In particular, treatment of hypertension, one of the systemic complications of chronic renal failure, can be life-saving.

Current perspectives on vaccines and vaccine development

Safe and effective vaccines are one of the most important advances in veterinary medicine in the last 60 years. Licensed inactivated vaccines against canine leptospirosis have been on the market since the 1960s. In the remainder of this review, we will discuss current scientific information will be discussed with regard to efficacy of commercial, inactivated leptospirosis bacterin vaccines; the need for

multivalent bacterins protective against newly emerging serovars; the risk of allergic reactions of dogs to immunization with these bacterins, particularly in small-breed dogs; growth media for bacterin vaccines; and the potential for recombinant vaccines.

Efficacy of current bacterin vaccines

Successful pioneering research on leptospirosis vaccines was performed a century ago in Japan⁴⁹ with guinea pigs. In the following period, a variety of chemical and physical inactivation methods was tested to develop effective bacterins. Some of these methods, in particular formaldehyde, thiomersal, or heat inactivation, have been used for decades for the development and licensing of commercial whole cell vaccines against leptospirosis in dogs, pigs, and cattle. In the 1970s, the focus of leptospirosis vaccine research shifted toward defining the primary structural component(s) responsible for protection.⁵⁰ Various groups tested the efficacy of the outer envelope of leptospires, mainly in hamsters. However, outer envelope vaccines and other inactivated acellular⁵¹ vaccines have not gained widespread support, the main reasons being lack of efficacy, lack of consistency of production, and high production costs. In this period of the 1970s, it had already been demonstrated that a higher dose of immunogen (whole cells, or outer envelope, or other cellular components) was required to protect hamsters or guinea pigs from renal infection than from death.^{52,53} Publications in the 1970s implicated vaccinated dogs as the source of leptospiral infections in humans and have expressed doubt as to the ability of commercial leptospirosis bacterins to protect dogs against renal infection and the carrier state.^{7,54} However, other studies with leptospirosis bacterins demonstrated protection from renal infection in dogs.^{54,55} Huhn et al,⁵⁶ using a commercial vaccine, showed that protection from renal infection in dogs with serovar Canicola or Icterohaemorrhagiae was vaccine dose-dependent. Apart from differences in design of efficacy studies and interpretation of results, an important reason for differences in efficacy results is the immunogenicity of the bacterins used.^{57,58} As in veterinary and human vaccines in general, adjuvants may increase the efficacy of canine leptospirosis vaccines. Since efficacy claims of licensed vaccines, specified in the “Summary of Product Characteristics” and the leaflet of the product,⁵⁹ have to comply with local regulatory requirements (eg, the 9CFR in the US and the European Pharmacopoeia in Europe),⁶⁰ veterinary practitioners are informed about the ability of current commercial vaccines to protect dogs from the renal carrier state.

Table 3 Treatment strategies in different clinical presentations of canine leptospirosis

Speed of onset and progress of clinical signs	Affected organ system	Pathology and disorders	Antibiotic therapy ^a	Supportive therapy ^{a,4,7,48,141-143} *Intravenous fluid replacement therapy/dialysis	Other treatments
Peracute, acute, subacute	Liver Kidneys	Acute hepatitis Acute interstitial nephritis, acute renal failure, uremia (also known as azotemia)	Vomiting/diarrhea (ie, oral medication not effective): ampicillin, 20 mg/kg BW IV every 6 hrs for 2 wks or penicillin G, 25,000–40,000 U/kg BW IV every 12 hrs for 2 wks After vomiting/diarrhea have subsided: doxycycline, 5 mg/kg BW orally every 12 hrs for 2 wks No vomiting/diarrhea: doxycycline, 5 mg/kg BW orally every 12 hrs for 2 wks	Hypoalbuminemia: plasma or colloids Lactate Ringer's solution (≥ 60 mL/kg BW/day) Oliguria: idem with diuretics (eg, furosemide or mannitol) Severe anuria: hemodialysis; hypoalbuminemia: plasma or colloids Pulmonary edema (secondary): plasma or colloids	N/A Pain medication: buprenorphine, 0.01 mg/kg BW IV every 6 hrs
	Lungs	Pulmonary hemorrhages, lung edema			Pulmonary hemorrhages: oxygen therapy (eg, with nasal tube) If severe: mechanical ventilation Uremic gastritis/vomiting: histamine receptor blockers or proton pump inhibitors, orally Intestinal intussusception: manual reduction or intestinal resection Pain medication: buprenorphine, 0.01 mg/kg BW IV every 6 hrs N/A
	Gastro-intestinal tract	Acute gastritis, acute enteritis, intestinal intussusception		N/A	
Chronic	Blood vessels, blood	Vasculitis, thrombocytopenia, disseminated intravascular coagulation, hemorrhages		Coagulation disorders: whole blood or plasma	
	Liver	Chronic hepatitis	When dog is still leptospiruric: doxycycline, 5 mg/kg orally every 12 hrs for at least 2 wks	N/A	N/A
	Kidneys	Chronic interstitial nephritis, chronic renal disease		Hypertension: antihypertensive agents (either against renin- or volume-dependent hypertension) Hypokalemia, hyperphosphatemia: treatment with phosphate-binding salts (preferably calcium) N/A N/A	N/A N/A
	Lungs	None			N/A
	Gastro-intestinal tract	Chronic (uremic) gastritis, chronic enteritis			Uremic gastritis/vomiting: histamine receptor blockers or proton pump inhibitors, orally Prolonged inappetence: enteral or parenteral nutrition N/A
	Blood vessels, blood	None		N/A	

Note: *Caveat: life-threatening fluid overload must be prevented.**Abbreviations:** BW, body weight; IV, intravenous; hrs, hours; wks, weeks; N/A, not applicable; idem, Lactate Ringer's solution.

A specific drawback of leptospirosis bacterins that cannot be solved by an increase of the amount of protective antigen is the efficacy spectrum; protection is directed only against the vaccine serovars or closely related serovars.^{18,61} Evidence of cross-protection by bacterins or LPS preparations against serovars from different serogroups has only rarely been published,⁶² although cross-protection following recovery from infection was reported in the 1970s.⁶¹ The restricted efficacy spectrum of bacterins requires a good knowledge of the regional epidemiology, which can be reliably gained only by culture and identification of locally prevalent serovars. This implies that, where multiple serovars are predominant in dogs, multivalent vaccines for dogs are necessary. This topic will be discussed in the following “Newly emerging serovars and multivalent bacterins” section.

A complicating factor in assessment of the onset and duration of immunity induced with vaccines is the unreliability of the MAT as an indicator of protection. In several vaccination-challenge studies in dogs using experimental infection, no correlation was found between protection and the titer of agglutinating antibodies prior to challenge.^{58,63–67} In bacterin-vaccinated dogs, MAT titers in general show a rapidly declining pattern, but in various studies, dogs without detectable agglutinating antibodies have been demonstrated to be protected, even 12 months after the last vaccination.^{64,65} Therefore, the MAT is unsuitable to assess the onset or duration of immunity elicited by vaccination of dogs. As long as there is no alternative laboratory assay correlating with protection, leptospiral challenge of dogs, unfortunately, is necessary to determine the efficacy of vaccines. In vaccination studies with cattle, cell-mediated immunity has been detected for at least 4 months after completion of a two-dose vaccination regimen.^{68,69} To the authors’ knowledge, since the work of Bey and Johnson in the 1980s,⁷⁰ who used a lymphocyte stimulation test to measure serovar-specific, cell-mediated responses in dogs vaccinated with several leptospiral vaccines, no cell-mediated immunity studies in dogs have been reported. Even when serovar-specific, cell-mediated responses could be measured in vaccinated dogs, this type of assay would be less suitable than serological assays for routine efficacy testing by vaccine manufacturers, since blood or cells have to be tested immediately after sampling from the animals. Therefore, it is necessary to continue the search for an alternative serological response that does show some correlation with protection, and, as is necessary for vaccines containing more than one serovar, is serovar-specific. So far, however, for dogs only *Leptospira* genus-specific and no serovar-specific antibody ELISAs have

been developed and validated for diagnostic purposes (see the “Laboratory diagnosis” section).

Studies of duration of immunity of leptospirosis bacterins in dogs, assessed by *Leptospira* spp. challenge, have been reported less frequently than onset of immunity studies, probably due to the higher costs of the former. Whereas a duration of immunity of less than 6 months after the primary course and 1 year after each annual re-vaccination was determined in a serological study in dogs,⁴⁵ vaccination-challenge studies performed by vaccine manufacturers show that protective immunity elicited by commercial bacterins lasts approximately 12 months.^{64,66} In humans, agglutinating (anti-LPS) antibodies are predominantly, but not exclusively, IgM. However, this is not necessarily the case in animals, where IgG agglutinating antibodies are often produced. Indeed, in revaccination studies in dogs with a novel tetravalent bacterin (Klaasen, unpublished data, 2006), an anamnestic MAT serological response was observed, with at least a fourfold difference between titers done after annual revaccination and those after primary vaccination (Klaasen, unpublished data, 2006).

Newly emerging serovars and multivalent bacterins

As described in the Canine leptospirosis: epidemiology and clinical characteristics section, an increased prevalence of dogs seropositive for *Leptospira* spp. has been reported in various countries around the world (EM Broens, personal communication, 2015).^{22–24} Changes in the epidemiology of canine leptospirosis in North America have led to the inclusion of serovars Grippotyphosa and Pomona in bacterins available there. In Europe, serovars other than the two “classical” bivalent bacterin serovars, Canicola and Icterohaemorrhagiae, have been reported as emerging serovars, particularly serovars Grippotyphosa and Bratislava.^{71,72} In recent years, trivalent (serogroups Canicola, Icterohaemorrhagiae, and Grippotyphosa) and tetravalent vaccines (serogroups Canicola, Icterohaemorrhagiae, Grippotyphosa, and Australis) have been licensed in European countries. In Australia, commercial bacterins for dogs containing strains of serogroups Icterohaemorrhagiae and/or Australis have been on the market for some time. The US Department of Agriculture, responsible for licensing of new animal vaccines in USA and involved in research into infectious diseases in animals, published a Center for Veterinary Biologics (CVB) notice *Requirements for Addition of Leptospira bratislava in Canine Bacterins*⁷³ describing “the minimum evidence required for inclusion of the *L. bratislava* antigen

in licensed leptospira-containing bacterins". The reason for this requirement was the lack of evidence by isolation for serovar Bratislava as a significant pathogen of dogs in the US. Nevertheless, in several European countries, strains of serovar Bratislava have been isolated from dogs with nephritis or reproductive diseases.⁷¹ Serovar Bratislava is associated with clinical and subclinical leptospirosis in several animal species, including reproductive disease.⁷⁴⁻⁷⁷ Since cases of mild or latent forms of canine leptospirosis are frequently missed by veterinary practitioners, either because the dog owners do not visit their veterinarian or due to misdiagnosis, the chance of underdiagnosis of serovar Bratislava infections in dogs is relatively high.

As long as no novel efficacious and broadly protecting leptospirosis vaccines have been developed, multivalent bacterins such as the tetravalent vaccines in the US and Europe will aid in the battle against canine leptospirosis. Vaccination with these bacterins, however, will only result in significant reduction of clinical disease and spreading by infected dogs when the major serovars of a given region are included in the vaccine. This limitation causes several hurdles in vaccine development. Apart from the difficulties in identifying the most important local serovars, the second hurdle in developing multivalent bacterins is to overcome the risk of insufficient efficacy with higher numbers of serovars in one vaccine. Here again, the use of appropriate adjuvants can minimize this possible risk. However, in the US and Europe (and countries that follow the US or European guidelines), efficacy claims of newly licensed canine leptospirosis bacterins are based on vaccine-challenge studies in dogs, by demonstrating protection against the relevant regional serovars. Therefore, non-efficacious vaccines will not be licensed.

Growth media for vaccines

Up to the present time, all licensed canine leptospirosis vaccines have been chemically or physically inactivated whole bacterial cell vaccines (bacterins) or "purified" bacterial cell wall vaccines. Early experimental or commercial bacterins were prepared by inactivating leptospires cultivated in media containing rabbit serum. Due to the use of rabbit serum and the variability in rabbit serum batches, the manufacturing processes were inconsistent, bacterial harvest often insufficient, and the vaccines unsafe for the target animals because of the allergenic effects of the foreign serum proteins. Clearly, any serum used must be free of anti-leptospirosis antibodies. Later, "serum-free", semi-defined, albumin-containing media were developed in which polysorbate 80 (Tween 80) replaced the essential fatty acids that were supplied by the rabbit

serum and that are required by the *Leptospira* spp. bacteria as the sole source of energy and carbon.⁴² These media (one of which is the Ellinghausen–McCullough–Johnson–Harris [EMJH] medium, named after Ellinghausen and McCullough and modified by Johnson and Harris) are still widely used by researchers and vaccine manufacturers, and contain essential vitamins, salts, and minerals, 0.125% v/v polysorbate 80, and up to 1% w/v bovine serum albumin (BSA).⁴² As *Leptospira* spp. bacteria in vitro do not utilize protein, the main function of the albumin component of EMJH is considered to be the detoxification of the fatty acids provided by the polysorbate 80 in the culture medium by reversibly complexing them, while keeping them biologically available. There is batch variation in the ability of BSA to support growth of leptospires. The delipidation of BSA with chloroform/methanol improves growth,⁴² but is not suitable for large-scale processes. During the 1960s and 1970s, scientists attempted to develop chemically defined media free of serum and/or albumin. These media did not gain wide acceptance by vaccine manufacturers because adaptation of the *Leptospira* spp. strains to the new medium was mostly required, and many strains could not be cultivated at all. Additionally, a method of detoxification of polysorbates using charcoal to enhance growth of leptospires in the absence of albumin, although feasible at lab scale, appeared not to be suitable for (large-scale) manufacturing purposes, because the detoxification was not consistent, and the processes were time-consuming and expensive, and resulted in inadequate antigen yields. In 2006, a patent application⁷⁸ was published by a manufacturer reporting a novel method to culture *Leptospira* spp. bacteria in a protein-free and fatty acid-free medium, whereby fatty acids were "fed" to the bacteria through continuous feeding of low amounts of untreated (non-detoxified) polysorbates.

Risk of allergic reactions in small-breed dogs

Adverse reactions frequently observed in canine vaccination are allergic reactions such as urticaria, pruritus, facial edema, weakness/depression, dyspnea, vomiting, diarrhea, and hypotension. In a study in the US⁷⁹ using electronic records of 1,226,159 dogs, a vaccine-associated adverse event (VAAE) rate was recorded as 38.2/10,000 dogs. Young adult, small-breed, neutered dogs that received multiple vaccines per office visit were at greatest risk of a VAAE. In a random sample of 400 dogs in this study, predominant adverse reactions (observed within 3 days of vaccine administration) consisted of facial or periorbital edema (31%), wheals or urticaria (21%), generalized pruritus (15%), and vomiting (10%).

A Japanese research group⁸⁰ suggested that immediate-type allergic reactions in dogs after vaccination were induced by type I hypersensitivity mediated by IgE directed against vaccine components such as fetal calf serum (FCS), gelatin, and casein. In their subsequent study,⁸¹ they detected IgE reactivity against BSA and other unknown bovine serum components, and they recommended elimination of FCS, BSA, and stabilizer proteins from the live and inactivated components of canine vaccines. Day,⁸² however, concluded that although there have always been adverse reactions to vaccinations in dogs and cats, the prevalence of such reactions is extremely low. Nevertheless, in a more recent study in Japan⁸³ with data from 57,300 dogs vaccinated with non-rabies combined vaccines, the VAAE rate was 62.7/10,000 dogs, which is almost twice as high as the VAAE rate in USA study. The authors suggested that the overrepresentation of small breeds in Japan and the proposed higher risk of VAAEs in dogs of small breeds (≤ 10 kg body weight) may have contributed to the higher VAAE rate in comparison with other countries. Despite the generally accepted view that small-breed dogs may be at higher risk of VAAEs, there is no consensus of whether or not the currently licensed canine leptospirosis vaccines are safe enough for small-breed dogs.⁶ Although the exact cause(s) of adverse reactions in dogs after vaccination with one of the available commercial leptospirosis vaccines are yet to be identified, some vaccine manufacturers have introduced refinements of their production processes to reduce the content of potentially allergenic compounds.^{84,85}

Potential for recombinant vaccines

As outlined in the previous “Risk of allergic reactions in small-breed dogs” section, bacterin vaccines induce immunity that is restricted to serovars that are closely related through surface-exposed, agglutinating, LPS antigens. Immunity following natural infection is likewise generally accepted as being serovar, or at best serogroup, specific. Nevertheless, there is substantial evidence that cross-protective immunity can be stimulated and that it is mediated by proteins (reviewed by Adler⁶¹). Vaccines based upon conserved leptospiral proteins would offer significant advantages, including broad coverage, ease and consistency of production, and reduced cost. There is now a substantial literature on protection studies performed with a large range of recombinant proteins in laboratory animals, most commonly hamsters, guinea pigs, or gerbils. Unfortunately, almost all of the claims of protection do not withstand rigorous scrutiny and are therefore dubious. The most common problems are inappropriate statistical analyses, lack of reproducibility, the use of incorrect

controls, and the use of inadequate challenge doses.⁶¹ The outer membrane protein LigA currently represents the most promising candidate antigen. However, there is evidence that it may not protect against some serovars; for example, clear protective homologous immunity in hamsters has been shown with serovar Copenhageni, while homologous LigA did not protect against Manilae or Canicola.⁶¹ The reasons for this disparity are unknown.

Alternative delivery methods such as adenovirus vector (*lipL32*)⁸⁶ and plasmid DNA (*lipL32*, *flaB*)^{87,88} have been reported; however, the results do not withstand biological and statistical scrutiny, and these approaches have not been developed further. A recent study⁸⁹ suggested that immunization with LipL32 reduced kidney invasion in hamsters, but notably, there was no protection from lethality, thus limiting the value of these results. Indeed, a recent re-evaluation indicated that LipL32, the main candidate in these and many other vaccine studies, is not exposed on the leptospiral surface.⁹⁰

A further caveat is that vaccination studies cannot necessarily be extrapolated to different animal species. Proper vaccination and challenge experiments in the target animal species are the only valid criteria for assessment of vaccine efficacy. Therefore, there is no prospect of a recombinant vaccine for canine leptospirosis in the foreseeable future.

Disclosure

EK is an employee of, and BA has acted as an expert adviser to, Merck, Sharp and Dohme Animal Health. The authors report no other conflicts of interest in this work.

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