Molecular and serological diagnostic of leptospirosis: a review (2014–2020)
Diagnóstico molecular e sorológico da leptospirose: uma revisão (2014–2020)
Diagnóstico molecular y serológico de la leptospirosis: una revisión (2014–2020)

Abstract
Leptospirosis is a zoonosis caused by pathogenic bacteria of the genus *Leptospira*. This disease affects several animals and humans. Symptoms of leptospirosis can range from mild to severe, may in some cases lead to death. For the diagnosis of leptospirosis, the microscopic agglutination test is considered the “gold standard”; however, it has limitations and studies are being conducted to develop alternative methods of screening and diagnosis of leptospirosis. Serological and immunochemical tests using *Leptospira* recombinant antigens in combination to molecular tests may be an alternative. In this study, we reported the advances achieved from 2014 until 2020 in controlling leptospirosis based on serological tests using recombinant antigens and molecular diagnosis.

Keywords: Serological tests; Immunochemical tests; Molecular diagnosis; Leptospirosis; Recombinant proteins.

Resumen
La leptospirosis es una zoonosis causada por bacterias patogénicas del género *Leptospira*. Esta enfermedad afecta a varios animales y humanos. Los síntomas de la leptospirosis pueden variar de leves a graves, podiendo en algunos casos llevar a la muerte. Para el diagnóstico de leptospirosis, la prueba de aglutinación microscópica es considerado el “estándar de oro”; sin embargo, tiene limitaciones y se están realizando estudios para desarrollar métodos alternativos de detección y diagnóstico de la leptospirosis. Las pruebas sorológicas e inmunocimétricas utilizando antígenos recombinantes de *Leptospira*, en combinación con pruebas moleculares, pueden ser una alternativa. En este estudio, relatamos los avances alcanzados de 2014 a 2020 en el control de la leptospirosis basado en pruebas serológicas con antígenos recombinantes y diagnóstico molecular.

Palabras clave: Pruebas sorológicas; Pruebas inmunocimétricas; Diagnóstico molecular; Leptospirosis; Proteínas recombinantes.

1. *Leptospira* and leptospirosis

Leptospirosis is a disease caused by pathogenic spirochetes of the genus *Leptospira*, family Leptospiraceae, and order Spirochaetales. These bacteria are spiral and helical in shape, having a length and width of 6–20 µm and 0.1–0.2 µm,
respectively (S. Faine, 1999). Furthermore, *Leptospira* spp. are classified into serovars according to the epitopes exposed on the surface in a mosaic of antigens, which can be proteins and lipopolysaccharides (LPS), and the specificity of epitopes is associated with sugar composition (Adler & de la Pena Moctezuma, 2010). To date, the genus *Leptospira* contains 64 species divided into saprophytes, intermediates, and pathogenic (Guglielmini et al., 2019; Ko, Goaran, & Picardeau, 2009; Philip et al., 2020). Regarding the serovars, approximately 300 have been reported, of which almost 270 are pathogenic (Gregoire et al., 2020; Guglielmini et al., 2019; Picardeau, 2017; Thibeaux et al., 2018).

Pathogenic *Leptospira* spp. can infect humans and animals. Moreover, the infection can be direct, i.e., through the urine of infected animals, or indirectly, i.e., through water or soil contaminated with the urine of infected animals (Jorge et al., 2018; S. Faine, 1999), despite this, however, the transmission of human-human leptospirosis remains unclear (Victoriano et al., 2009). Climatic conditions strongly influence the transmission of leptospires, which require hot and humid conditions for survival, bacteria can persist for weeks to months after excretion in water or moist soil (Mwachui, Crump, Hartskeerl, Zinsstag, & Hattendorf, 2015; Sun, Liu, & Yan, 2020).

Leptospirosis is an emerging zoonotic disease leading to more than one million of serious cases and 60,000 deaths each year worldwide, mainly in tropical countries (Grippi et al., 2020; Guglielmini et al., 2019; Lam, Low, & Chee, 2020; Philip et al., 2020). In addition, leptospirosis is classified as a neglected disease of global distribution, which can affect a wide variety of domestic animals such as pets and those of economic interest, including dogs, cattle, and pigs, as well as wild animals, fish, and humans (Bharti et al., 2003; Mwachui et al., 2015; S. Faine, 1999; Vinetz, 2001). In humans, leptospirosis is considered a public health problem associated with a series of clinical signs affecting multiple organs, such as the liver, kidneys, lungs, and brain (Adler & de la Pena Moctezuma, 2010; Gouveia et al., 2008; S. Faine, 1999). In animals, leptospirosis is associated with poor reproduction, abortions, premature births, and stillbirths, which lead to economic damage to the agricultural sector (Levett, 2001; Padilha, Simao, Oliveira, & Hartwig, 2019; Petrakovsky, Bianchi, Fisun, Najera-Aguilar, & Pereira, 2014; S. Faine, 1999).

At present, the diagnosis of leptospirosis is based on both clinical examination and serological tests. Among all the tests used, the microscopic agglutination test (MAT) is considered the “gold standard” (Rajapakse et al., 2020). MAT test have as a principle to expose human or animal sera to a series of different strains of live leptospires and thus detects antibodies present in the sera samples (S. Faine, 1999). Although MAT is used as a reference method for the diagnosis of leptospirosis, it has several limitations, including low sensitivity in the acute phase of the disease (when antibodies are difficult to detect and there is a higher level of IgM) and incapable of differentiating IgM from IgG antibodies (highly present in other phases of the disease) (McBride, Athanazio, Reis, & Ko, 2005; Rajapakse, Rodrigo, Handunnetti, & Fernando, 2015). In addition, many serological variants produce highly diverse strains, thus making it extremely difficult to identify them using this serological approach, resulting in several cross-reactions (Adler & de la Pena Moctezuma, 2010). Also, MAT is an extremely laborious and expensive technique because of the need to maintain bacteria in cultivation (Caimi, Repetto, Varni, & Ruybal, 2017).

Thus, alternative methods for screening and diagnosis of leptospirosis are being developed. Serological and immunochemical tests using *Leptospira* recombinant antigens, as well as molecular tests, have been described as alternatives for the diagnosis of leptospirosis.

### 2. Study Criteria

This review included all studies published in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) from 2014 until 2020, which contained the following keywords: “Immunodiagnosis leptospirosis,” “Serological diagnosis leptospirosis,” “Detection *Leptospira* recombinant protein,” “Diagnosis leptospirosis recombinant protein,” “Immunodiagnosis leptospirosis recombinant protein,” and “Molecular diagnosis leptospirosis,” leading to 157, 103, 12, 32, 9, and 104 articles, respectively. The abstracts
of these articles were analyzed, and all studies aimed at developing molecular and immunological diagnostic methods for leptospirosis were included in this review. Studies with other purposes were excluded. Thus, 67 articles were included in this review.

3. Antigens of *Leptospira* spp. used in Diagnosis

Several antigens of *Leptospira* spp. have been evaluated, many of them with potential for vaccine development (Conrad et al., 2017; da Cunha et al., 2019; Ghazali-Bina et al., 2019; Oliveira et al., 2019; Oliveira et al., 2018; Raja et al., 2018) or diagnosis (Alizadeh et al., 2014; Deneke et al., 2014; Nagalingam et al., 2015; Padiilha et al., 2019; Shiokawa et al., 2016; Ye et al., 2014). As reported in other spirochetes, the *Leptospira* spp. codes more lipoproteins than other bacteria, containing approximately 145 genes for likely lipoproteins and outer membrane proteins (OMPs) (Setubal, Reis, Matsunaga, & Haake, 2006; Viratyosin, Ingrisrisang, Pacharaowongvakada, & Palittapongarnpin, 2008; Yang et al., 2006). Several proteins are already described and confirmed to be present on the surface of *Leptospira* spp. Among these, the well-known ones are the Lig, Loa22, LipL32, OmpL1, LenA, LenD, OmpL36, OmpL37, OmpL47, and OmpL54 (Ko et al., 2009; Lo, Cordwell, Bulach, & Adler, 2009). Lipoproteins and OMPs are the main targets in the development of vaccine and diagnostic tests to leptospirosis control. All the cited antigens were frequently found in studies evaluating new diagnostic techniques (Table 1).

![Table 1. Antigens evaluated in the development of diagnostic tests for leptospirosis.](http://dx.doi.org/10.33448/rsd-v11i2.25471)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Characteristic</th>
<th>Serovar used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LipL32</td>
<td>Lipoprotein (outer membrane)</td>
<td><em>L. interrogans</em> serovar Copenhageni</td>
<td>(Alizadeh et al., 2014; Shiokawa et al., 2016; Ye et al., 2014)</td>
</tr>
<tr>
<td>LigB</td>
<td>Leptospiral immunoglobulin-like protein (outer membrane)</td>
<td><em>L. interrogans</em> serovar Pomona; <em>L. borgpetersenii</em> serovar Hardjo</td>
<td>(Deneke et al., 2014; Nagalingam et al., 2015)</td>
</tr>
<tr>
<td>LipL21</td>
<td>Lipoprotein (outer membrane)</td>
<td><em>L. interrogans</em> serovar Pomona; <em>L. interrogans</em> serovar Autumnalis</td>
<td>(Anita et al., 2016; Ye et al., 2014)</td>
</tr>
<tr>
<td>Lsa63</td>
<td>Protein of adhesion (outer membrane)</td>
<td><em>L. interrogans</em> serovar Copenhageni</td>
<td>(Alizadeh et al., 2014)</td>
</tr>
<tr>
<td>Loa22</td>
<td>Lipoprotein (outer membrane)</td>
<td><em>L. interrogans</em> serovar Pomona</td>
<td>(Ye et al., 2014)</td>
</tr>
<tr>
<td>LigA</td>
<td>Leptospiral immunoglobulin-like protein (outer membrane)</td>
<td><em>L. interrogans</em> serovar Pomona</td>
<td>(Ye et al., 2014)</td>
</tr>
<tr>
<td>ErpY-like</td>
<td>Lipoprotein (outer membrane)*</td>
<td><em>L. interrogans</em> serovar Copenhageni</td>
<td>(Padiilha et al., 2019)</td>
</tr>
</tbody>
</table>

* Location not yet described. Source: Authors (2021).

4. Diagnosis of leptospirosis

4.1 Polymerase chain reaction

Methods based on DNA amplification, such as polymerase chain reaction (PCR), are crucial tools for the detection of *Leptospira* spp. during the acute phase of the disease wherein antibodies are below the detection limit of the most serological tests (Ahmed, van der Linden, & Hartskeerl, 2014; Denipitiya et al., 2016). The PCR is a fast, sensitive, and specific technique; however, it remains expensive (Courdure et al., 2017; Waggoner & Pinsky, 2016).

Several types of PCR have been used in the diagnosis of leptospirosis (Table 2). For example, conventional PCR (Gokmen, Soyal, Kalayci, Onlen, & Koksal, 2016; Waggoner et al., 2015), real-time quantitative reverse transcription PCR...
(qRT-PCR) (Denipitiya et al., 2016; Nhan, Teissier, Roche, & Musso, 2014; Waggoner et al., 2015; Waggoner & Pinsky, 2016), real-time PCR with high resolution melting analysis (qRT-PCR-HRM) (Esteves et al., 2018), recombinase polymerase amplification (RPA) (Ahmed et al., 2014), PCR followed by restriction fragment length polymorphism (PCR-RFLP) (Gokmen et al., 2016), and assay loop-mediated isothermal amplification (Suwancharoen, Sittiwicheanwong, & Wiratsudakul, 2016). The biological samples used in most of these studies were blood or its fractions (serum or plasma) (Ahmed et al., 2014; Denipitiya et al., 2016; Gokmen et al., 2016; Nhan et al., 2014; Waggoner et al., 2015) and urine (Esteves et al., 2018; Podgorsek et al., 2020; Suwancharoen et al., 2016).

Table 2. PCR-based methods used to detect *Leptospira* spp.

<table>
<thead>
<tr>
<th>DNA target</th>
<th>Variant of PCR</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part of gene lipL32</td>
<td>RPA</td>
<td>Human</td>
<td>(Ahmed et al., 2014)</td>
</tr>
<tr>
<td>Gene secY</td>
<td>qRT-PCR</td>
<td>Human</td>
<td>(Denipitiya et al., 2016)</td>
</tr>
<tr>
<td>Genes lipB; secY</td>
<td>qRT-PCR-HRM</td>
<td>Human</td>
<td>(Esteves et al., 2018)</td>
</tr>
<tr>
<td>Gene rrs; Gene lipL32; Gene ompL1</td>
<td>16S rRNA-PCR followed by 16S rRNA-PCR-RFLP; LipL32-PCR; OmpL1-PCR</td>
<td>Human and bovine</td>
<td>(Gokmen et al., 2016)</td>
</tr>
<tr>
<td>Gene rrs</td>
<td>Taqman qPCR</td>
<td>Human</td>
<td>(Mohd Ali et al., 2018)</td>
</tr>
<tr>
<td>Gene lipL32</td>
<td>qRT-PCR</td>
<td>Human</td>
<td>(Nhan et al., 2014)</td>
</tr>
<tr>
<td>Gene lipL32; Gene lipL41</td>
<td>LAMP; qRT-PCR</td>
<td>Bovine</td>
<td>(Suwancharoen et al., 2016)</td>
</tr>
<tr>
<td>Gene rrs</td>
<td>Pathogenic qRT-PCR</td>
<td>Human</td>
<td>(Waggoner et al., 2015)</td>
</tr>
<tr>
<td>Gene 16S; Gene lipL32</td>
<td>qRT-PCR; PCR conventional</td>
<td>Human</td>
<td>(Waggoner &amp; Pinsky, 2016)</td>
</tr>
<tr>
<td>Gene lipL32</td>
<td>Multiplex PCR</td>
<td>Human</td>
<td>(Sea-Liang et al., 2019)</td>
</tr>
<tr>
<td>Gene 16S</td>
<td>Multiplex qPCR</td>
<td>Wild and domestic animals</td>
<td>(Perez, Lanka, DeShambo, Fredrickson, &amp; Maddox, 2020)</td>
</tr>
<tr>
<td>Gene lipL32</td>
<td>qRT-PCR</td>
<td>Human</td>
<td>(Podgorsek et al., 2020)</td>
</tr>
<tr>
<td>Gene rrs</td>
<td>PCR-nested PCR</td>
<td>Human</td>
<td>(Podgorsek et al., 2020)</td>
</tr>
<tr>
<td>Gene rrs</td>
<td>Duplex RT-PCR</td>
<td>Human</td>
<td>(Mohd Ali et al., 2019)</td>
</tr>
</tbody>
</table>

LAMP: loop-mediated isothermal amplification method; qRT-PCR-HRM: real-time PCR high resolution melting; qRT-PCR: real-time PCR; RFLP: restriction fragment length polymorphism; RPA: recombinase polymerase amplification. Source: Authors (2021).

Different PCR formats are developed to be used in both characterization and identification of *Leptospira* spp. in suspected samples. The molecular detection of *Leptospira* DNA is commonly based on marker genes such as 16S rRNA, flaB, lipL32, lipL41, ligA, and ligB. The lipL32 gene encodes an outer membrane lipoprotein that is present in pathogenic *Leptospira* species but absent in nonpathogenic species (Haake et al., 2000; Chang et al., 2016). Therefore, a wide range of studies has based on the lipL32 gene (Gokmen et al., 2016; Waggoner et al., 2015) as the main test gene or as the method of comparison with the new test developed, as PCR, real-time loop-mediated isothermal amplification (RealAmp), real-time PCR, loop-mediated isothermal amplification method (LAMP) and reverse-transcriptase PCR (Gokmen et al., 2016; Monica, Rathinasabapathi, & Ramya, 2019; Nhan et al., 2014; Suwancharoen et al., 2016; Waggoner et al., 2015).

Techniques such as real-time PCR are promising, faster, highly sensitive, and require less product handling than conventional PCR (Denipitiya et al., 2016). In general, real-time PCR is performed on the basis of the 16S gene, which can be amplified only in the presence of pathogenic *Leptospira* DNA (Waggoner et al., 2015). However, it has been suggested that *Leptospira* spp. that are nonpathogenic, such as *L. fainei*, can cause infection; thus, excluding them from the detection panel can lead to detection errors (Mohd Ali et al., 2018). Therefore, Mohd Ali and colleagues (2017) developed and validated a pan-*Leptospira* real-time PCR using the rrs gene which can detect the presence of pathogenic and nonpathogenic *Leptospira* in the
samples. In addition, to increase the accuracy of real-time PCR, Esteves and colleagues (2018) developed the qRT-PCR-HRM, which proved to be faster, more sensitive, specific, and cheaper, with 90% sensitivity and 97% specificity.

As an alternative to the high cost of implementing real-time PCR in the routine, the RPA technique (Ahmed et al., 2014) proved to be accurate, with sensitivity and specificity of 94.7% and 97.7%, respectively. The 16SrRNA-PCR-RFLP (Gokmen et al., 2016) and RealAmp (Monica et al., 2019) techniques showed promising results, with an early and accurate diagnosis, which distinguished pathogenic and nonpathogenic species from Leptospira spp. Moreover, a novel multiplex PCR assay has been developed for identifying Orientia tsutsugamushi, Rickettsia typhi and Leptospira spp., causative agents of scrub typhus, murine typhus, and leptospirosis (Sea-Liang et al., 2019). The duplex TaqMan probe-based qPCR has been used for the simultaneous detection of Burkholderia pseudomallei and Leptospira spp. (Mohd Ali et al., 2019).

### 4.2 Serological and immunochemical tests

Serological tests are based on the detection of antibodies that are produced because of the stimulation of the host's humoral immune response generated by Leptospira spp. infection (S. Faine, 1999). The incubation period of bacteria in the development of leptospirosis can range between 2 to 30 days. The onset of symptoms typically begins with the appearance of agglutinating antibodies, which increases with disease progression (Lessa-Aquino et al., 2017). This type of response occurs against LPS and several proteins that are constitutively expressed or upregulated during infection (Adler & de la Pena Moctezuma, 2010; Raja et al., 2018). However, IgM isotype antibodies appear approximately four days after symptom onset; this time varies according to the incubation period of the pathogen. The IgG isotype antibodies appear approximately 10 to 14 days, as they are more specific (Lessa-Aquino et al., 2017). Some serological diagnostic tests aim at an early result with the detection of IgM in the acute phase of the disease (Alizadeh et al., 2014; Courdurie et al., 2017; Kitashoji et al., 2015; Wynwood et al., 2016). Nonetheless, other tests aim for a more specific response, with the detection of IgG in the convalescent phase (Alizadeh et al., 2014; Anita et al., 2016; Brownlow et al., 2014; Nagalingam et al., 2015; Padilha et al., 2019; Shiokawa et al., 2016; Wynwood et al., 2016; Ye et al., 2014).

The LipL32 protein is the lipoprotein expressed in higher quantity in the outer membrane of leptospires, thus making it extremely immunogenic (Chang et al., 2016; Haake et al., 2000). In addition, LipL32 is conserved among pathogenic species and can be used as a distinctive marker for leptospirosis (Haake & Matsunaga, 2002; Shiokawa et al., 2016). Another protein that has been widely used in the development of serological tests is LipL21, a crucial immunodominant protein expressed during infection (Nally, Whitelegge, Bassilian, Blanco, & Lovett, 2007). Thus, these proteins are considered crucial targets in the serological diagnosis of leptospirosis (Table 3).
As shown in Table 3, most serological tests being developed are on the basis of enzyme-linked immunosorbent assay (ELISA) (Alizadeh et al., 2014; Courdurie et al., 2017; Deneke et al., 2014; Kitashoji et al., 2015; Padihya et al., 2019; Shiokawa et al., 2016; Ye et al., 2014) or in the latex agglutination test (LAT) (Brownlow et al., 2014; Deneke et al., 2014; Nagalingam et al., 2015). In addition, Wynwood and colleagues (2016) validated a microsphere immunosassay, which can simultaneously test many samples against a large number of serovars, as well as determine individual IgG and IgM titers. Overall, these factors would be extremely beneficial in laboratory diagnostics and in epidemiological studies of leptospirosis. Vanithamani and colleagues (2015) developed a lateral flow type assay based on LPS immunochromatography for the detection of IgM antibodies, which was sensitive and specific (>90%). Furthermore, this test is simple and fast and allows diagnosis of serogroup in endemic regions.

Raja and colleagues (2019) used gold and silver nanoparticles associated with different proteins (some well-known and others are little studied) to develop a dot blot that was fast, reliable, and sensitive for the early detection of leptospirosis. Moreover, the lipL32 gene was described in an optimized DNA-based bioassay for the detection of *Leptospira interrogans*, which used gold nanoparticle-embedded carboxylated multiwalled carbon nanotubes electrode (Nagraik et al., 2019). Nanotechnology has enhanced our ability to diagnose, treat, and prevent infectious diseases. However, there are concerns to be addressed and studied with respect to its limitations, as their toxicity, their effect on the environment and effective cost of this technology.

### Table 3. Serological tests for the diagnosis of leptospirosis.

<table>
<thead>
<tr>
<th>Protein target</th>
<th>Assay</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLsa63; rLipL32</td>
<td>Indirect ELISA</td>
<td>Human</td>
<td>(Alizadeh et al., 2014)</td>
</tr>
<tr>
<td>Unknown</td>
<td>LAT</td>
<td>Human</td>
<td>(Brownlow et al., 2014)</td>
</tr>
<tr>
<td>rLigB</td>
<td>Indirect ELISA and LAT</td>
<td>Bovine</td>
<td>(Deneke et al., 2014)</td>
</tr>
<tr>
<td>rLigA; Whole-cell</td>
<td>Capture ELISA</td>
<td>Human</td>
<td>(Kitashoji et al., 2015)</td>
</tr>
<tr>
<td>rLipL21; rLipL21; Whole-cell</td>
<td>IgM ELISA and dot blot</td>
<td>Human</td>
<td>(Anita et al., 2016)</td>
</tr>
<tr>
<td>rLigB</td>
<td>LAT</td>
<td>Bovine</td>
<td>(Nagalingam et al., 2015)</td>
</tr>
<tr>
<td>rErpY-like</td>
<td>Indirect ELISA</td>
<td>Swine</td>
<td>(Padihya et al., 2019)</td>
</tr>
<tr>
<td>rLipL32; rLigA; rArgC; rRecA; rLruC; rLruD</td>
<td>Silver-enhanced nanoparticles</td>
<td>Human</td>
<td>(Raja et al., 2019)</td>
</tr>
<tr>
<td>rLipL32</td>
<td>Indirect ELISA</td>
<td>Rodents</td>
<td>(Shiokawa et al., 2016)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Lateral flow based on specific LPS immunochromatography</td>
<td>Human</td>
<td>(Vanithamani et al., 2015)</td>
</tr>
<tr>
<td>Whole-cell</td>
<td>MIA</td>
<td>Human</td>
<td>(Wynwood et al., 2015)</td>
</tr>
<tr>
<td>Whole-cell</td>
<td>MIA</td>
<td>Bovine</td>
<td>(Wynwood et al., 2016)</td>
</tr>
<tr>
<td>rLipL21; rLoa22; rLipL32; rLigACon4-8</td>
<td>Indirect ELISA</td>
<td>Canine</td>
<td>(Ye et al., 2014)</td>
</tr>
<tr>
<td>rLipL32</td>
<td>LAT</td>
<td>Human</td>
<td>(Thongsukkaeng &amp; Boonyom, 2018)</td>
</tr>
</tbody>
</table>

ELISA: enzyme-linked immunosorbent assay; LAT: latex agglutination test; LPS: lipopolysaccharides; MIA: microsphere immunoassay.

Source: Authors (2021).

5. Final Considerations

Controlling leptospirosis is still a challenge and, therefore, several studies have been evaluated with the aim to develop more sensitive and applicable diagnostic tests. Serological methods based on specific recombinant antigens are widely studied. However, molecular methods have been widely used because they can be effective as the acute phase of the disease anticipates treatment and prevents evolution and its complications. Considering the importance of leptospirosis both in public health and the agricultural sector encourages the scientific community to identify methods that aim to improve and facilitate
the control of the disease. Therefore, several tests have been described to improve diagnostic accuracy, and several studies have shown promising results, bringing new perspectives for the diagnosis of leptospirosis.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References


