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Short communication

Vaccination with LiESP/QA-21 (CaniLeish[®]) reduces the intensity of infection in *Phlebotomus perniciosus* fed on *Leishmania infantum* infected dogs—A preliminary xenodiagnosis study



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ABSTRACT

Ten Beagle dogs at different stages of *Leishmania infantum* infection, among which 6 had received a full course of LiESP/QA-21 (CaniLeish[®]; Virbac) vaccination, were exposed to the bites of reared *Phlebotomus perniciosus* to assess their infectiousness potential. This was found to be negligible/nil in 2 seronegative dogs with subpatent infection. Among the 8 dogs with active infection (=positive serology, bone-marrow qualitative PCR and lymph node culture), 2/5 vaccinated (40.0%) and 2/3 nonvaccinated dogs (66.7%) were infectious to the sand flies ($p = 0.5$). However significantly fewer of the sand flies which fed on the vaccinated dogs were infected when compared to those which fed on the control dogs (10/82 compared to 30/49) (chi-squared test, $p < 0.0001$; mixed binomial model with the dog identity included as a random effect, $p = 0.03$). Furthermore, there was a significant difference in the proportion of sand flies with >500 parasites in their gut (i.e. a higher risk for subsequent transmission): 3.7% for vaccinated dogs compared with 28.6% for nonvaccinated dogs (Fisher's exact test, $p < 0.0001$; binomial mixed model, $p = 0.006$). Although preliminary, these results suggest value in further investigations on *L. infantum* transmissibility parameters in LiESP/QA-21 vaccinated dogs.

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1. Introduction

Canine leishmaniosis (CanL) is a potentially fatal disease caused by the kinetoplastid protozoan *Leishmania infantum* widespread in the Mediterranean basin (Franco et al., 2011). In this region dogs are the main reservoir

of the parasite which is transmitted to other dogs and humans by the bite of phlebotomine sand flies of the *Phlebotomus (Larroussius)* subgenus. Despite the availability of several topical insecticide preparations with good trial data for dogs (reviewed by Maroli et al., 2010) these products cannot prevent all potential infectious sand fly bites and there is still a need for further control measures (Solano-Gallego et al., 2009). The objective of vaccination against CanL is two-fold: a reduction in the risk of an individual vaccinated dog suffering from the disease, and a reduction in the risk of subsequent transmission of the parasite. Canine vaccination with LiESP/QA-21 (CaniLeish[®]; Virbac, France), recently licensed in Europe, was found to induce

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an appropriate Th1-profile cell-mediated response shortly after completing the primary course, and this response effectively reduced the *L. infantum* load in pre-infected macrophages *in vitro* (Moreno et al., 2012). When administered to dogs exposed to natural *L. infantum* infection, the vaccine was shown to decrease approximately four-fold the risk of an individual dog progressing to symptomatic active infection (CaniLeish®: EPAR – Public assessment report – <http://www.ema.europa.eu>; Oliva et al., 2012). This reduction in the number of highly infectious dogs in the later stages may be useful on an epidemiological scale, as it is generally assumed that early or chronic subpatent infection stages have limited or nil transmission potential (Courtenay et al., 2002), but there is an additional benefit if dogs which progress to develop the disease despite vaccination are also less infectious to sand flies.

2. Methods

Ten 3-year-old Beagle dogs at different stages of *L. infantum* infection were selected to be included in the study. The animals, which were born in an area of northern Europe where the infection is not endemic, were hosted together in an open-air kennel sited in a territory highly endemic for CanL in Naples province, south Italy. Six dogs had received a primary 3-dose course of LiESP/QA-21 before natural exposure to *L. infantum*, and a year later they were boosted with a single vaccine dose. Four dogs had been left untreated during the whole period. Serology, molecular detection of *Leishmania* DNA and culture were used to characterize the infection stage in each dog. Detection of anti-*Leishmania* IgG antibodies was performed by an in-house IFAT assay using *L. infantum* promastigotes as antigen and following the protocol recommended by the Office International des Epizooties (Gradoni and Gramiccia, 2008). Bone-marrow (BM) aspirate material was examined by a nested (n)-PCR assay. Briefly, BM DNA was subjected to two consecutive PCR amplifications using the kinetoplastid-specific primers R221 and R332 in the first run, and the *Leishmania*-specific primers R223 and R333 in the second run (van Eys et al., 1992). Popliteal lymph-node (LN) aspirates were cultured in Evans' Modified Tobie's medium and cultures were periodically examined for promastigote growth during one month.

At the time of the study, two dogs (one each from vaccinated and nonvaccinated group) showed a subpatent infection (Oliva et al., 2006), characterized by positive BM n-PCR but negative serology and LN culture, and by the absence of clinical signs. The remaining eight dogs were in an active infection stage (=positive serology, BM nested-PCR and LN culture), which was asymptomatic in three vaccinated and one control dogs, and symptomatic in two each from vaccinated and nonvaccinated groups (Table 1). Clinical signs exhibited by the latter four dogs consisted of early clinicopathological and external signs such as moderate pancytopenia, gammaglobulin increase, popliteal lymph node enlargement and moderate weight loss.

Xenodiagnosis, consisting in the controlled exposure of dogs to unfed reared *Phlebotomus perniciosus* females, was performed in the same kennel where the dogs lived. A small

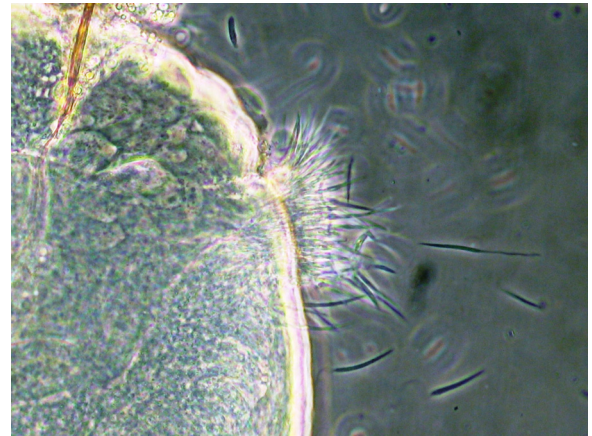


Fig. 1. Grade 1 infection of the sand fly found infected after feeding on vaccinated dog A.

room was equipped for dog sedation and for controlled temperature (26–28 °C) and humidity (75–85%). Female sand fly specimens from a colony routinely maintained at the facilities of Istituto Superiore di Sanità in Rome were transported to the kennel site on the same day of xenodiagnosis in plaster-lined plastic pots used for larval rearing. The dogs were sedated with medetomidine chlorhydrate (Domitor) plus butorphanol tartrate (Dolorex), followed by propofol (Rapinovel) administered slowly iv during 1 h. The dog's head was inserted into a fine net cage containing 60 female sand flies and exposed to bites in the dark for 1 h. When insects were reluctant to feed on a dog, a second exposure to another batch of 60 sand flies was performed one week later. The sand flies which fed were individually retained and fed thereafter with only saturated sucrose solution. From day 7 post-blood meal surviving flies were dissected, the gut examined microscopically for the presence of promastigotes and scored for development and burden of parasites (Sádllová et al., 2003) (see infection grade definitions in footnote of Table 1).

Infection rate/burden data were analyzed using chi-squared, Fisher's and Mann Whitney tests where appropriate (SPSS Answer Tree version 3.1). Furthermore, a mixed binomial model with the dog identity included as a random effect was analyzed using the GENMOD procedure (SAS version 9.1.3).

3. Results

Xenodiagnosis performance and results are detailed in Table 1. Dogs' attractiveness to flies varied greatly, as observed in simultaneous exposures of 2 dogs using the same batch of sand flies randomly split into different exposure cages (data not shown). Four dogs (2 from each group) required a second exposure, for a total of 120 sand flies. Altogether, the cohort of vaccinated dogs infected 11/105 specimens (10.5%) and that of control dogs 30/75 (40.0%) (chi-squared test, $p < 0.0001$). As regards the two dogs with subpatent infection, the vaccinated one (dog A) infected one specimen out of 23 examined, at very low intensity (<100 parasites with no infection of foregut) (Fig. 1),

Table 1

Laboratory and clinical parameters detected in LiESP/QA-21 vaccinated and control dogs, and xenodiagnosis results. Dogs A (vaccinated) and G (control) had a sub-patent infection; dogs B–D (vaccinated) and H (control) had an asymptomatic active infection; dogs E, F (vaccinated), I and J (control) had a symptomatic active infection.

Group	Dog	IFAT titre ^a	BM-nPCR	LN culture	Clinical signs ^b	Sand flies (<i>Phlebotomus perniciosus</i>)					
						Fed	Positive/dissected (%)	Infection grade ^c			
								1	2	3	4
Vaccine	A	Neg	Pos	Neg	No	27/60	1/23 (4.3)	1			
	B	Low	Pos	Pos	No	28/60	0/28				
	C	Low	Pos	Pos	No	12/120	0/8				
	D	Low	Pos	Pos	No	7/60	0/7				
	E	High	Pos	Pos	Yes	19/120	1/19 (5.3)	1			
	F	High	Pos	Pos	Yes	26/60	9/20 (45.0)	6	1	1	1
						Pooled results	11/105 (10.5)	7	1	2	1
Control	G	Neg	Pos	Neg	No	32/60	0/26				
	H	Low	Pos	Pos	No	9/120	0/9				
	I	High	Pos	Pos	Yes	32/60	25/31 (80.6)	7	4	7	7
	J	High	Pos	Pos	Yes	9/120	5/9 (55.5)	1	4		
							Pooled results	30/75 (40.0)	8	8	7

BM: bone marrow; LN: lymph node.

The use of bold characters is warranted for the “core” results, represented by positive specimens and rates recorded by xenodiagnosis.

^a Negative: <1/40; low: 1/40–1/320; high: 1/640–1/5120.

^b At least 2 clinicopathological signs plus 1 external signs recorded (Paltrinieri et al., 2010).

^c Grade = 1: less than 100 promastigotes (p); 2: 100–500 p; 3: 500–1000 p; 4: more than 1000 p.

whereas the control animal (dog G) infected none of the 26 flies examined (no significant difference between groups; Fisher's exact test, $p = 0.5$).

Because LiESP/QA-21 vaccine efficacy is associated to the prevention of active infections, comparative analyses have been particularly addressed to the eight dogs showing this condition. Of them, only those exhibiting signs of CanL disease – two of the five vaccinated (40.0%) and two of the three control dogs (66.7%) – were infectious to the sand flies, with no significant difference between groups (Fisher's exact test, $p = 0.5$). However significantly fewer of the blood-fed sand flies which fed on the vaccinated dogs were infected when compared to those which fed on the control dogs: 10/82 (12.2%) compared to 30/49 (61.2%), respectively (chi-squared test, $p < 0.0001$). In light of the possible variation in the intensity of the infection in the individual dogs we also performed an additional test using a mixed binomial model with the dog identity included as a random effect. In this analysis the difference between groups remains significant ($p = 0.03$). The proportion of blood-fed sand flies which developed high parasite burdens was also considered, as these represent higher risk for subsequent transmission. First, infection scores were compared between the 2 cohorts of dogs and found to be significantly higher in control dogs (Mann Whitney test, $p < 0.0001$). Second, there was a significant difference in the proportion of blood-fed sand flies with >500 parasites in their gut (Fig. 2): 3/82 (3.7%) for vaccinated dogs compared with 14/49 (28.6%) for control dogs (Fisher's exact test, $p < 0.0001$; binomial mixed model, $p = 0.006$).

4. Discussion

A limitation of our work consisted in the low number of sand flies tested for some of the dogs examined by xenodiagnosis, because the assay had to be performed

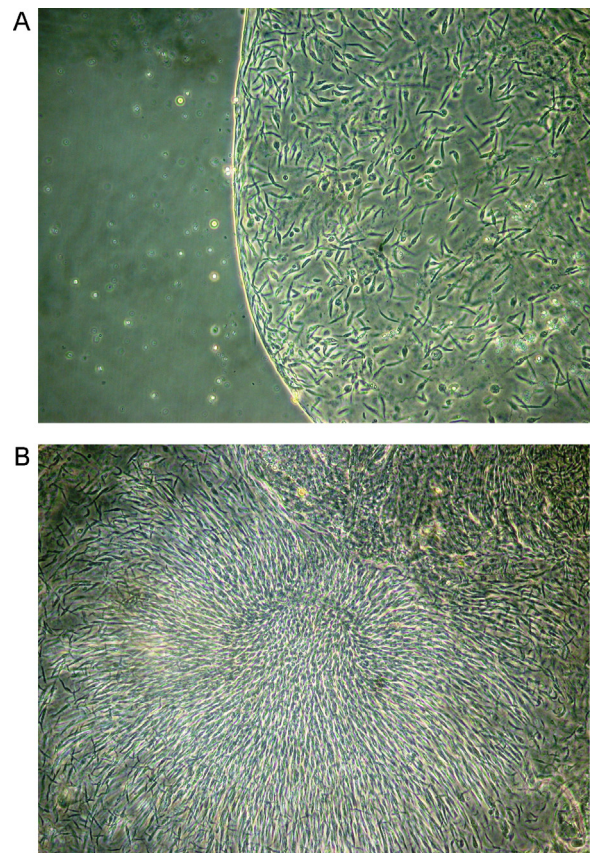


Fig. 2. Examples of grade 4 infection detected in sand flies fed on nonvaccinated dog I. A: heavy gut infection; B: “Rose” of promastigotes emerging from the dissected stomodeal valve.

under field conditions and the colonized flies had to be transported from the laboratory to the study site. Because the natural attractiveness of individual dogs can be variable, the test conditions may have exacerbated the sand fly reluctance to feed on some animals. It should be noted, however, that a number of examined sand flies as low as <10 was equally represented in experiments performed on vaccinated and non-vaccinated animals: dogs C and D, and dogs H and J, respectively (Table 1). On the other hand, the study dogs have been well characterized as regards infection/disease stage, thus having animals representative of all stages of infection in both vaccinated and control groups. We failed to confirm that seropositive but asymptomatic dogs can be infectious to the competent vector (Molina et al., 1994), whereas this study confirms earlier work demonstrating that the infectiousness burden of dogs increases as they progress to the symptomatic stages, as recorded in Mediterranean (Gradoni et al., 1987; Molina et al., 1994; Guarga et al., 2000) as well as in New World CanL, where *Lutzomyia* phlebotomines are the vector (Travi et al., 2001; Courtenay et al., 2002; da Costa-Val et al., 2007; Verçosa et al., 2008). Of note, this is the first record of xenodiagnosis performed on animals with subpatent infection, i.e. a seronegative asymptomatic stage. One of the 2 dogs presenting such condition was infectious to 1/23 sand flies which fed on it, although the infection was at very low intensity. From an epidemiological point of view this finding is a matter of concern as it showed that seronegative dogs may also play a role in the transmission of *L. infantum*.

A preliminary study like this involving a small number of dogs and sand flies, is unable to conclusively answer the question of the impact of the canine vaccine on the transmission of the parasite. However some significant observations are noteworthy: on one hand, we have shown that dogs progressing to active *L. infantum* infection despite LiESP/QA-21 vaccination can be infectious to competent vectors in proportions similar to those of nonvaccinated dogs, which shows that combined prophylaxis against CanL (i.e. vaccination and topical insecticides) should be performed in dogs living in endemic regions. On the other hand, the infectiousness burden of these vaccinated dogs appears significantly reduced, somehow mirroring the *Leishmania* burden control exerted by the canine response to LiESP/QA-21-vaccination (Moreno et al., 2012). Because this vaccine does not prevent all cases of disease, there is an additional benefit if dogs which progress to develop the disease despite vaccination are less infectious to sand flies. These initial results are encouraging and suggest value in further investigation of this effect.

Conflict of interest statement

The authors have read the journal's policy and have the following conflict: AMC is employee of Virbac.

Ethical statement

The study design and technical protocol were approved by the Veterinary Board of the Italian Ministry of Health

following the European Directive 86/609/EEC, adopted by the Italian Government with the Law 116/1992.

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