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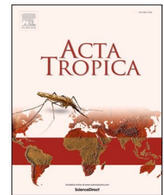

Evaluation of five different rapid immunochromatographic tests for canine leishmaniosis in Spain

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ABSTRACT

Canine leishmaniosis is a vector-borne disease caused by *Leishmania* parasites. Serological methods are the most common tests used for the diagnosis. This study aimed to evaluate and compare different serological commercial immunochromatographic rapid tests available in Spain to detect anti-*Leishmania* canine antibodies. The immunochromatographic tests were evaluated in different groups of dogs (healthy seronegative dogs (n = 21), naturally-sick dogs with moderate anti-*Leishmania* antibodies (n = 39), naturally-sick dogs with high anti-*Leishmania* antibodies (n = 37), dogs with the serological result of other pathogens infection (n = 20) and exposed dogs (n = 33)) admitted to the Veterinary Teaching Hospital of the University of Zaragoza (Spain) according to the clinical information sent with the sample to the laboratory for diagnostic purposes. The serology status was also routinely recorded through an in-house enzyme-linked immunosorbent assay (ELISA) and an in-house indirect immunofluorescence test (IFAT). The qualitative commercial serological immunochromatographic tests used were: FASTest LEISH, Uranotest *Leishmania*, Uranotest *Leishmania* 2.0, Speed Leish K, Witness *Leishmania*, and DFV Test *Leishmania*. Performance measures analyzed for each test were: sensitivity, specificity, and area under the receiver-operating (ROC) curve. The maximum specificity (1.00) was attained for Uranotest *Leishmania* and DFT Test *Leishmania*, followed by FASTest LEISH (0.98), Uranotest *Leishmania* 2.0 (0.98), Speed Leish K (0.98), and Witness *Leishmania* (0.95). The maximum sensitivity was attained for FASTest LEISH (1.00), followed by Uranotest 2.0 (0.97), Speed Leish K (0.97), Uranotest (0.96), and the lowest results with Witness (0.84) and DFV Test (0.59). Regarding the ROC curve, the maximum value was attained with the FASTest LEISH (0.99), followed by Uranotest (0.98), Uranotest 2.0 (0.97), Speed Leish K (0.97), Witness (0.90), and the lowest result with DFV Test (0.79). Efforts in the field of diagnosis should focus on establishing a commercial immunochromatographic test with high sensitivity and specificity with a reasonable cost-benefit balance.

1. Introduction

Canine leishmaniosis (CanL) is a vector-borne disease caused by the obligate intracellular protozoan parasite *Leishmania*. In European Mediterranean countries, natural transmission is primarily via Phlebotomus spp, being dogs naturally exposed to *P. perniciosus* bites (Vlkova et al., 2011). In dogs, clinical disease is characterized by non-specific clinicopathological abnormalities and clinical signs observed during the physical examination. However, most of the infected dogs do not develop any clinical signs being classified as seropositive animals with a negative result by PCR and/or cytology for exposed dogs or, by contrast, healthy infected dogs but positive PCR and/or cytology in bone marrow, lymph node, spleen, skin, or peripheral blood (Paltrinieri et al., 2016). In endemic areas of CanL, identifying seropositive dogs is necessary as a part of the prevention measures, including early detection of the infection (Solano-Gallego et al., 2011). In this sense, higher anti-*Leishmania*

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antibodies are linked to clinical disease (Proverbio et al., 2014). Other measures based on an integrative approach are the use of topical insecticides with proven activity for bite prevention in dogs and humans, environmental measures focused on reducing sand fly number, anti-*Leishmania* treatment of dogs with active leishmaniosis, immunotherapy, and vaccinations as complementary measures to use in dogs. All these measures are focused on limiting transmission from dogs to humans as a parasite reservoir (Miró et al., 2017; Miró and López-Vélez, 2018).

Different confirmatory tests are available to detect the presence of the parasite, including parasitological methods based on direct observation of the parasite (Maia and Campino, 2008). Other confirmatory techniques focus on detecting the presence of *Leishmania* spp. DNA in different biological samples with real-time PCR, conventional PCR, and nested PCR (Maia and Campino, 2018). Finally, a wide range of confirmatory tests is represented by serological methods based on detecting anti-*Leishmania* antibodies (IgGs) in serum samples. Serological methods include IFAT, ELISA, Western Blot (WB), and immunochromatographic rapid tests (ICTs) using a nitrocellulose matrix with recombinant antigens (Maia and Campino, 2008). However, significant differences can be observed among the serological methods; some of these techniques can quantify the level of anti-*Leishmania* antibodies obtaining an antibody titer for IFAT or an optical density for ELISA (Maia and Campino, 2018). By contrast, the WB technique is characterized by immunoreaction of anti-*Leishmania* antibodies with polypeptide fractions from the *Leishmania* antigen, and different positive kDa bands are observed (Alcover et al., 2021). The ELISA, WB, and IFI techniques require special equipment and trained personnel resulting in diagnostic delays. In the case of ICTs, these tests are easy to perform, obtaining a rapid diagnosis without quantifying the anti-*Leishmania* antibodies in the sample with variable sensitivity and high specificity compared to the reference laboratory techniques (Maia and Campino, 2018). ICTs have been used as an essential first step in diagnostic algorithms; however, as the dichotomic result obtained by these types of techniques, serodiagnosis interpretation in treated animals and seropositive healthy dogs living in highly endemic areas is difficult to interpret, being better diagnostic approaching quantitative serological techniques (Cavalera et al., 2021; Solano-Gallego et al., 2016).

In Spain, there are no recent comparative studies to determine the diagnostic performance of the most common ICTs that are commercially available (DFV Test *Leishmania*, FASTest LEISH, Speed Leish K, Uranotest *Leishmania*, Uranotest *Leishmania* 2.0, Witness *Leishmania*) in a clinical setting. Therefore, the serum samples originating from the archives of the Clinical Immunology Laboratory, Veterinary Faculty, University of Zaragoza, Spain, were used to evaluate the current ICTs available in Spain.

2. Material and methods

2.1. Serum samples

A total of 150 serum samples from dogs living in a leishmaniosis endemic area were selected for this study (Zaragoza, Spain) based on STARD guidelines (Standards for the Reporting of Diagnostic Accuracy Studies). These samples were collected for *Leishmania* diagnostic confirmation from June 2020 to December 2020, including two aliquots of each sample stored at −20 °C until testing. Each dog was classified into one group, including seronegative healthy dogs, clinically-sick infected dogs with moderate and high anti-*Leishmania* antibodies, dogs infected by some other pathogens or with other clinical conditions, and naturally exposed dogs without infection (Table 1). This classification was based on clinical evaluation and routine red blood cell count, clinical chemistry, urinalysis, serum protein electrophoresis, and the serology of *Leishmania* status based on in-house ELISA and in-house IFAT results as reported elsewhere (Solano-Gallego et al., 2014).

Table 1
Group of dogs included in the study.

Serum samples classification	Clinical signs were observed, and laboratory abnormalities detected	*Leishmania* serological status based on reference tests	*Leishmania* molecular status by PCR in lymph node	Number of samples included
Seronegative healthy dogs	Absence of clinical manifestations during a physical examination and no laboratory alterations	Seronegative	Not applicable	21
Naturally-sick dogs with moderate anti-*Leishmania* antibodies	Presence of variable clinical manifestations and/or laboratory alterations	Seropositive with medium anti-*Leishmania* antibodies detected	Not applicable	39
Naturally-sick dogs with high anti-*Leishmania* antibodies	Presence of variable clinical manifestations and/or laboratory alterations	Seropositive with high anti-*Leishmania* antibodies detected	Not applicable	37
Infected dogs by some other pathogens or with other clinical conditions	Presence of variable clinical manifestations and/or laboratory alterations *Neospora caninum* ($n = 1$, IFAT antibody titer of 1:200) *Dirofilaria immitis* ($n = 4$, positive result to heartworm antigen test) *Anaplasma platys* ($n = 6$, IFAT antibody titers ranging from 1:40 to 1:160) *Toxoplasma gondii* ($n = 1$, IFAT antibody titer of 1:160) *Babesia canis* ($n = 1$, IFAT antibody titer of 1:100). *Ehrlichia canis* ($n = 1$, IFAT antibody titer of 1:160) Demodicosis ($n = 1$) Canine Distemper ($n = 1$) Canine Parvovirus ($n = 1$) Lymphoma ($n = 1$) Sterile nodular panniculitis ($n = 1$) Multiple myeloma ($n = 1$)	Seronegative	Negative	20
Naturally exposed dogs without infection	Absence of clinical manifestations during a physical examination and no laboratory alterations	Seropositive with low anti-*Leishmania* antibodies detected	Negative	33

2.2. Immunochromatographic tests

Five ICTs were used to detect anti-*Leishmania* antibodies, including DFV Test *Leishmania* with a sensitivity of 0.93 and specificity of 0.95 as indicated in the manufacturer's instructions (Divasa, Barcelona, Spain). FASTest LEISH with a sensitivity of 0.99 and specificity of 0.98 as indicated in the manufacturer's instructions (MEGACOR Diagnostik, Hörbranz, Austria). Speed Leish K with a sensitivity of 0.98 and specificity 1.00 as indicated in the manufacturer's instructions (Virbac, La Seyne Sur Mer, France). Uranotest *Leishmania* with a sensitivity of 0.97 and specificity of 0.99 as indicated in the manufacturer's instructions (UranoVet, Barcelona, Spain). Uranotest *Leishmania* 2.0 with a sensitivity of 0.99 and specificity of 0.99 as indicated in the manufacturer's instructions (UranoVet, Barcelona, Spain) and finally, Witness *Leishmania* with a sensitivity of 0.58 and specificity of 1.00 as indicated in the literature (Rodríguez-Cortes et al., 2013) (Zoetis, New Jersey, USA). All assays were carried out according to the manufacturer's instructions, and they were performed independently. Two laboratory members read all tests. If discrepancies arose between results, a third observer participated. The operators were blinded to the results of the quantitative serological tests.

2.3. Reference serological tests

Anti-Leishmania antibodies were detected by in-house ELISA (sensitivity 0.99 and specificity 0.98) and in-house IFAT (sensitivity 0.97 and specificity 0.92 described in the internal protocol of the laboratory) as reference tests using a whole antigen of in-house cultured *L. infantum* promastigotes (MHOM/FR/78/LEM75 zymodeme MON-1), as described by Alcover et al. (2021) with some modifications. The cut-off value of ELISA was set to 30 EU (mean+4 standard deviations of values from 70 healthy dogs from a non-endemic area). Sera with EU $\geq$ 200 were classified as high positive, with EU $\geq$ 100 and $<$ 200 as medium positive, and with EU $\geq$ 30 and $<$ 100 as low positives. In the case of the IFAT technique, the methodology was performed following the protocol described by Alcover et al. (2021). The cut-off value was set at$\geq$ 1:80. Sera with an antibody titer $\geq$ 1:80 and $<$ 1:320 were classified as low positive, with an antibody titer $\geq$ 1:320 and $<$ 1:1280 as medium positive, and with an antibody titer $\geq$ 1:1280 as highly positive. Selected samples included have concordant results between ELISA and IFAT in this study. Each quantitative technique was performed independently for different technicians, and the operators did not know the results obtained by the other technique. The operators were blinded to the results of the ICTs.

The statistical software R was used to construct two $\times$ two tables (R core team, 2013). The epiR package (https://cran.r-project.org/web/packages/epiR/index.html) was used to determine sensitivity, specificity, positive (PPV), and negative (NPV) predictive values, and likelihood ratio (LR). Binomial confidence limits were calculated for each measure. Receiver operating characteristic (ROC) curves were constructed on Stata 15 software (StataCorp, 2017). The confidence level was designated as 95%, and differences with $p < 0.05$ were statistically significant.

3. Results and discussion

The ICTs evaluated showed variable sensitivity; the maximum sensitivity was attained for FASTest LEISH (1.00), followed by Uranotest *Leishmania* 2.0 (0.97), Speed Leish K (0.97), Uranotest *Leishmania* (0.96), and the lowest results with Witness *Leishmania* (0.84) and DFV Test *Leishmania* (0.59) (Table 2 and Fig. 1A). By contrast, all tests showed high values of specificity, near to 1.00, except the FASTest LEISH (0.98), Uranotest *Leishmania* 2.0 (0.98), Speed Leish K (0.98), and Witness *Leishmania* (0.95) (Table 2, Table 3 and Fig. 1B). A similar pattern was obtained in the PPV, where the tests showed values ranging between 0.98 and 1.

Table 2
ICTs results by serological status obtained by the reference tests.

Test	Result	Serological results obtained by reference tests			
		Negative ($n = 41$)	Low positive ($n = 33$)	Medium Positive ($n = 39$)	High positive ($n = 37$)
DFV Test *Leishmania*	Test +	0	10	19	35
	Test -	41	23	20	2
FASTest LEISH	Test +	1	33	39	37
	Test -	40	0	0	0
Speed Leish K	Test +	1	33	36	37
	Test -	40	0	3	0
Urano test *Leishmania* 2.0	Test +	1	31	38	37
	Test -	40	2	1	0
Urano test *Leishmania*	Test +	0	32	36	37
	Test -	41	1	3	0
Witness *Leishmania*	Test +	2	27	28	37
	Test -	39	6	11	0

It is highlighted that some LR could not be calculated due to the absence of false negatives and false positives in the tests (Table 2). Regarding the relationship between the serological status obtained by the reference tests and the result of the ICTs evaluated, it was observed that the ICTs tests do not present contradictory results when the antibody titers are absent or are significantly elevated. In contrast, some tests showed marked differences concerning the results obtained by the reference tests, especially in those samples classified as medium positive (Table 3).

ROC curve analysis revealed statistically significant differences in the areas under the test curve for Witness *Leishmania* ($p = 0.0002$) and DFV test *Leishmania* ($p = 0.0001$) and the rest of the ICTs (FASTest Leish, Speed Leish K, Uranotest *Leishmania*, Uranotest *Leishmania* 2.0) (Table 3, Fig. 2).

To increase the sample population included in this study and evaluate the diagnostic performance of serological tests concerning cross-reaction phenomenon, we further selected confirmed seronegative samples to *L. infantum* with varying immunological exposures, including other vector-borne pathogens protozoan agents and immunopathological conditions which exacerbate the formation of immunoglobulins. In our study, a false-positive result was obtained by 2 ICTs, namely Fast Test Leish with a dog with myeloma multiple and Speed Leish K with a dog with heartworm disease. These findings did not highlight cross-reaction between *Leishmania* and other pathogens such as vector-borne helminths and tick-borne agents, critical circumstances in areas where exposure to ectoparasites is frequent such as European countries.

The present study was conducted to increase the performance of the most common ICTs in Spain and to compare the sera from different groups of dogs with different titers of anti-*Leishmania infantum* antibodies to reflect the situation in veterinary practice. Different rapid tests can be commercially available based on the immunochromatography dipstick method or dot enzyme-linked immunosorbent assay (DOT-ELISA) technique. The main difference among these two techniques is that the DOT-ELISA has an extra step with the presence of an integrated wash step that removes debris from the result window. Further research is needed to assess whether the performance of the DOT-ELISA is superior to it.

Despite the availability of several ICTs and the easy-to-use format, these techniques alone are not always sufficient to identify all seropositive cases because differences among ICTs can be observed. In general, most of the ICTs were highly specific ($>$0.95), while sensitivity was variable with low sensitivity values obtained from Witness *Leishmania* and DFV Test *Leishmania*. These situations should be considered, and

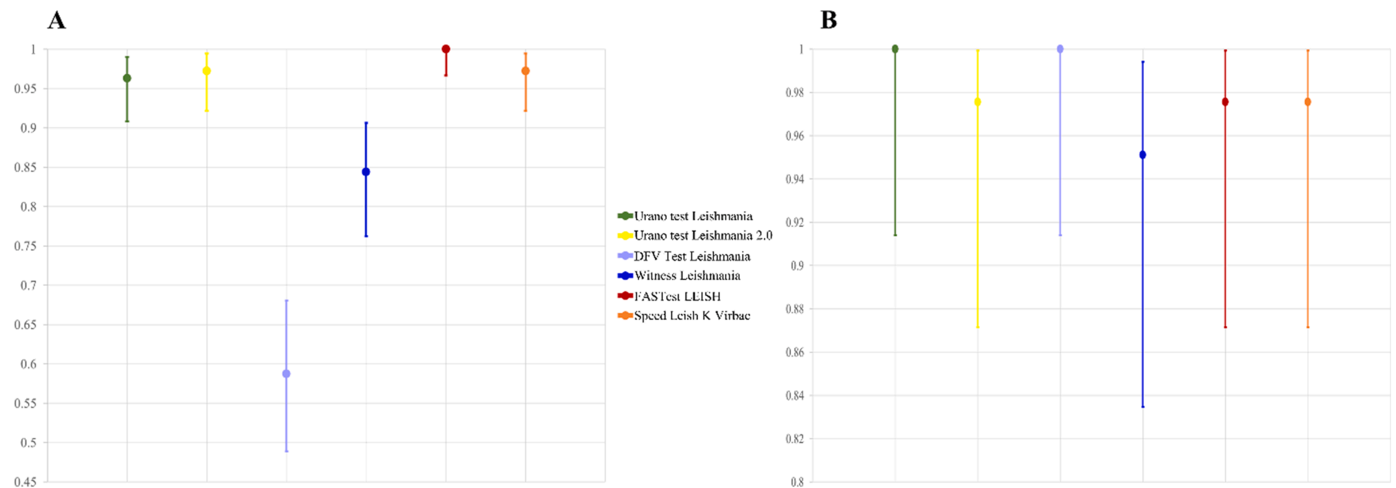

Fig. 1. Graphical comparison of the diagnostic performance of the tests evaluated A) Sensitivity results with confidence intervals. B) Specificity with confidence intervals.

Table 3
Diagnostic performance of ICTs on canine samples from Spain (NA: Not applicable).

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Likelihood ratio +	Likelihood ratio -
DFV Test *Leishmania*	0.59 (0.49 – 0.68)	1 (0.91 – 1)	1 (0.94 – 1)	0.48 (0.37 – 0.59)	NA	0.41 (0.33 –0.52)
FASTest LEISH	1 (0.97 – 1)	0.98 (0.87 – 1)	0.99 (0.95 – 1)	1 (0.91 – 1)	41 (5.9 – 284.1)	NA
Speed Leish K	0.97 (0.92 – 0.99)	0.98 (0.87 – 1)	0.99 (0.95 – 1)	0.93 (0.81 –0.98)	39.9 (5.8 – 276.4)	0.02 (0.009 – 0.09)
Uranotest *Leishmania* 2.0	0.97 (0.92 – 0.99)	0.98 (0.87 – 1)	0.99 (0.95 – 1)	0.93 (0.81 –0.98)	39.9 (5.8 – 276.4)	0.02 (0.009 – 0.09)
Uranotest *Leishmania*	0.96 (0.91 – 0.99)	1 (0.91 – 1)	1 (0.96 – 1)	0.91 (0.79 – 0.98)	NA	0.04 (0.014 – 0.096)
Witness *Leishmania*	0.84 (0.76 – 0.91)	0.95 (0.83 –0.99)	0.98 (0.92 – 1)	0.7 (0.56 – 0.81)	17.3 (4.46 –67)	0.16 (0.1 –0.26)

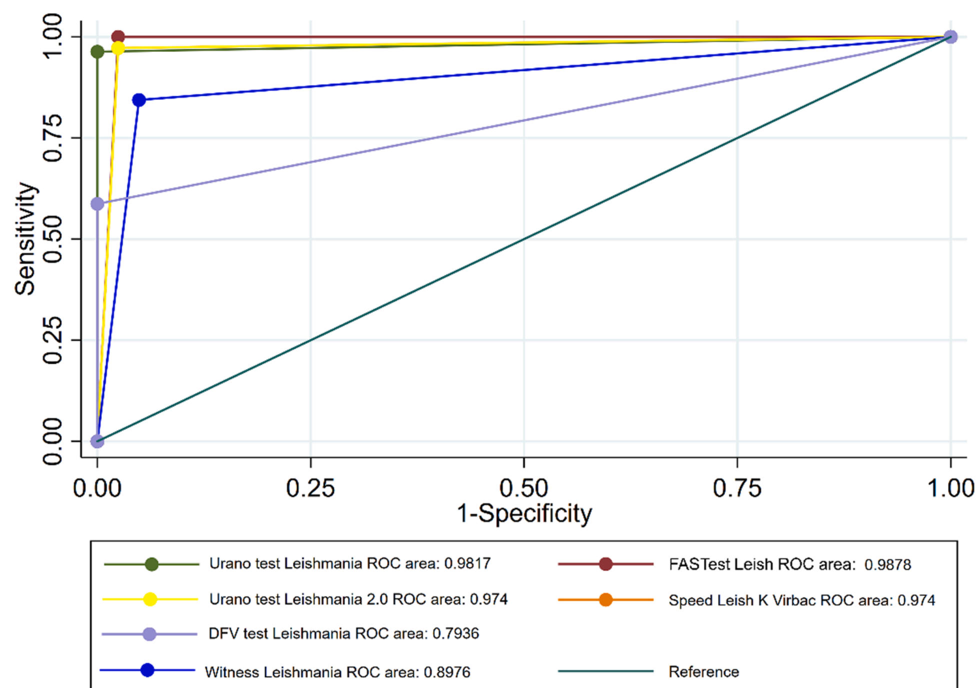

Fig. 2. ROC curve analysis of the ICTs evaluated.

these circumstances could lead to underestimating the number of seropositive dogs. These differences could probably be associated with the type of antigen used in each ICT. Most of the ICTs detected a higher number of seropositive samples compared with DFV Test *Leishmania*. This apparent lower sensitivity of DFV *Leishmania* does not agree with its claimed sensitivity as indicated in the manufacturer's instructions. The same situation has been published previously with other ICT (Solano-Gallego et al., 2014), and this fact could be important because this may be considered a less valuable screening test, and it may result in no detection seropositive sick dogs in some cases.

Serological methods are helpful tools in different settings, including seroepidemiological studies with a large-scale screening of serum

samples to determine the seroprevalence in a geographical region or for surveillance programs, for clinical diagnosis to confirm clinically suspected cases and to detect dogs with subclinical infection. In the case of subclinical infection, molecular techniques could be complementary techniques to detect the presence of the parasite DNA in the dog (Solano-Gallego et al., 2011). Depending on the application for the serological test, sensitivity or specificity should be prioritized: serological diagnosis in suspected dogs, high specificity should be considered, while seroprevalence studies and surveillance programs to detect the presence of seropositive dogs, the sensitivity should be considered as the most critical parameter.

Although quantitative serological techniques represent the reference serologic test, FASTest LEISH was the only test able to classify all seropositive samples correctly, followed by Speed Leish K Virbac, Urano test *Leishmania* 2.0, and Urano test *Leishmania*. Despite differences in diagnostic measures, confidence intervals are overlapped based on the ROC curve, which means that there are no differences in the use of these tests compared to Witness *Leishmania*. In our study, the possibility of a rapid test with optimal sensitivity and specificity in comparison to quantitative techniques can lead to advantages including the absence of special storage conditions for reagents, results obtained within a short time, ease-of-use, the lack of the operator's experience and of special technical requirement to perform the test, among others.

Evaluating serological diagnostic techniques for canine leishmaniosis in Europe is essential because there is an increasingly evident risk of introducing *Leishmania* species such as *Leishmania tropica* to neighboring areas where other *Leishmania* species are introduced; for example, *L. infantum*, is the predominant parasite. In the case of *L. tropica* and Spain and other European Mediterranean countries such as France or Italy, this parasite is present in some areas of North Africa, being dogs and small rodents potentially infected by this species (Ready et al., 2010). The introduction of this *Leishmania* species into these European countries should be considered.

The main limitations of this study are the retrospective nature of the study, the absence of negative samples from the non-endemic area or serum samples from dogs infected by other *Leishmania* species phylogenetically similar, the low number of used sera in the study, as well as and the inclusion of other rapid tests available in other European countries, while the employment of multiple serological tests together with a serum sample characterization in different groups with detailed clinical and anamnestic data are its strength.

4. Conclusions

These results indicate the existence of some ICTs that may be a reliable alternative to the quantitative serological techniques in circumstances where laboratory serological techniques are not accessible. In general, these tests are simple, fast, and easy to perform under practice conditions.

CRediT authorship contribution statement

Sergio Villanueva-Saz: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Marivi Martínez:** Methodology, Writing – original draft. **Juan David Ramirez:** Methodology, Software, Writing – review & editing. **Giovanny Herrera:** Methodology, Software, Writing – review & editing. **Diana Marteles:** Investigation, Methodology. **Marina Servián:** Investigation, Methodology. **Maite Verde:** Funding acquisition, Investigation, Resources. **Jacobo Giner:** Resources, Validation, Writing – review & editing. **Delia Lacasta:** Investigation, Methodology, Resources. **Héctor Ruíz:** Investigation, Methodology, Resources. **Andrés Yzuel:** Resources, Writing – review & editing. **Antonio Fernández:** Project administration, Resources, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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