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Validation of the diagnostic performance of conjunctival swab *Leishmania* nested-PCR in dogs from different settings of Mediterranean canine leishmaniasis

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Recent studies have established that conjunctival swab (CS) molecular analysis can be useful for Canine Leishmaniasis (CanL) diagnosis in naturally *Leishmania*-exposed dogs because both sensitive and non-invasive (Leite et al, 2010, Vet Parasitol; Lombardo et al, 2012, Vet Parasitol). These findings suggested a careful evaluation of the diagnostic performance of CS nested(n)-PCR analysis during EDENext project. A comparative performance was evaluated by performing a larger validation study in dog groups from Mediterranean endemic settings of Italy, Portugal, Turkey, and Georgia.

Methods

A validated protocol suggested by EDENext PhBD group was used in all surveys. Exfoliative epithelial cells were collected from the right and left conjunctiva of each animal using sterile cotton swabs manufactured for bacteriological isolation. The swabs were rubbed robustly back and forth once in the lower conjunctival sac. They were then immersed in 2 ml of sterile saline in 20-ml plastic tubes and stored at 4°C for 24 h. After the manual stirring of swabs, the saline containing the eluted exfoliating cells was transferred into sterile vials pending DNA extraction; n-PCR was applied to detect *Leishmania* DNA in canine samples. Samples from the right and left eyelid conjunctivas were processed separately.

In Italy, CS n-PCR method was previously applied for diagnosing CanL in different stages of infection in comparison to the performance of the indirect immunofluorescence antibody test (IFAT), lymph node microscopy, and buffy coat n-PCR (Di Muccio et al, 2012, J Clin Microbiol). CS n-PCR was evaluated as an early diagnostic marker at different stages of infection in a heterogeneous canine population (273 dogs) living in 4 CanL endemic areas of central Italy. Among the 72 animals that were positive by at least one test (28.45%), CS n-PCR showed the best relative performance (76.4%), with **high concordance to standard IFAT serology (k 0.75**).

The highest positivity rates were found in **asymptomatic infected dogs (84.2%)** and **sick dogs (77.8%)** (Table 1). Further, n-PCR alone was able to identify an additional group of dogs that was IFAT negative. However, the performance of CS n-PCR did not depend on the animal's clinical condition, given that the presence of *Leishmania* DNA in CS was similar when comparing dogs with overt clinical signs (75.5%) and asymptomatic dogs (78.9%)(Table 2).

Table 1. Positivity rate using CS n-PCR, by stage of CanL infection

Test[b]	No. of dogs (%, 95% CI)[c] Negative	E	I	S
IFAT[d]	0 (0, 0–18.5)	7 (100, 59–100)	38 (100, 90.7–100)	9 (100, 66.4–100)
LN-CE	0 (0, 0–18.5)	0 (0, 0–41)	27 (71.1, 54.1–84.6)	9 (100, 66.4–100)
BC n-PCR	3 (16.7, 3.6–41.4)[e]	0 (0, 0–41)	16 (42.1, 26.3–59.2)	3 (33.3, 15.6–48.7)
CS n-PCR	16 (88.9, 65.3–98.6)[e]	0 (0, 0–41)	32 (84.2, 68.7–94.0)	7 (77.8, 40.0–97.2)
	18	7	38	9

[a] Stages of CanL infection were determined as proposed by Paltrinieri et al. (35). Note that according to these authors, attribution to various CanL stages does not include CS examination results.
[b] IFAT, indirect immunofluorescence antibody test; LN-CE, cytological examination of lymph node aspirate; BC n-PCR, n-PCR on buffy coat from peripheral blood; CS n-PCR, n-PCR on conjunctival swab.
[c] CI, confidence interval; E, exposed (low IFAT titer plus negative cytology and negative PCR on relevant tissue); I, infected (low IFAT titer plus positive cytology and/or positive PCR on relevant tissue and without clinical signs); S, sick (high IFAT titer plus positive cytology with at least one clinical sign).
[d] IFAT titer ranges were 1:80 to 1:160 for exposed dogs; 1:320 to 1:640 for infected dogs; and ≥1:1,280 for sick dogs.
[e] n-PCR positives in otherwise negative dogs.

Table 2. Positivity rate using serologic and parasitologic assay in CanL-positive dogs

Clinical signs (no. of dogs)	No. of positive dogs (%, 95% CI)[a] IFAT	CS n-PCR	BC n-PCR	LN-CE
Present (53)	49 (92.45, 81.8–97.9)	40 (75.5, 61.7–86.2)	19 (35.84, 23.1–50.2)	35 (66.03, 49.8–76.9)
Absent (19)	5 (26.31, 9.1–51.2)	15 (78.9, 54.4–93.9)	3 (15.78, 3.4–39.6)	1 (5.26, 1–26)

[a] CI, confidence interval; IFAT, indirect immunofluorescence antibody test; CS n-PCR, n-PCR on conjunctival swab; BC n-PCR, n-PCR on buffy coat; LN-CE, cytological examination of lymph node aspirate.

In Portugal, 320 randomly selected dogs from 4 kennels of the Metropolitan Lisbon Region were enrolled during October 2011. ELISA prevalence was 13.4%, whereas CS n-PCR prevalence was 35.6%. CS n-PCR scored the **most sensitive assay in asymptomatic animals (36.6%).** 179 dogs (55.9%) resulted positive for CanL by at least one test. The relative performance of the tests in the 179 Can-L positive dogs resulted 45.2% for CS n-PCR, 19.5% for BC n-PCR, and 12.8% for ELISA (Table 3). Only 2 dogs were found positive by all test. CS n-PCR identifies a group of dogs that resulted seronegative, BC n-PCR negative and without clinical signs. The other 38 animals were positive to different combinations of tests.

Table 3. Comparative performance of three tests in 179 dogs shown to be positive for CanL diagnosis by at least one test

N° of Dogs (%)	ELISA (%)	BC-PCR (%)	CS-PCR (%)
81 (45.25)	-	-	+
35 (19.55)	-	+	-
23 (12.84)	+	-	-
20 (11.17)	-	+	+
11 (6.14)	+	-	+
7 (3.91)	+	+	-
2 (1.11)	+	+	+
179	43 (24.02)	64 (35.75)	114 (63.68)

In Georgia, 72 domestic dogs were enrolled from veterinarian clinics. All dogs in this group were classified as sick with a variety of clinical signs. Diagnosis of CanL was confirmed by parasitological testing (microscopy of bone marrow samples) in 67 out of 72 sampled dogs. CS n-PCR positive results were shown in 46 animals, of which 1 dog was negative and the rest 45 (98%) positive by parasitological test. In total, **concordance** in positivity of two tests (**CS n-PCR** and **bone marrow microscopy**) was shown **in 73% of dogs**.

In Turkey, 100 dogs were randomly chosen in 6 villages of Cukurova Plain; 4 samplings were done before and after transmission seasons 2011 and 2013. The overall **concordance** between **IFAT and CS n-PCR** tests was **65%** (Table 4). Moreover, a group of seronegative asymptomatic dogs showed CS n-PCR positivity and they were found seropositives in subsequent samplings. In the 1st sampling, 16 dogs were seronegative in IFAT but positive in CS n-PCR. Fourteen (87.5%) out of 16 dogs became seropositive in subsequent samplings. In whole study period, a total of 26 seronegative/CSn-PCR positive dogs turned to seropositive in subsequent samplings. This is most important finding showing the ability of **CS n-PCR for CanL diagnosis in the early stage**.

Table 4. The comparative results of IFAT and CS-n-PCR

		IFAT							
		1st Sampling		2nd Sampling		3rd Sampling		4th Sampling	
		POS	NEG	POS	NEG	POS	NEG	POS	NEG
CS n-PCR	POS	11	16	12	25	12	24	13	10
	NEG	15	58	13	43	7	44	12	57

Conclusion

Taken together, our findings can be summarized as follows:

1. CS n-PCR positivity appears to occur at high rates in dogs living in settings with elevated CanL prevalence;
2. CS n-PCR seems to be effective in early detecting *Leishmania* contacts in dogs exposed to parasite transmission before seroconversion or specific clinical signs;
3. CS n-PCR showed the best performance in comparison with other non-invasive tests;
4. CS n-PCR performance applied on random canine populations did not differ significantly from standard serological evaluation, but in asymptomatic dogs CS n-PCR resulted the most sensitive assays.

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