SENSITIVITY AND SPECIFICITY OF IN SITU HYBRIDIZATION FOR THE 
DIAGNOSIS OF CUTANEOUS INFECTION BY Leishmania infantum IN DOGS

Running title: In situ hybridization of L. infantum in dogs

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ABSTRACT

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

INTRODUCTION

Leishmaniasis is a worldwide disease caused by protozoa of the genus *Leishmania*, which infect wild and domestic mammals including humans (38). The spectrum of clinical forms of leishmaniasis can vary from focal cutaneous to disseminated visceral disease (38). There are
twelve *Leishmania* species infecting dogs: *L. donovani*, *L. infantum* (syn. *L. chagasi*), *L. major*, *L. arabica* and *L. tropica* in the Old World, and *L. infantum*, *L. colombiensis*, *L. panamensis*, *L. mexicana*, *L. braziliensis*, *L. peruviana*, *L. pifanoi* and *L. amazonensis* in the New World (7, 8, 9, 11, 24, 34, 38). Although many species of *Leishmania* infect dogs, dogs are considered a proven reservoir only for *L. infantum* (7, 38). The species *L. infantum* can also infect humans, cats and wild mammals and is the cause of zoonotic visceral leishmaniasis (VL) (7, 21, 38).

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

The clinical diagnosis of canine VL is difficult, with many animals being asymptomatic. Therefore, a variety of serological, parasitological and molecular laboratory methods have been developed to detect infection by *L. infantum* in dogs (20, 23). Serological assays, polymerase chain reaction (PCR) and parasitological culture are the most sensitive methods for the diagnosis of *L. infantum* infection (13, 15, 17, 19, 28, 30, 37). However, these three methods do not allow visualization of the intact amastigotes within the tissue and correlation of the parasites with associated lesions, which is possible by histopathology (HP) and immunohistochemistry (IHC) (10). HP and IHC are frequently used in the current routine for the histological diagnosis of *L. infantum* in dogs, but these methods have limited accuracy and do not allow speciation (37, 28, 29). The speciation of *Leishmania* is currently only possible by parasitological culture followed by multilocus enzyme electrophoresis (MLEE), which is the reference method, and by PCR (38).
Hence, alternative histological methods are necessary to improve the accuracy of diagnosing infection of dogs with *L. infantum*. A recently established chromogenic in situ hybridization (ISH) technique is a promising method for the diagnosis of canine VL because it permits the highly specific identification of *Leishmania* in formalin-fixed, paraffin-embedded surgical biopsies of dogs (10). However, the previously published probe for ISH was unable to speciate *Leishmania* (10). In addition, the published ISH protocol was based on manual labeling, which presents lower efficiency and productivity than automation (32). Thus, the present study aimed to evaluate the sensitivity and specificity of automated ISH for the diagnosis of canine cutaneous infection caused by *L. infantum* on routinely processed surgical biopsy samples using the previously published generic probe for *Leishmania* and a newly designed probe specific for *L. infantum*. The results of both ISH tests were compared to IHC and HP in the same samples, using parasitological culture as the reference standard technique.

**MATERIAL AND METHODS**

**Samples**

A prospective study was designed using randomly selected samples from 2,066 surgical skin biopsies of dogs collected between the years 2008 and 2012. The dogs originated from seven cities in Brazil with endemic canine VL: Niterói-RJ, Rio de Janeiro-RJ, Bauru-SP, Brasília-DF, Cuiabá-MT, Palmas-TO and Fortaleza-CE. Skin samples were selected for this study since they are easy to obtain and have been shown to be a good target for the confirmation of canine VL by parasitological culture (17).
For the collection of samples, one 3 mm punch biopsy was obtained from the intact skin over the scapula, after disinfection with 70% alcohol and local anesthesia with 2% lidocaine. Each obtained specimen was divided into two samples. One of them was immersed in sterile saline with antimicrobials (1) and submitted for parasitological culture. The other fragment was fixed in 10% neutral buffered formalin and processed for routine paraffin embedding (5). The paraffin blocks were processed for ISH, IHC and HP. The IHC, HP and parasitological culture with identification of the species of trypanosomatids by MLEE were performed at the Evandro Chagas Clinical Research Institute, FIOCRUZ, Brazil. The ISH was performed at the Diagnostic Center for Population and Animal Health, Michigan State University, USA. The biopsy procedure performed on animals was approved by the Ethics Committee on the Use of Animals, FIOCRUZ, Brazil (license L-038/08).

**Study Design**

ISH using a previously published generic (ISH-GP) and a newly developed specific (ISH-SP) oligoprobe for the diagnosis of *L. infantum* infection in dogs were evaluated. In order to calculate the number of samples required for this study, the estimated values for the ISH were 70% of sensitivity/specificity according to preliminary tests, 17% of absolute error in sensitivity/specificity and 5% of alpha. Considering the loss of samples during processing, the calculated number was increased by 4%. As a result, 51 dogs positive for *L. infantum* infection in the parasitological culture and 51 negative dogs for *L. infantum* infection in the parasitological culture were randomly selected from 2,066 surgical skin biopsies of dogs. Ten of the 51 samples of the *L. infantum* negative group were from dogs positive for the protozoon *Trypanosoma caninum* based on parasitological culture. Thirty formalin-fixed, paraffin-embedded (FFPE)
samples (8 positive and 22 negative for *L. infantum* based on parasitological culture) were consumed after the testing with ISH-GP. They were replaced by new randomly selected samples (8 positive and 22 negative for *L. infantum* based on parasitological culture) for the testing with ISH-SP. All 132 samples were tested by IHC and HP and a total of 102 dogs was tested with ISH-GP as well as with ISH-SP. Parasitological culture was used as the reference standard to evaluate the sensitivity and specificity of ISH-GP, ISH-SP, IHC and HP. The microscopic examination of the ISH, IHC and HP stained slides was performed blindly by a single pathologist with experience in microscopic diagnosis of *Leishmania* (RCM).

**Parasitological Culture and Characterization by MLEE**

Skin samples collected in saline were seeded in the biphasic culture medium NNN (Novy, MacNeal and Nicolle)/Schneider’s Insect Medium (Sigma-Aldrich Co., St Louis, MO) containing 10% fetal bovine serum and were incubated at 26-28 °C. The *Leishmania* promastigotes isolated were identified by MLEE using five enzymatic systems (6).

**Immunohistochemistry and Histopathology**

For immunohistochemistry, serial sections of 5 µm were obtained on silane-treated slides and processed according to a previously described protocol (29), with some modifications. The antigen exposure was performed by incubation of the sections in a sodium citrate buffer (pH = 6.0) at 100 °C for 20 min in steam. Then, the sections were incubated with rabbit anti-*Leishmania* polyclonal serum at the dilution of 1:500. Histological sections with numerous *Leishmania* amastigote forms were incubated with homologous non-immune serum as negative
control and with the rabbit anti-*Leishmania* polyclonal serum as positive control. For histopathology, serial sections of 5 μm were stained by hematoxylin-eosin (5).

**Probe Design for ISH**

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

**ISH Technique**

Several preliminary tests were done to define the best protocol and concentration of each probe in order to achieve the best signal-to-noise ratio and thus the maximum sensitivity and specificity. Considering that each probe had different properties of melting temperature at the
NaCl concentration of 50 mM, of guanine-cytosine (GC) content and molecular weight, and also different targets and specificities, a different protocol and concentration were used for each probe.

Serial tissue sections of the selected FFPE skin samples were prepared at 5 μm thickness and placed on positively charged slides. These slides were then submitted to deparaffinization and fixation using the Discovery XT automated slide processing system (Ventana Medical Systems, Inc., Tucson, AZ), as programmed in the protocol for RiboMap in situ hybridization reagent system (Ventana Medical Systems). Proteolytic treatment was performed using Protease 3 (0.02 units/ml alkaline protease, Ventana Medical Systems) for 12 min at 37 ºC. Thereafter, the slides received pre-treatment through mild cell conditioning using the citrate buffer-based RiboCC reagent (Ventana Medical Systems) for 4 min at 95 ºC. The slides were then submitted to denaturation for 4 min at 37 ºC, followed by hybridization with the antisense oligonucleotide probe for *Leishmania* suspended in hybridization buffer (RiboHybe, Ventana Medical Systems). The time of hybridization was 1 hour at 37 ºC for the generic probe and at 47 ºC for the specific probe. The concentration used for the generic probe was 93 ng/ml (1:10,000 dilution) and for the specific probe was 893 ng/ml (dilution 1:1,000). For the generic probe, three stringency washing steps were performed using 0.5× RiboWash (Ventana Medical Systems; equivalent to 0.5× saline sodium citrate) each for 4 min at 42 ºC. For the specific probe, four stringency washing steps were performed at 42 ºC using 0.1× RiboWash (Ventana Medical Systems; equivalent to 0.1× saline sodium citrate) for 4 min for the first three and for 8 min for the forth washing step. After the stringency washes, the slides were incubated with anti-digoxigenin antibody for 32 min at 37 ºC. The anti-digoxigenin antibody for the generic probe was a rabbit polyclonal serum (Sigma-Aldrich Co., St. Louis, MO) at the dilution of 1:20,000. For the specific probe, a rabbit
monoclonal anti-digoxigenin antibody (Invitrogen Corporation, Frederick, MD) was used at the
dilution of 1:10,000. After streptavidin-alkaline phosphatase conjugate (UMap anti-Rb AP,
Ventana Medical Systems) incubation for 16 minutes at 37 °C, the signal was detected
automatically using the BlueMap NBT/BCIP substrate kit (Ventana Medical Systems) for 2
hours at 37 °C. Finally, the sections were counterstained with the nuclear fast red equivalent
reagent Red Counterstain II (Ventana Medical Systems) for 4 min before coverslipping. Sections
of FFPE skin and lymph node samples of dogs infected with numerous *L. infantum* amastigote
forms were used as controls. Infection by *L. infantum* in these controls had been confirmed by
parasitological culture and MLEE. For the negative reagent controls, sections were treated only
with RiboHybe hybridization buffer. The total duration of slide processing for ISH-GP was 8:17
hours and for ISH-SP was 8:25 hours using the Discovery XT.

**Probe Validation**

Before testing the ISH on the selected canine skin samples, validation of each probe was
performed to confirm the specificity of the generic probe to *Leishmania* and the specificity of the
specific probe to *L. infantum* by excluding cross-hybridization with selected pathogenic fungi
and protozoa. For this purpose, FFPE tissues infected with various protozoa and fungi and FFPE
pellets of some of these microorganisms obtained by centrifugation of cultured pathogens were
tested. Validation samples of protozoa consisted of *L. infantum* (skin of dog; pellet of
promastigote forms), *L. braziliensis* (skin of dog and hamster; pellet of promastigote forms), *L.
amazonensis* (skin of mouse; pellet of promastigote forms), *Trypanosoma caninum* (pellet of
epiprostigote, spheromastigote and trypomastigote forms), *T. cruzi* (heart of mouse; pellet of
epiprostigote and trypomastigote forms), *Neospora caninum* (lung of rat; pellet of tachyzoites),
Toxoplasma gondii (lung and heart of dog; brain of mouse; intestine and lymph nodes of wallaby), Sarcocystis neurona (brain of horse) and Rangelia vitalii (heart and kidney of dog). The methods used for the diagnosis of protozoa were isolation in parasitological culture and MLEE, except for T. gondii and N. caninum, which were identified by PCR and IHC, and R. vitalii, which was detected by HP in tissues of a dog with the characteristic clinical and pathological alterations caused by this protozoon (14). Validation samples of fungi consisted of Blastomyces dermatitidis (lung of dog), Cryptococcus neoformans (nasal mucosa of horse), Sporothrix (skin of dog and cat) and Histoplasma capsulatum (spleen and kidney of dog; pellet of mycelial form). The diagnosis of all these fungi was based on mycological culture and Grocott’s methenamine silver stain. In all pellets and tissue samples used for validation, microorganisms were easily visible by light microscopy.

Statistical Analysis

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

RESULTS

The ISH using both the generic (ISH-GP) and specific (ISH-SP) probes clearly detected amastigote forms of Leishmania with a dark blue signal that was slightly stronger for the ISH-GP (Fig. 1A). The ISH-SP showed instead less background and a better signal-to-noise ratio (Fig.
There was no cross-hybridization of either probe with any of the other microorganisms tested. However, there was cross-reaction of the polyclonal anti-digoxigenin antibody (Sigma-Aldrich) used for ISH-GP with cysts and tachyzoites of *Toxoplasma gondii*. This problem was solved by replacing this antibody with the same monoclonal anti-digoxigenin antibody (Invitrogen) used for ISH-SP. All species of *Leishmania* tested were detected by ISH-GP (Fig. 1A, D). The ISH-SP detected only *L. infantum* (Fig. 1B, E, F).

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

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

Of the 51 skin samples positive for *L. infantum* by parasitological culture, 36 were detected by ISH-GP. Fifteen skin samples positive by parasitological culture were not detected by ISH-GP. Of the 51 skin samples positive for *L. infantum* by parasitological culture, 38 were detected by ISH-SP. Thirteen skin samples positive by parasitological culture were not detected by ISH-SP.

**DISCUSSION**

The ISH-GP and ISH-SP were both sensitive and specific methods for the diagnosis of *Leishmania* in dogs, showing satisfactory accuracy compared to the reference standard. The values of sensitivity of both ISH methods were very close to those of IHC, with ISH-SP being the most sensitive, followed by ISH-GP, IHC and HP. The somewhat higher sensitivity of ISH-SP in spite of the slightly lower signal intensity compared to ISH-GP may be due to the better signal-to-noise ratio of ISH-SP. The use of ISH and IHC increased the number of correctly diagnosed...
The results of the current study confirm ISH as an accurate method for the diagnosis of *L. infantum* infection in dogs. Nonetheless, it is less sensitive for the diagnosis of *L. infantum* infection in dogs than parasitological culture, PCR on frozen skin samples and some serological assays (13, 17, 19, 28, 30, 37). ISH failed to detect between 25.5% and 29.4% of the parasitological culture positive cases in the current study. In the previous study using the same generic ISH probe (10), ISH was negative in 3 dogs out of 6 positive for *L. infantum* by PCR. In a systematic review (30), the majority of studies on serological assays for the diagnosis of *L. infantum* infection in dogs found sensitivities higher than 75%. Nonetheless, parasitological culture, PCR and serological assays present disadvantages that prevent their use alone for the routine diagnosis of *L. infantum* infection in dogs. The parasitological culture is time-consuming, because it takes from 5 to 30 days (in average 15 days) to be completed and there are worldwide only a small number of reference centers currently using MLEE (1, 38). In addition, this method is susceptible to microbiologic contamination, which in many cases prevents its use in samples collected in the field, where proper storage and sterile conditions may be difficult to attain (38, 20). Furthermore, parasitological culture may be difficult to perform due to poor adaptation of some isolates to the medium (38, 20). The drawbacks of PCR are lack of standardization of the different protocols used among laboratories, possibility of contamination and the fact that it does not necessarily indicate live infection with *Leishmania* (2, 15, 37, 38). Serologic assays may yield false-positive results due to cross-reactivity with sera of dogs infected with *L. braziliensis*, *T. cruzi*, *T. caninum* and *Ehrlichia canis* (3, 19, 27, 36). Also, they do not necessarily indicate...
current infection (20) and do not differentiate positive results produced by natural infection from
those induced by vaccines (30).

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

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

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

\section*{ACKNOWLEDGEMENTS}

The authors would like to thank the histopathology technicians Tom Wood and Kelli Cicinelli from DCPAH, Michigan State University, USA and Luiz Claudio Ferreira from IPEC, FIOCRUZ, Brazil for their technical assistance; Mr. Rodrigo Méxas from IOC, FIOCRUZ, Brazil for processing the figures; and Dr. Jitender P. Dubey from USDA, USA, Dr. Rafael A.
Fighera from UFSM, Brazil and Dr. Léa C. Finkelstein from FIOCRUZ, Brazil for generously providing the paraffin-embedded tissues infected with *N. caninum*, *R. vitalii* and *L. amazonensis*, respectively. RCM has a fellowship from CAPES Foundation, Ministry of Education of Brazil, process number: BEX 6925/10-3.

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FIGURE LEGENDS

FIG 1. In situ hybridization using A) the generic probe for *Leishmania* spp. on the skin of a dog showing numerous intrahistiocytic *Leishmania infantum* amastigotes labeled with a dark blue signal, and B) the specific probe for *L. infantum* on the skin of a dog showing numerous intrahistiocytic *L. infantum* amastigote forms labeled with a dark blue signal. Note that the signal is slightly weaker than that of the generic probe and there is less background, which allowed clearer visualization of individualized amastigote forms. C) Immunohistochemistry of the skin of a dog showing numerous dark-brown stained *L. infantum* amastigote forms within macrophages. In situ hybridization using D) the generic probe for *Leishmania* spp. on the skin of a dog showing numerous intrahistiocytic *L. braziliensis* amastigote forms labeled with a dark blue signal, E) the specific probe for *L. infantum* on the skin of a dog infected by *L. braziliensis* demonstrating that amastigote forms (arrows) were not labeled, and F) the same specific probe on the skin of a mouse experimentally infected with *L. amazonensis* showing numerous intrahistiocytic amastigote forms not labeled. Bar of all figures = 33 µm.
Table 1. Sensitivity and accuracy of in situ hybridization using a specific probe for *Leishmania infantum* (ISH-SP), in situ hybridization using a generic probe for *Leishmania* (ISH-GP), immunohistochemistry (IHC) and histopathology (HP) for detecting *L. infantum* in formalin-fixed, paraffin-embedded surgical skin biopsies of dogs.

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<th>Accuracy in %</th>
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<tr>
<td>ISH-SP (N=102)</td>
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<td>87.2</td>
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<tr>
<td>ISH-GP (N=102)</td>
<td>70.6 (61.7 - 79.4)</td>
<td>85.3</td>
</tr>
<tr>
<td>IHC (N=132)</td>
<td>69.5 (61.1 - 77.9)</td>
<td>86.4</td>
</tr>
<tr>
<td>HP (N=132)</td>
<td>57.6 (49.2 - 66.1)</td>
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N = number of skin samples tested; CI = confidence interval.
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