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1 **SENSITIVITY AND SPECIFICITY OF IN SITU HYBRIDIZATION FOR THE**
2 **DIAGNOSIS OF CUTANEOUS INFECTION BY *Leishmania infantum* IN DOGS**
3
4 Running title: In situ hybridization of *L. infantum* in dogs
5
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23 **ABSTRACT**

24 An accurate diagnosis of infection by *Leishmania infantum* in dogs is fundamental for the
25 control of zoonotic visceral leishmaniasis (VL). Histopathology (HP) and immunohistochemistry
26 (IHC) are frequently used for the histological diagnosis of *L. infantum* in dogs, but have shown
27 limited accuracy. To improve the sensitivity and specificity of the histological diagnosis of VL, we
28 evaluated automated in situ hybridization (ISH) using a generic probe for *Leishmania* and a specific
29 probe for *L. infantum* in surgical skin biopsies of dogs. The ISH results were compared with those
30 of HP and IHC, using parasitological culture as the reference standard. Samples of skin from 51
31 dogs with cutaneous *L. infantum* infection and 51 non-infected dogs were randomly selected from
32 samples of dogs from various cities in Brazil where canine VL is endemic. These samples were
33 processed for parasitological culture, HP, IHC and ISH using both probes. The sensitivities of ISH
34 using the specific probe, ISH using the generic probe, IHC and HP were, respectively, 74.5%,
35 70.6%, 69.5% and 57.6%. The specificity of both ISH probes tested was 100% and there was no
36 cross-hybridization of the generic and specific probes with selected pathogenic fungi and protozoa.
37 The specific probe discriminated *L. infantum* from the other species of *Leishmania* that infect dogs
38 in the New World. ISH is highly sensitive and specific for the diagnosis of *L. infantum* in histologic
39 samples of skin from infected dogs and can be used on routine biopsy material to make a diagnosis
40 of leishmaniasis.

41

42 **INTRODUCTION**

43 Leishmaniasis is a worldwide disease caused by protozoa of the genus *Leishmania*, which
44 infect wild and domestic mammals including humans (38). The spectrum of clinical forms of
45 leishmaniasis can vary from focal cutaneous to disseminated visceral disease (38). There are

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46 twelve *Leishmania* species infecting dogs: *L. donovani*, *L. infantum* (syn. *L. chagasi*), *L. major*,
47 *L. arabica* and *L. tropica* in the Old World, and *L. infantum*, *L. colombiensis*, *L. panamensis*, *L.*
48 *mexicana*, *L. braziliensis*, *L. peruviana*, *L. pifanoi* and *L. amazonensis* in the New World (7, 8, 9,
49 11, 24, 34, 38). Although many species of *Leishmania* infect dogs, dogs are considered a proven
50 reservoir only for *L. infantum* (7, 38). The species *L. infantum* can also infect humans, cats and
51 wild mammals and is the cause of zoonotic visceral leishmaniasis (VL) (7, 21, 38).

52 In many countries, zoonotic VL constitutes a significant public health problem, especially
53 due to its prevalence, high mortality rates, mainly in children, and emergency rate in patients
54 infected by the human immunodeficiency virus (22, 38). Transmission in endemic areas is
55 usually via bites of infected sand flies, with dogs as the main domestic reservoir of the parasite
56 (22, 38). Therefore, a rapid and accurate diagnosis of the infection of dogs with *L. infantum* is
57 fundamental for the control of zoonotic VL transmission.

58 The clinical diagnosis of canine VL is difficult, with many animals being asymptomatic.
59 Therefore, a variety of serological, parasitological and molecular laboratory methods have been
60 developed to detect infection by *L. infantum* in dogs (20, 23). Serological assays, polymerase
61 chain reaction (PCR) and parasitological culture are the most sensitive methods for the diagnosis
62 of *L. infantum* infection (13, 15, 17, 19, 28, 30, 37). However, these three methods do not allow
63 visualization of the intact amastigotes within the tissue and correlation of the parasites with
64 associated lesions, which is possible by histopathology (HP) and immunohistochemistry (IHC)
65 (10). HP and IHC are frequently used in the current routine for the histological diagnosis of *L.*
66 *infantum* in dogs, but these methods have limited accuracy and do not allow speciation (37, 28,
67 29). The speciation of *Leishmania* is currently only possible by parasitological culture followed
68 by multilocus enzyme electrophoresis (MLEE), which is the reference method, and by PCR (38).

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69 Hence, alternative histological methods are necessary to improve the accuracy of diagnosing
70 infection of dogs with *L. infantum*.

71 A recently established chromogenic in situ hybridization (ISH) technique is a promising
72 method for the diagnosis of canine VL because it permits the highly specific identification of
73 *Leishmania* in formalin-fixed, paraffin-embedded surgical biopsies of dogs (10). However, the
74 previously published probe for ISH was unable to speciate *Leishmania* (10). In addition, the
75 published ISH protocol was based on manual labeling, which presents lower efficiency and
76 productivity than automation (32). Thus, the present study aimed to evaluate the sensitivity and
77 specificity of automated ISH for the diagnosis of canine cutaneous infection caused by *L. infantum*
78 on routinely processed surgical biopsy samples using the previously published generic probe for
79 *Leishmania* and a newly designed probe specific for *L. infantum.* The results of both ISH tests were
80 compared to IHC and HP in the same samples, using parasitological culture as the reference
81 standard technique.

82

83 **MATERIAL AND METHODS**

84 **Samples**

85 A prospective study was designed using randomly selected samples from 2,066 surgical skin
86 biopsies of dogs collected between the years 2008 and 2012. The dogs originated from seven
87 cities in Brazil with endemic canine VL: Niterói-RJ, Rio de Janeiro-RJ, Bauru-SP, Brasília-DF,
88 Cuiabá-MT, Palmas-TO and Fortaleza-CE. Skin samples were selected for this study since they
89 are easy to obtain and have been shown to be a good target for the confirmation of canine VL by
90 parasitological culture (17).

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91 For the collection of samples, one 3 mm punch biopsy was obtained from the intact skin
92 over the scapula, after disinfection with 70% alcohol and local anesthesia with 2% lidocaine.
93 Each obtained specimen was divided into two samples. One of them was immersed in sterile
94 saline with antimicrobials (1) and submitted for parasitological culture. The other fragment was
95 fixed in 10% neutral buffered formalin and processed for routine paraffin embedding (5). The
96 paraffin blocks were processed for ISH, IHC and HP. The IHC, HP and parasitological culture
97 with identification of the species of trypanosomatids by MLEE were performed at the Evandro
98 Chagas Clinical Research Institute, FIOCRUZ, Brazil. The ISH was performed at the Diagnostic
99 Center for Population and Animal Health, Michigan State University, USA. The biopsy
100 procedure performed on animals was approved by the Ethics Committee on the Use of Animals,
101 FIOCRUZ, Brazil (license L-038/08).
102
103 **Study Design**
104 ISH using a previously published generic (ISH-GP) and a newly developed specific (ISH-
105 SP) oligoprobe for the diagnosis of *L. infantum* infection in dogs were evaluated. In order to
106 calculate the number of samples required for this study, the estimated values for the ISH were
107 70% of sensitivity/specificity according to preliminary tests, 17% of absolute error in
108 sensitivity/specificity and 5% of alpha. Considering the loss of samples during processing, the
109 calculated number was increased by 4%. As a result, 51 dogs positive for *L. infantum* infection in
110 the parasitological culture and 51 negative dogs for *L. infantum* infection in the parasitological
111 culture were randomly selected from 2,066 surgical skin biopsies of dogs. Ten of the 51 samples
112 of the *L. infantum* negative group were from dogs positive for the protozoon *Trypanosoma*
113 *caninum* based on parasitological culture. Thirty formalin-fixed, paraffin-embedded (FFPE)

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114 samples (8 positive and 22 negative for *L. infantum* based on parasitological culture) were
115 consumed after the testing with ISH-GP. They were replaced by new randomly selected samples
116 (8 positive and 22 negative for *L. infantum* based on parasitological culture) for the testing with
117 ISH-SP. All 132 samples were tested by IHC and HP and a total of 102 dogs was tested with
118 ISH-GP as well as with ISH-SP. Parasitological culture was used as the reference standard to
119 evaluate the sensitivity and specificity of ISH-GP, ISH-SP, IHC and HP. The microscopic
120 examination of the ISH, IHC and HP stained slides was performed blindly by a single pathologist
121 with experience in microscopic diagnosis of *Leishmania* (RCM).
122
123 **Parasitological Culture and Characterization by MLEE**
124 Skin samples collected in saline were seeded in the biphasic culture medium NNN (Novy,
125 MacNeal and Nicolle)/Schneider's Insect Medium (Sigma-Aldrich Co., St Louis, MO)
126 containing 10% fetal bovine serum and were incubated at 26-28 $^{o}$C. The *Leishmania*
127 promastigotes isolated were identified by MLEE using five enzymatic systems (6).
128
129 **Immunohistochemistry and Histopathology**
130 For immunohistochemistry, serial sections of 5 μm were obtained on silane-treated slides
131 and processed according to a previously described protocol (29), with some modifications. The
132 antigen exposure was performed by incubation of the sections in a sodium citrate buffer (pH =
133 6.0) at 100 $^{o}$C for 20 min in steam. Then, the sections were incubated with rabbit anti-
134 *Leishmania* polyclonal serum at the dilution of 1:500. Histological sections with numerous
135 *Leishmania* amastigote forms were incubated with homologous non-immune serum as negative

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136 control and with the rabbit anti-*Leishmania* polyclonal serum as positive control. For
137 histopathology, serial sections of 5 µm were stained by hematoxylin-eosin (5).
138

139 **Probe Design for ISH**

140 The generic probe is a digoxigenin-labeled oligonucleotide probe that detects a 5.8S
141 ribosomal RNA sequence specific to all relevant *Leishmania* species (10). The specific probe
142 was developed based on previous published sequences from GenBank using the computer
143 program Oligo 6 (31) and following previously described parameters (10, 12). It is an
144 oligonucleotide probe (5'-GCCCCTACCCGGAGGACCAGAAAAGTT-3') labeled with
145 digoxigenin at the 5' end (Integrated DNA Technologies, Coralville, IA) and that targets a
146 fragment of the kinetoplast minicircle DNA (kDNA) gene. The specific probe was designed to
147 discriminate *L. infantum* from the other species of *Leishmania* that infect dogs in the New World
148 such as *L. colombiensis*, *L. panamensis*, *L. mexicana*, *L. braziliensis*, *L. peruviana*, *L. pifanoi* and
149 *L. amazonensis* (8). The in silico analysis using the Basic Local Alignment Search Tool
150 (www.ncbi.nlm.nih.gov/blast.cgi) showed that the specific probe cross reacted only with *L.*
151 *donovani*, *L. tropica*, *L. major*, which are species that do not occur in the New World. Following
152 labeling with digoxigenin at the 5' end, both oligoprobes were purified by high performance
153 liquid chromatography (HPLC) (Integrated DNA Technologies, Coralville, IA).
154

155 **ISH Technique**

156 Several preliminary tests were done to define the best protocol and concentration of each
157 probe in order to achieve the best signal-to-noise ratio and thus the maximum sensitivity and
158 specificity. Considering that each probe had different properties of melting temperature at the

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159 NaCl concentration of 50 mM, of guanine-cytosine (GC) content and molecular weight, and also
160 different targets and specificities, a different protocol and concentration were used for each
161 probe.

162 Serial tissue sections of the selected FFPE skin samples were prepared at 5 µm thickness and
163 placed on positively charged slides. These slides were then submitted to deparaffinization and
164 fixation using the Discovery XT automated slide processing system (Ventana Medical Systems,
165 Inc., Tucson, AZ), as programed in the protocol for RiboMap in situ hybridization reagent
166 system (Ventana Medical Systems). Proteolytic treatment was performed using Protease 3 (0.02
167 units/ml alkaline protease, Ventana Medical Systems) for 12 min at 37 ºC. Thereafter, the slides
168 received pre-treatment through mild cell conditioning using the citrate buffer-based RiboCC
169 reagent (Ventana Medical Systems) for 4 min at 95 ºC. The slides were then submitted to
170 denaturation for 4 min at 37 ºC, followed by hybridization with the antisense oligonucleotide
171 probe for *Leishmania* suspended in hybridization buffer (RiboHybe, Ventana Medical Systems).
172 The time of hybridization was 1 hour at 37 ºC for the generic probe and at 47 ºC for the specific
173 probe. The concentration used for the generic probe was 93 ng/ml (1:10,000 dilution) and for the
174 specific probe was 893 ng/ml (dilution 1:1,000). For the generic probe, three stringency washing
175 steps were performed using 0.5× RiboWash (Ventana Medical Systems; equivalent to 0.5× saline
176 sodium citrate) each for 4 min at 42 °C. For the specific probe, four stringency washing steps
177 were performed at 42 °C using 0.1× RiboWash (Ventana Medical Systems; equivalent to 0.1×
178 saline sodium citrate) for 4 min for the first three and for 8 min for the forth washing step. After
179 the stringency washes, the slides were incubated with anti-digoxigenin antibody for 32 min at 37
180 °C. The anti-digoxigenin antibody for the generic probe was a rabbit polyclonal serum (Sigma-
181 Aldrich Co., St. Louis, MO) at the dilution of 1:20,000. For the specific probe, a rabbit

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182 monoclonal anti-digoxigenin antibody (Invitrogen Corporation, Frederick, MD) was used at the
183 dilution of 1:10,000. After streptavidin-alkaline phosphatase conjugate (UMap anti-Rb AP,
184 Ventana Medical Systems) incubation for 16 minutes at 37 °C, the signal was detected
185 automatically using the BlueMap NBT/BCIP substrate kit (Ventana Medical Systems) for 2
186 hours at 37 °C. Finally, the sections were counterstained with the nuclear fast red equivalent
187 reagent Red Counterstain II (Ventana Medical Systems) for 4 min before coverslipping. Sections
188 of FFPE skin and lymph node samples of dogs infected with numerous *L. infantum* amastigote
189 forms were used as controls. Infection by *L. infantum* in these controls had been confirmed by
190 parasitological culture and MLEE. For the negative reagent controls, sections were treated only
191 with RiboHybe hybridization buffer. The total duration of slide processing for ISH-GP was 8:17
192 hours and for ISH-SP was 8:25 hours using the Discovery XT.
193
194 **Probe Validation**
195 Before testing the ISH on the selected canine skin samples, validation of each probe was
196 performed to confirm the specificity of the generic probe to *Leishmania* and the specificity of the
197 specific probe to *L. infantum* by excluding cross-hybridization with selected pathogenic fungi
198 and protozoa. For this purpose, FFPE tissues infected with various protozoa and fungi and FFPE
199 pellets of some of these microorganisms obtained by centrifugation of cultured pathogens were
200 tested. Validation samples of protozoa consisted of *L. infantum* (skin of dog; pellet of
201 promastigote forms), *L. braziliensis* (skin of dog and hamster; pellet of promastigote forms), *L.*
202 *amazonensis* (skin of mouse; pellet of promastigote forms), *Trypanosoma caninum* (pellet of
203 epimastigote, spheromastigote and trypomastigote forms), *T. cruzi* (heart of mouse; pellet of
204 epimastigote and trypomastigote forms), *Neospora caninum* (lung of rat; pellet of tachyzoites),

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205 *Toxoplasma gondii* (lung and heart of dog; brain of mouse; intestine and lymph nodes of
206 wallaby), *Sarcocystis neurona* (brain of horse) and *Rangelia vitalii* (heart and kidney of dog).
207 The methods used for the diagnosis of protozoa were isolation in parasitological culture and
208 MLEE, except for *T. gondii* and *N. caninum*, which were identified by PCR and IHC, and *R.*
209 *vitalii,* which was detected by HP in tissues of a dog with the characteristic clinical and
210 pathological alterations caused by this protozoon (14). Validation samples of fungi consisted of
211 *Blastomyces dermatitidis* (lung of dog), *Cryptococcus neoformans* (nasal mucosa of horse),
212 *Sporothrix* (skin of dog and cat) and *Histoplasma capsulatum* (spleen and kidney of dog; pellet
213 of mycelial form). The diagnosis of all these fungi was based on mycological culture and
214 Grocott's methenamine silver stain. In all pellets and tissue samples used for validation,
215 microorganisms were easily visible by light microscopy.

216

217 **Statistical Analysis**

218 Data obtained were stored in the EpiData software and then analyzed using the Statistical
219 Package for Social Sciences software (version 16.0) for Windows. The sensitivity and its
220 respective 95% confidence interval (CI), the specificity and accuracy of ISH-GP, ISH-SP, IHC
221 and HP were compared to the reference standard (parasitological culture). The comparison
222 between the sensitivity and specificity of ISH-GP, ISH-SP, IHC and HP was descriptive.

223

224 **RESULTS**

225 The ISH using both the generic (ISH-GP) and specific (ISH-SP) probes clearly detected
226 amastigote forms of *Leishmania* with a dark blue signal that was slightly stronger for the ISH-GP
227 (Fig. 1A). The ISH-SP showed instead less background and a better signal-to-noise ratio (Fig.

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228 1B). There was no cross-hybridization of either probe with any of the other microorganisms
229 tested. However, there was cross-reaction of the polyclonal anti-digoxigenin antibody (Sigma-
230 Aldrich) used for ISH-GP with cysts and tachyzoites of *Toxoplasma gondii*. This problem was
231 solved by replacing this antibody with the same monoclonal anti-digoxigenin antibody
232 (Invitrogen) used for ISH-SP. All species of *Leishmania* tested were detected by ISH-GP (Fig.
233 1A, D). The ISH-SP detected only *L. infantum* (Fig.1B, E, F).

234 The IHC clearly detected amastigote forms of *Leishmania* with a dark brown signal and a
235 good signal-to-noise ratio (Fig. 1C).

236 The results of sensitivity and accuracy of ISH-SP, ISH-GP, IHC and HP for detecting *L.*
237 *infantum* are listed in Table 1. The specificity of ISH-SP, ISH-GP, IHC and HP tested was 100%.

238 Of the 51 skin samples positive for *L. infantum* by parasitological culture, 36 were detected by
239 ISH-GP. Fifteen skin samples positive by parasitological culture were not detected by ISH-GP.

240 Of the 51 kin samples positive for *L. infantum* by parasitological culture, 38 were detected by
241 ISH-SP. Thirteen skin samples positive by parasitological culture were not detected by ISH-SP.

242

243 **DISCUSSION**

244 The ISH-GP and ISH-SP were both sensitive and specific methods for the diagnosis of
245 *Leishmania* in dogs, showing satisfactory accuracy compared to the reference standard. The
246 values of sensitivity of both ISH methods were very close to those of IHC, with ISH-SP being
247 the most sensitive, followed by ISH-GP, IHC and HP. The somewhat higher sensitivity of ISH-SP
248 in spite of the slightly lower signal intensity compared to ISH-GP may be due to the better signal-
249 to-noise ratio of ISH-SP. The use of ISH and IHC increased the number of correctly diagnosed

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250 positive cases compared to HP similar to what has been reported by other authors when using
251 IHC (28, 33, 35, 37).

252 The results of the current study confirm ISH as an accurate method for the diagnosis of *L.*
253 *infantum* infection in dogs. Nonetheless, it is less sensitive for the diagnosis of *L. infantum*
254 infection in dogs than parasitological culture, PCR on frozen skin samples and some serological
255 assays (13, 17, 19, 28, 30, 37). ISH failed to detect between 25.5% and 29.4% of the
256 parasitological culture positive cases in the current study. In the previous study using the same
257 generic ISH probe (10), ISH was negative in 3 dogs out of 6 positive for *L. infantum* by PCR. In
258 a systematic review (30), the majority of studies on serological assays for the diagnosis of *L.*
259 *infantum* infection in dogs found sensitivities higher than 75%. Nonetheless, parasitological
260 culture, PCR and serological assays present disadvantages that prevent their use alone for the
261 routine diagnosis of *L. infantum* infection in dogs. The parasitological culture is time-consuming,
262 because it takes from 5 to 30 days (in average 15 days) to be completed and there are worldwide
263 only a small number of reference centers currently using MLEE (1, 38). In addition, this method
264 is susceptible to microbiologic contamination, which in many cases prevents its use in samples
265 collected in the field, where proper storage and sterile conditions may be difficult to attain (38,
266 20). Furthermore, parasitological culture may be difficult to perform due to poor adaptation of some
267 isolates to the medium (38, 20). The drawbacks of PCR are lack of standardization of the
268 different protocols used among laboratories, possibility of contamination and the fact that it does
269 not necessarily indicate live infection with *Leishmania* (2, 15, 37, 38). Serologic assays may
270 yield false-positive results due to cross-reactivity with sera of dogs infected with *L. braziliensis*,
271 *T. cruzi*, *T. caninum* and *Ehrlichia canis* (3, 19, 27, 36). Also, they do not necessarily indicate

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272 current infection (20) and do not differentiate positive results produced by natural infection from
273 those induced by vaccines (30).

274 The main advantage of ISH over parasitological culture, PCR, serological assays, IHC and
275 HP for the diagnosis of *Leishmania* infection in dogs is that it simultaneously allows
276 visualization of the intact amastigotes within the tissue and speciates them as *Leishmania*
277 *infantum*, as demonstrated in the present study by ISH-SP. This observation of amastigotes
278 within the tissue, which is not possible by parasitological culture, PCR and serological assays,
279 offers the possibility to correlate parasites with the associated lesions and also to semi-quantify
280 the parasite load (10, 26, 28). Similarly to ISH, IHC and HP also link amastigotes of *Leishmania*
281 to lesions (28); however, they are not able to discriminate *L. infantum* from other *Leishmania*
282 species. An important advantage of ISH compared to IHC and HP is therefore the higher
283 specificity of ISH. Extensive testing of the specificity of ISH has shown no cross-reaction of the
284 *Leishmania* probes with other histomorphologically similar organisms (10), which was
285 confirmed in the current study. Although IHC in the present study was specific for the diagnosis
286 of *Leishmania*, cross reactivity with histomorphologically similar fungi such as *Histoplasma*
287 *capsulatum* has been demonstrated (29). In addition, parasitic organisms such as *Histoplasma*
288 and *Trypanosoma* are difficult to be differentiated from *Leishmania* by HP; thus, wrong or
289 inconclusive etiologic diagnoses may occur using this method (4, 10). Furthermore,
290 commercially available anti-*Leishmania* antibodies for IHC that work well on FFPE tissues are
291 currently lacking (4, 10). The possibility of automation is another advantage of ISH (32), which
292 was tested with success in the present work, but has not yet been evaluated for IHC in the
293 diagnosis of *L. infantum* infection in dogs. This automation reduced the time to one day for the
294 labeling of the slides in comparison to the two days needed for the manual protocols of the IHC

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295 used in the present study and of the previously reported ISH (10). In addition to productivity,
296 automation will improve the reproducibility of ISH.

297 The use of ISH-SP for the specific detection of *L. infantum* will be particularly important in
298 some areas in south-eastern Brazil, where this protozoal organism co-occurs with *L. braziliensis*
299 (18, 19). In these areas, many dogs afected only by *L. braziliensis* are unnecessarily euthanatized
300 as a method for controlling VL (22) due to serological cross reaction with *L. infantum*. Dogs
301 parasitized by species of *Leishmania* other than *L. infantum* do not have to be euthanatized in
302 Brazil because the dog is not a proven reservoir of the other *Leishmania* species and thus is not
303 considered to be involved in their zoonotic transmission (22). Considering that the specific probe
304 cross reacted in silico with *L. donovani*, *L. tropica*, *L. major,* its use is not as useful in areas
305 where these species occur in dogs such as in the Middle East, Africa and Asia (9, 11, 16, 24, 25),
306 possibly warranting the design of additional probes.

307 The current study demonstrated that ISH-GP and ISH-SP have a high sensitivity and
308 specificity, improving the histological diagnosis of *L. infantum* in routinely processed, formalin-
309 fixed surgical skin biopsies of dogs. Hence, the concurrent use of ISH-GP and ISH-SP should be
310 implemented in the laboratory as a useful tool to not only detect *Leishmania* in general, but to
311 differentiate *L. infantum* in surgical samples.

312

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322

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453 **FIGURE LEGENDS**

454

455 FIG 1. In situ hybridization using A) the generic probe for *Leishmania* spp. on the skin of a dog
456 showing numerous intrahistiocytic *Leishmania infantum* amastigotes labeled with a dark blue
457 signal, and B) the specific probe for *L. infantum* on the skin of a dog showing numerous
458 intrahistiocytic *L. infantum* amastigote forms labeled with a dark blue signal. Note that the signal
459 is slightly weaker than that of the generic probe and there is less background, which allowed
460 clearer visualization of individualized amastigote forms. C) Immunohistochemistry of the skin of
461 a dog showing numerous dark-brown stained *L. infantum* amastigote forms within macrophages.
462 In situ hybridization using D) the generic probe for *Leishmania* spp. on the skin of a dog
463 showing numerous intrahistiocytic *L. braziliensis* amastigote forms labeled with a dark blue
464 signal, E) the specific probe for *L. infantum* on the skin of a dog infected by *L. braziliensis*
465 demonstrating that amastigote forms (arrows) were not labeled, and F) the same specific probe
466 on the skin of a mouse experimentally infected with *L. amazonensis* showing numerous
467 intrahistiocytic amastigote forms not labeled. Bar of all figures = 33 µm.

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476 **TABLE**

477 Table 1. Sensitivity and accuracy of in situ hybridization using a specific probe for *Leishmania*
478 *infantum* (ISH-SP), in situ hybridization using a generic probe for *Leishmania* (ISH-GP),
479 immunohistochemistry (IHC) and histopathology (HP) for detecting *L. infantum* in formalin-
480 fixed, paraffin-embedded surgical skin biopsies of dogs.

| Method | Sensitivity in % (95% CI) | Accuracy in % |
|---|---|---|
| ISH-SP (N=102) | 74.5 (66.1 - 83.0) | 87.2 |
| ISH-GP (N=102) | 70.6 (61.7 - 79.4) | 85.3 |
| IHC (N= 132) | 69.5 (61.1 - 77.9) | 86.4 |
| HP (N= 132) | 57.6 (49.2 - 66.1) | 81.1 |

481 N = number of skin samples tested; CI = confidence interval.

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1 **TABLE**

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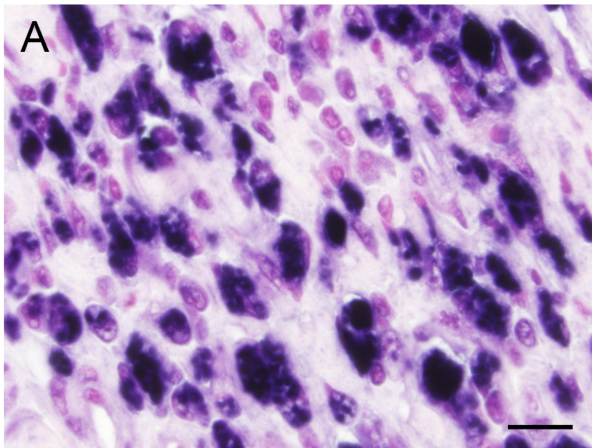


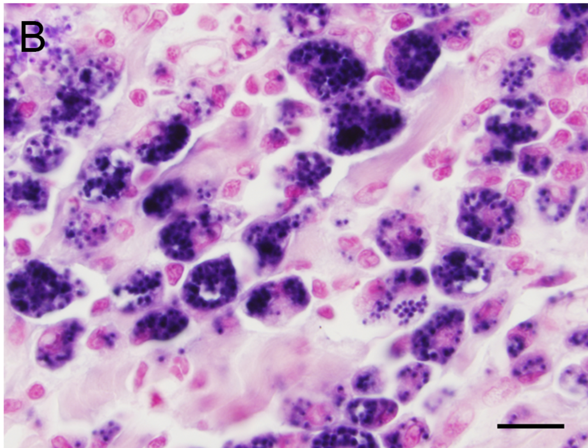


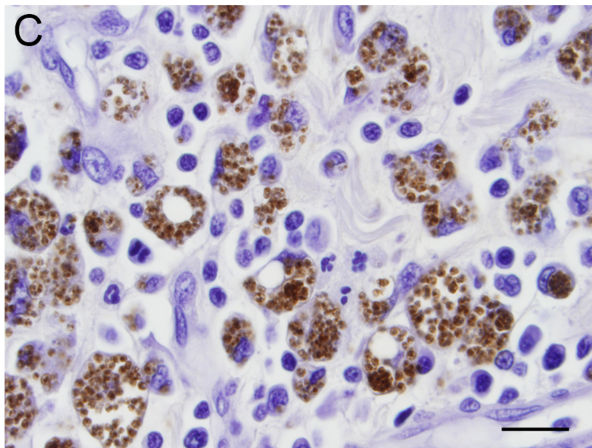


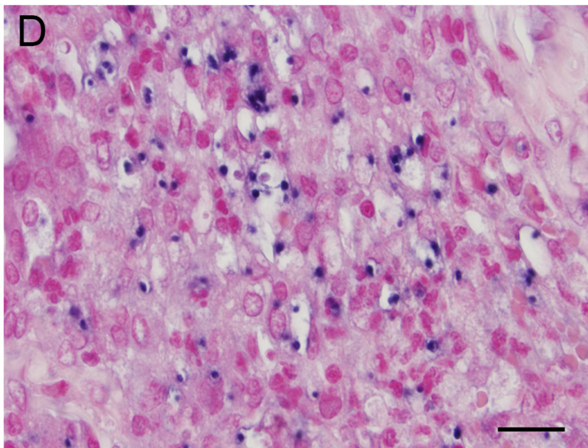


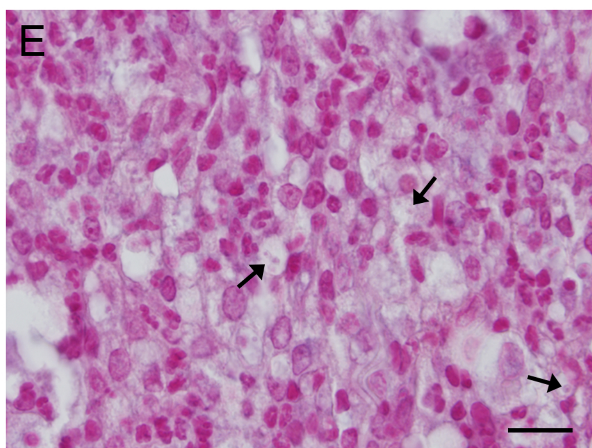


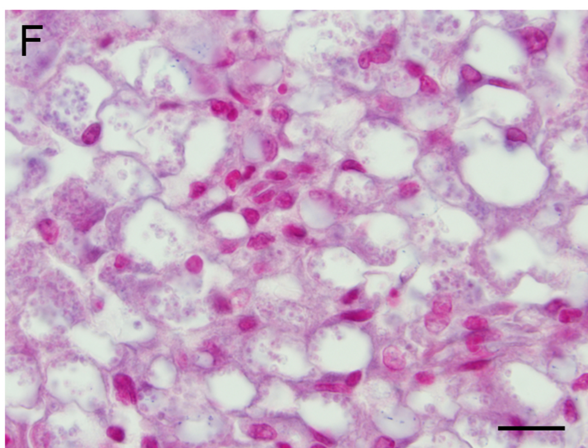


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