Review

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Posted Date: 28 April 2024

doi: 10.20944/preprints202404.1812.v1

Keywords: Rickettsia rickettsii; Rocky Mountain spotted fever; Brazilian spotted fever; diagnosis

 doi:10.20944/preprints202404.1812.v1

Review

A Mini-Review on Diagnostic Methods for Antigen and Antibody Detection of Rocky Mountain and Brazilian Spotted Fever

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Abstract: Rocky Mountain or Brazilian spotted fever, caused by *Ricketsia ricketssi*, is a fulminant, seasonal, and neglected disease that occurs in focal points of North America and South America. Rapid detection are essential for a better prognosis and survival rate of the infected individual. However, disease diagnosis still faces challenges as the accuracy of many of the available laboratory tests fluctuates. Therefore, this review aimed to identify methods for antibody or antigen detection, its gaps, and evolution over time. A search was conducted to find all studies in the Pubmed database that described antibody or antigen detection for *Ricketsia ricketsii* infection. Initially, a total of 403 articles were screened. Of these articles, only 19 fulfilled the pre-established inclusion criteria and were selected. Among the different methods applied, the IFA technique was the one most frequently found in the studies. However, it presented varied results such as low specificity using the indirect method. Other techniques, such as ELISA and immunohistochemistry, were also found, although in smaller numbers and with their own limitations. Although some studies showed promising results, there is a pressing need to find new techniques to develop a rapid and effective *Ricketsia ricketssi infection* diagnosis.

 doi:10.20944/preprints202404.1812.v1

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1. Introduction

Rickettsia rickettsii [1] is an important infectious agent that is part of the rickettsiosis group. The disease caused by this intracellular gram-negative coccobacillus bacterium is known as Rocky Mountain spotted fever (RMSF) or Brazilian spotted fever (BSF), depending on its geographical location [2,3]. RMSF was first described by Wood in 1896, where he reported clinical data suggesting "spotted-fever" as a distinct disease of unknown origin [4,5]. A hyperendemic outbreak in Montana's Bitterroot Valley in the late 19th and early 20th centuries triggered more interest and research, giving rise to the name Rocky Mountain spotted fever [6,7]. Subsequently, cases were recorded in other regions of the United States and throughout the Americas, in countries such as Colombia, Brazil, Mexico, Costa Rica, Argentina, and Panama [8].

R. rickettsii is transmitted through tick bites, and due to its extensive distribution throughout the Americas, each region has a different species of tick as the main vector. In North America, transmission occurs through *Dermacentor variabilis* [9], and *Dermacentor andersoni* [10]. In South America, especially in Brazil, *Amblyomma sculptum* [11] is considered the most important *R. rickettsii* transmission vector [12,13]. Wild and domestic animals, such as capybaras, horses, and dogs, play an important role in the disease's epidemiological chain since they are the main reservoir of spotted fever transmitters, with humans as incidental hosts [14,15]. In addition, animals can also be susceptible to infection. For instance, dogs exhibit fever, lethargy, anorexia, anemia, thrombocytopenia, and potential vestibular dysfunction when exposed to the the disease [16].

Symptoms usually begin on the 1st to 4th day after contact with the infected vector. During this period, the infected subject may experience fever, headaches, and photophobia, along with other milder symptoms. Rashes occur in approximately 85% of infected individuals, usually occurring on the 2nd to 4th day and spreading across the body from the wrists and ankles [17]. In more severe cases, interstitial pneumonia, meningoencephalitis, acute kidney injury, and multiple organ failure may occur after the 5th day [18]. In cases where symptoms worsen and there is no adequate treatment, the condition can be fatal [19].

Despite being essential for a better prognosis, diagnosing the infection still faces challenges. In the acute phase, nonspecific symptoms and low bacteremia are obstacles to an accurate laboratory diagnosis [20]. Moreover, infected individuals rarely display measurable IgM antibodies in the early stages, while the presence of IgG antibodies is less specific in the late stages, as they can remain detectable in the blood for months or even years [21]. In this sense, diagnosis mainly depends on a clinical evaluation and epidemiological background. Molecular and serological laboratory tests are applied to diagnose the infection, with the ideal test depending on the stage of the disease and the type of sample available for testing [22].

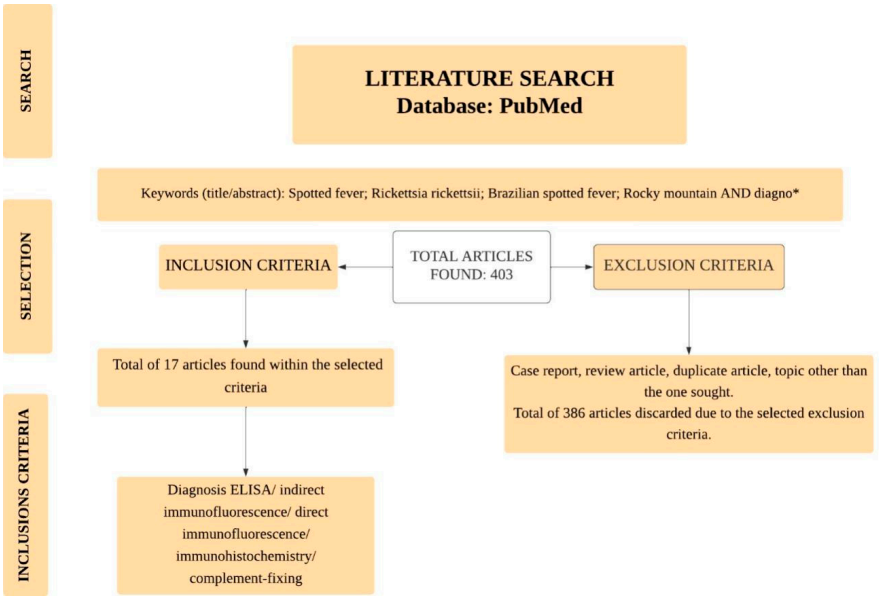
Molecular detection of rickettsial DNA by polymerase chain reaction (PCR) has become widely used for accurate confirmation of rickettsial infections. However, due to the need for advanced resources, the use of PCR in some endemic environments is limited to reference and research laboratories [23]. Furthermore, test sensitivity will depend on the sample type and the time of infection at which the sample was collected [20]. Regarding serological methods, indirect immunofluorescence antibody (IFA) assays using paired acute and convalescent sera are the reference standard for serological confirmation of rickettsial infection [24]. This method is superior to other techniques, such as complement fixation (CF), the latex agglutination test (LAT), and the Weil-Felix test. However, IFA can also face problems in relation to sensitivity and specificity [25]. The enzyme-linked immunosorbent assay (ELISA) can be used to overcome these limitations since it shows reliable performance when diagnosing diagnosis variousl infections. In addition to methods aimed at detecting antibodies, others are used for detecting antigens in samples, such as direct IFA and immunohistochemistry.

 doi:10.20944/preprints202404.1812.v1

Nonetheless, *R. rickettsii* detection remains a challenge, requiring improved laboratory diagnostic techniques to offer the best prognosis. This review set out to identify methods for detecting antigens or antibodies of RMSF and BSF infection and then monitor their progress. Identifying gaps in current knowledge can help guide new research with that in mind.

2. Materials and Methods

The search for scientific articles was conducted using the PubMed database. The search included all papers published to date. The descriptors were: (Rocky Mountain spotted fever[Title/Abstract]) AND (diagno*[Title/Abstract]); (brazilian spotted fever[Title/Abstract]) AND (diagno*[Title/Abstract]); (*rickettsia rickettsii* [Title/Abstract]) AND (diagno*[Title/Abstract]). The selected articles were screened using inclusion and exclusion criteria. Only those using antigen or antibody detection methods for diagnosing RMSF or BSF were selected. Bibliographical reviews, case studies, epidemiological reviews, molecular diagnosis and serological diagnosis of other diseases, editorials, duplicate articles, and articles related to other subjects were excluded.



3. Antigen and Antibody Detection for Diagnosing RMSF and BSF Infections

3.1. Serological Tests Used to Detect RMSF and BSF

Serological methods offer several advantages compared to other diagnostic methods [26,27]. Noninvasive sampling, in which the necessary material can be obtained with minimal discomfort to the individual, is less aggressive than methods that require tissue biopsies [26]. These tests are often highly specific and can detect low antibody or antigen concentrations, making them useful for the early detection of diseases or immune responses [26]. Serological tests are generally cheaper than molecular methods, making them accessible for large-scale screening or in resource-limited settings [27].

Many serological tests can be quickly performed and do not require specialized equipment or highly trained personnel, facilitating their use in different healthcare settings [27,28]. In addition, these tests can be easily scaled up, being particularly useful in epidemiological surveys or for screening blood donations. Moreover, they can be used to monitor the prevalence of diseases in a population or assess levels of immunity against certain infections, such as after vaccination campaigns [28]. However, serological methods also have limitations, such as the window period during which antibodies may not be detectable after initial infection, potential cross-reactivity leading to false positives, and inability to provide information about the presence of live pathogen [28]. It is also essential to consider the context in which these methods are used, as their performance may vary depending on the disease, the stage of infection, and the population tested [28]. Among the

 doi:10.20944/preprints202404.1812.v1

available serological tests, the indirect IFA is the standard one used for diagnosing RMSF and BSF [22,29]. However, other serological tests, such as ELISA, are also used.

3.1.1. ELISA

ELISA is a widely used diagnostic and laboratory tool [30], allowing the detection and quantification of antigens or antibodies and used to guide the diagnosis and monitoring of diseases [31]. ELISA can be highly sensitive, detecting very low antigen or antibody levels in a sample. Moreover, due to the use of antibodies that specifically bind to target antigens, ELISA tests can present high specificity for the substance they were designed to detect [32]. In this sense, ELISA is a valuable tool for diagnosing a wide range of diseases. However, it is still rarely applied for a *R. rickettsii* infection diagnosis.

Radulovic et al. (1993) tested an immunodominant B cell epitope of *R. rickettsii* in a human humoral immune response to RMSF. In their study, the epitope-blocking ELISA (DEB-EIA) used an antigen from *R. rickettsii*. A serological panel of 35 positive serum samples was used. In addition, 50 serum samples from Mediterranean spotted fever, collected in different countries, 8 serum samples from endemic typhus individuals, 16 serum samples from individuals with Q fever pneumonia, and 477 serum samples from individuals living along the Adriatic Sea, where RMSF does not occur, were also used. DEB-EIA results showed that only RMSF positive serum samples were able to perform epitope blocking, resulting in 100% sensitivity and specificity [33].

3.1.2. Indirect Immunofluorescence Assay

Indirect IFA is an advanced technique used to identify specific antibodies in samples through the use of two antibodies: an unlabeled primary and a secondary conjugated to a fluorophore. The primary antibody specifically binds to the target antigen, while the secondary antibody, specific to the primary antibody, is responsible for the fluorescence [24]. The indirect method has the advantage of being widely used due to its high sensitivity, signal amplification, and ability to detect multiple targets in the same sample [34].

McQuiston et al. (2014) analyzed data from a surveillance program in Tennessee, USA, in which *R. rickettsii* was detected in 77% of individuals using indirect IFA. In this work, 13 volunteers underwent visits during the acute phase of the disease,0 to 2 weeks after the onset of symptoms, and serum was collected for IFA tests. Serum was also collected at 2 to 4 weeks, 4 to 8 weeks, and one year after symptom onset. IFA tests for IgG and IgM were performed according to the standard method using a specific antibody for *R. rickettsii*. Based on the serological results, recent infection could not be confirmed in any infected individual, but anti-*R. rickettsii* IgM and/or IgG were present in at least one serum collection in 77% of the volunteers, occurring in 10 of 13. Anti- *R. rickettsii* IgM antibodies were commonly observed, occurring in nine of 13 RMSF-infected individuals. However, the pattern of reactivity among them was not as expected. The first collections did not demonstrate development of IgG while, when present, IgM was often elevated during the first collection and did not increase in the first few weeks of infection. The last collection, one year later, showed IgM antibodies in three of 10 infected individuals, while IgG was present in five of 13. Twenty-three percent (three of 13) showed both IgM and IgG antibodies one year later. These results reveal the possibility of false positives when using IgM [35].

Straily et al. (2020) compared the level of antibody reactivity among healthy individuals in two regions of the United States and assessed the impact of the prevalence of antibodies against *R. ricketsii* on public health surveillance in one of these regions. Blood donations were collected between May and July of 2016, a time of year that coincides with the peak of reported cases. The samples were evaluated using an indirect IFA aimed at identifying the presence of IgG. As a result, 11.1% (166/1493) of donors from Georgia and 6.3% (95/1511) of donors from Oregon and Washington demonstrated reactive antibody titers, at titers ≥64. Moreover, only 3.1% (93/3004) of all donors had titers ≥128. The results suggest that a single IgG antibody titer is an unreliable measure for RMSF diagnosis [36].

 doi:10.20944/preprints202404.1812.v1

3.1.3. Complement Fixation

The CF technique was first described in 1901 [37,38], and is an immunological method used to detect the presence of antigens or antibodies in a biological sample [39]. The technique involves adding serum containing antibodies or antigens to a mixture of complement and antigen or antibody. The presence of an antigen or antibody in the sample results in complement fixation, which can be detected through a hemolysis reaction [39]. The quantitative CF technique is a variation that allows quantifying the amount of antigen or antibody present in the sample [40], and its accuracy depends on the use of a satisfactory antigen and the ideal amount of complement [41,42]. This technique can be used to diagnose different types of disease-causing microorganisms, including viruses, such as influenza [43], and herpes [44]; bacteria, such as *R. Ricketsii* [45] and *Treponema pallidum* [46]; and protozoa, such as *Leishmania* spp [47] and *Trypanosoma Cruzi* [48].

Shepard et al (1976) tested sera from 137 individuals suspected of rickettsial infection, including RMSF, using CF. One hundred two infected individuals were identified, in which only those whose antibody titers were equal to or greater than 1:16 were included. Among the infected individuals, only 9.5% showed maximal titers detection, while 50% had titer detection greater than 256. The main observation in this study was the slow appearance of antibodies in the CF test for the diagnosis of *Rickettsia*. Therefore, other tests may be more efficient in terms of rapid detection of IgM or other antibodies [49].

Lundgren, Thorpe & Haskell (1966) studied the development and persistence of CF antibodies and rickettsiosis in 183 birds of different species, from chickens to falcons, inoculated with *R. rickettsii*. Results showed that 10 birds were positive and 173 birds were negative for *R. rickettsii*. The authots concluded that certain bird species may contribute to the spread of the etiological agent of RMSF in nature and that CF may be an inappropriate test for the serological diagnosis of *R. rickettsii* in birds [50].

3.2. Direct Immunofluorescence Assay

Direct IFA is a technique characterized by the immunohistochemical detection of rickettsiae in skin biopsy samples taken from bedsores or exanthematic lesions and considered to be a reliable assay for diagnosis [51] The best sample and evolution time of the lesions for carrying out the examination varies. Furthermore, the procedure should cause minimal trauma to the sample, which is generally 4 mm long and involves both the epidermis and dermis [52]. The technique consists of fixing a biopsy tissue in formalin and embedded in paraffin, thus being used to detect the antigen directly in the sample. The direct technique involves the use of primary antibodies directly labeled with fluorophores, allowing rapid identification of the antigen. This method has the advantage of being faster compared to the indirect IFA [34].

Hall and Bagley (1978) described the IFA by examining fixed tissue sections rather than unfixed tissue. A modification of a trypsin digestion procedure for fixed tissues was made. In their study, rhesus monkey, guinea pig, and chicken embryo yolk sacs were experimentally infected, and tissues were then fixed in formalin and embedded in paraffin. Results showed that it was possible to identify R. rickettsii by IFA through both methods. Moreover, staining intensity using the formalin-fixed method was slightly decreased as compared to fresh tissues. Conversely, there was a notable improvement in the morphology of the formalin-fixed and paraffin-embedded tissues used in this procedure, facilitating the identification of cells and tissues that harbor rickettsiae by becoming more apparent [53].

A study conducted by Walker & Cain (1978) investigated an IFA for the specific RMSF diagnosis using fixed tissues embedded in paraffin. Autopsy samples from 10 probable cases of RMSF were analyzed. The results showed that structures with the size and shape of rickettsiae were specifically stained in the endothelium and vascular walls of renal capillaries, veins, and arteries in the kidneys of seven out of 10 probable RMSF cases. However, the time between treatment and tissue collection may affect the results [54].

Fleischer, Lennette & Honig. (1979) performed an IFA to diagnose RMSF by identifying R. rickettsii in skin biopsy tissue from two individuals, aged 3 and 6 years, with suspected disease. Tissue staining was done with rabbit anti-R. rickettsii globulin labeled with fluorescein

 doi:10.20944/preprints202404.1812.v1

isothiocyanate. After all preparation steps had been made, a fluorescent microscopy was performed. Tissues from the 3-year-old individual showed supposedly positive identification in all sections and areas of moderate fluorescence, with bright green coloration, and a coccobacillary morphology clearly visible in several locations. However, no sections from the 6-year-old individual exhibited fluorescence [55].

Davidson et al. (1989) described the animal diagnosis of R. rickettsii. In their study, 14 laboratory beagles without reactive antibodies for R. rickettsii were used in the experiment. Among them, nine dogs were inoculated intradermally with R. rickettsii (Shelia Smith strain), while five dogs served as controls and were similarly inoculated with a equal volume of diluent. Tissue samples were obtained before inoculation and post-inoculation on days 3, 6, 9, 12, 15, 17, and 19. The results suggest that unaffected cutaneous inguinal skin is inferior to affected cutaneous lesions in detecting rickettsial antigen in tissues from infected dogs [56]. Melles, Colombo and Lemos (1999) evaluated the presence of R. rickettsii through direct IFA in different cultures grown with concentrations of 3% and 5% of bovine serum. For this purpose, a standard sample of the R. rickettsii Sheyla Smith strain, cultivated in the Vero cell line, a petechial lesion sample from a patient with suspected BSF, and capybara and Amblyomma cooperi ticks were used. Positive results were determined by observing green fluorescent rickettsia-like microorganisms in the cells, demonstrating that when using a higher percentage of fetal bovine serum for culture, such as 5%, the more sensitive the technique becomes compared to adding 3% fetal bovine serum [57].

3.3. Immunohistochemistry

Immunohistochemical staining (IHC) is the set of methods in which antibodies are used as specific reagents capable of identifying and establishing a connection with tissue constituents that function as antigens. The method detects and localizes the protein of interest within a tissue section using a specific antibody that will be detected by an enzymatic reaction (such as peroxidase or alkaline phosphatase), generating a colored chromogenic product [58]. Co-marking can also be performed using this technique, being a qualitative or semi-quantitative method. This connection enables the location and identification of the presence of various substances in cells and tissues through the color associated with the antigen-antibody complexes formed in the meantime [59]. IHC is a powerful tool for specifically binding an antibody to an antigen to detect and localize specific antigens in cells and tissues, being widely used in clinical diagnosis in anatomic pathology [60].

Paddock et. al. (1999) described 16 fatal RMSF cases between 1996-1997, serologically unconfirmed, for which a diagnosis of RMSF was established by IHC of tissues obtained at autopsy. Serum and tissue samples from individuals with a fatal disease compatible with RMSF were also tested by indirect IFA, where no serum demonstrated IgG or IgM antibodies reactive with *R. rickettsii*. However, IHC staining confirmed the RMSF diagnosis in all individuals. These data suggest that IHC staining is underrecognized and underutilized as a diagnostic tool, and that many, if not most, deaths caused by *R. rickettsii* in the United States are overlooked, unconfirmed, or unreported [61].

3.4. Comparative Studies

Philip et al. (1977) evaluated the CF, microimmunofluorescence (micro-IF), microagglutination (MA), and hemagglutination (HA) results for antibody detection against R. rickettsii using sera from 324 individuals. The study demonstrates that a total of 30% (97/324) were diagnosed by micro-IF as having RMSF, 26% (85/324) were seropositive for RMSF by MA, 30% (98/324) were confirmed with RMSF by HA, and only 13% (43/324) were confirmed with RMSF by CF. Of the total number of positive individuals, 86% (93/108) were seropositive for two or more methods, and only 37% (40/108) were positive for all four. There was good agreement between the micro-IF, MA, and HA tests, but the CF test was less sensitive than the others. Only half of the individuals considered to have RMSF by micro-IF, MA, and HA were positive for CF, whereas almost all who were positive for CF were also positive on all other tests [62].

The Weil-Felix test is a serological agglutination method used to detect the presence of antibodies against bacteria of the Rickettsia genus in serum [63]. Hechemy (1979) carried out a

 doi:10.20944/preprints202404.1812.v1

comparative study between the Weil-Felix test and CF, considering micro-IF as a confirmatory standard for the diagnosis of RMSF. Of the 335 reactive individuals in the Weil-Felix test, only 21 were detected by micro-IF. Of the 21 individuals positive for micro-IF, only three were positive for CF. A low specificity for the Weil-Felix test was observed, as well as low sensitivity for CF. Finally, the author warned against uncritically trusting the positive results of the Weil-Felix test or the negative results of the CF test [64].

Walker et al. (1980) explored diagnostic methods for RMSF aiming to assess the specificity and sensitivity of various techniques, including skin biopsy IFA, HA, CF, and Weil-Felix (Proteus Ox-2 and Proteus Ox-19 agglutination). The analysis involved 142 serum samples and 16 skin biopsies obtained from individuals with clinical suspicion of RMSF infection. The sensitivity of IFA was 70% (7/10), HA 19% (3/16), CF 0% (0/4), Proteus Ox-2 18% (3/17), and Proteus Ox-19 65% (11/17). Regarding the specificity of these diagnostic tests for RMSF, reflecting the occurrence of false-positive results, HA and skin biopsy IFA demonstrated the best specificity results with values of 99% and 100%, respectively [65].

Clements et al. (1983) evaluated IgM and IgG antibody responses in the RMSF infection using ELISA techniques and IFA. Initially, the IFA was used to measure specific immunoglobulins of the Rickettsia class in sera obtained (before and after vaccination) from volunteers using a new formalin-inactivated vaccine for RMSF. However, the tests' low sensitivity for detecting IgM antibodies in post-vaccination and post-infection sera led to the adaptation of an ELISA for comparison. An IFA negative healthy volunteer was considered as a negative control for ELISA, Regarding the positive controls for the IgM ELISA and the IgG ELISA, paired serum samples from early convalescent (IgM antibody titer by IFA test, 1:80) and late convalescent were used as reference standards. Overall, the IgM ELISA and IFA IgM test results agreed in only 13 (52%) of the 25 paired sera, while the IgG ELISA and the IFA test for total immunoglobulins gave concordant results in 85 (84%) of 101 paired sera and the concordance of the IgG ELISA and the IFA test for IgG was 19 of 25 paired sera. The authors stated that the ELISA test and the IFA test were uniformly specific, but the ELISA was more sensitive than the IFA test for detecting low levels of antibodies present after vaccination and during late convalescence [66].

Hechemy et al. (1983) conducted a comparative study on the results of the latex-Rickettsia rickettsii test and micro-IF for detecting antibodies against RMSF in 11 laboratories across nine U.S. states where the disease is endemic. The study was conducted during the 1980 RMSF season and used a double-blind study design, dividing the laboratories into two groups: the New York State Laboratory (NYL) and Collaborating Laboratories (CL). The authors considered serum samples positive for R. rickettsii by the micro-IF method as the standard for a positive result. The latex-R. rickettsii test successfully diagnosed active RMSF at high titers in a single serum from individuals with an active infection. However, the test failed to detect antibodies in individuals with a previous infection. When compared with the micro-IF, the results of the latex-R. rickettsii method demonstrated a sensitivity of 84.45% for tests conducted in the NYL and 79.20% for tests conducted in the CL. Moreover, the efficiency of the latex-R. rickettsii test was 96.79% for the NYL and 93.30% for the CL. Both tests were capable of detecting antibodies one week after the onset of infection. However, with micro-IF, titers appeared to remain above the minimum significant reactivity levels for R. rickettsii in comparison to latex-R. Rickettsii titers [67].

White, Patrick & Miller (1994) evaluated 23 individuals with suspected RMSF using direct IFA and immunoperoxidase tests. During tissue preparation, part of the tissues was frozen for IFA and the other part was fixed in formalin, routinely processed, and embedded in paraffin for staining with immunoperoxidase, hematoxylin, and eosin. Ten of the 23 individuals were identified with RMSF, of which nine were positive for both tests. Immunoperoxidase on paraffin-embedded tissue provided results essentially identical to direct immunofluorescence on fresh-frozen biopsy material. In the only case that presented a false negative, the individual had received anti-rickettsial antibiotics 72 hours before the biopsy. Therefore, no significant differences were observed between the two methods [68].

Table 1. DATA FROM PUBLISHED ANTIBODY OR ANTIGEN DETECTION STUDIES.

Disease stage	Infected host	Sample used	Method	Results	Author/Country
Acute	Animal	Serum	CF	CF was not able to detect antibodies from infected chickens, pheasants, sparrow hawks, magpies, or ravens CF detected antibodies from infected pigeons with maximal detection between the 3rd and 5th infection-week CF detected antibodies from one red-tailed hawk and one marsh hawk in the 2nd and the 3rd infection-week	Lundgren, Thorpe & Haskell (1966) / USA [50]
-	Human	Serum	CF	9.5% of infected individuals showed maximal titers detection 64% of serum samples from infected individuals showed cross-reaction with typhus antigens	Shepard et al., (1976) / USA [49]
Acute and convalescent-phase	Human	Serum	Micro-IF MA HA CF	Reactivity - Micro-IF: 30% MA: 26% HA: 30% CF: 13%	Philip et. al., (1977) / USA [62]

doi:10.20944/preprints202404.1812.v1

Convalescent-phase	Animal	Kidney and skin	Direct IFA	Direct IFA detected 7 (7/10) samples from infected individuals. Samples from individuals negative for RMSF did not show immunofluorescence	Walker & Cain (1978) / USA [54]
-	Human and animal	Lung, heart, epididymis, and testis	Direct IFA	The formalin-fixed method showed a slightly reduced intensity staining, with improved morphology. Normal tissues showed no staining	Hall and Bagley (1978) / USA [53]
-	Human	Serum	Weil-Felix Micro-IF CF	Weil-Felix: only 6% of similarity with micro-IF. Agreement between these two tests was higher when considering paired serums. CF: 86% false-negative	Hechemy (1979) / USA [64]
Acute	Human	Skin	Direct IFA	Direct IFA was able to detect the presence of *R. rickettsii* in only 1 (1/2) positive sample	Fleischer, Lennette & Honig (1979) / USA [55]
Acute	Human	Serum and skin	Direct IFA CF HA Weil-Felix	Direct IFA - Sensitivity: 70% Specificity: 100% CF - Sensitivity: 0% Specificity: 0% HA -	Walker et. al., (1980) / USA [65]

doi:10.20944/preprints202404.1812.v1

				Sensitivity: 19% Specificity: 99% Weil-Felix Proteus Ox-2 - Sensitivity: 18% Specificity: 96% Weil-Felix Proteus Ox-19 - Sensitivity: 65% Specificity: 78%	
Acute and convalescent-phase	Human	Serum	ELISA and Indirect IFA	ELISA - Sensitivity: 100% Specificity: 100% IFA - Sensitivity: 100% Specificity: 83%	Clements et. al., (1983) / USA [66]
-	Human	Serum	Latex-*Rickettsia rickettsii* test and micro-IF	New York State laboratory - Sensitivity: 84.45% Specificity: 99.98% Collaborating laboratories - Sensitivity: 79.20% Specificity: 95.81%	Hechemy et. al., (1983) / USA [67]
-	Animal	Skin	Direct IFA	Direct IFA was able to detect the presence of *R. rickettsii* in 18 (18/23) samples from erythematous macules	Davidson et. al, (1989) / USA [56]

doi:10.20944/preprints202404.1812.v1

				Using normal inguinal skin, direct IFA was unable to detect the presence of *R. rickettsii*	
-	Human	Serum	ELISA	Sensitivity:100% Specificity: 100%	Radulovic et. al., (1993) / USA [33]
-	Human	Serum Skin biopsy and autopsy sample	Direct IFA and immunoperoxidase test	Direct IFA and immunoperoxidase test detected the presence of *R. rickettsii* in 9 (9/10) positive samples	White, Patrick & Miller (1994) / USA [68]
-	Human and animal	Skin	Direct IFA	Sensitivity was improved when using a higher concentration of fetal bovine serum	Melles, Colombo & Lemos (1999) / Brazil [57]
-	Human	Serum Whole blood Liver, myocardium, spleen, kidney, lung, adrenal gland, pancreas, cerebral cortex, cerebellum, skin, stomach, colon, bone marrow, lymph node, small intestine, trachea,	IHC	IHC was able to detect the presence of *R. rickettsii* in (12 (12/16) samples	Paddock et. al., (1999) / USA [61]

doi:10.20944/preprints202404.1812.v1

		skeletal muscle, thymus, thyroid, coronary artery, aorta, hippocampus, medulla, pons, pineal gland, choroid plexus, ovary, tongue, and appendix			
Acute and convales-cent-phase	Human	Serum	Indirect IFA	Anti- *R. rickettsii* IgM and/or IgG were detected in at least one collected serum sample from 10 (10/13) infected individuals	McQuiston et. al., (2014) / USA [35]
Acute and convales-cent-phase	Human	Serum	Indirect IFA	Only 11.1% of Georgia donors and 6% of Pacific Northwest donors had IgG titers ≥64	Straily et. al., (2020) / USA [36]

CF: complement fixation; IFA: immunofluorescence assay; IHC: Immunohistochemical staining; HA: hemagglutination; MA: microagglutination; Micro- IF: microimmunofluorescence.

doi:10.20944/preprints202404.1812.v1

 doi:10.20944/preprints202404.1812.v1

4. Discussion

The disease caused by the bacteria *R. ricketsii* occurs in different parts of the world, primarily in the Americas [8]. Although it is not a highly reported disease, the mortality rate can reach 20% to 30% without immediate treatment. However, early treatment may be hampered by the fact that the disease presents nonspecific symptoms and may be confused with other diseases, such as dengue, malaria, or ehrlichiosis [69]. Moreover, the recommended laboratory diagnosis still has negative points, which acts as a barrier to an accurate infection diagnosis. Due to these bottlenecks, there is a pressing need to develop a highly accurate and specific method capable of diagnosing the disease and clearly identifying it for the best treatment of the infected individual [3,70].

In response, several researchers have conducted tests with different techniques over the years, especially in the last century, including the IFA, which is still considered the gold standard method for detecting RMSF and BSF infections. Studies that used both direct and indirect IFA have demonstrated promising results in diagnosing the disease. Other techniques, such as immunohistochemistry and ELISA, were also successfully used, demonstrating the ability to distinguish between negative and positive serum samples. However, since only a limited number of studies have used these techniques, it is not possible to infer whether they are reliable when it comes to diagnosing RMSF or BSF infection. Furthermore, other method, such as the CF and Weil-Felix techniques, did not demonstrate good diagnostic performance, both having low detection results. Indeed, those studies that compared different diagnostic tests showed that CF was one of the least promising techniques, followed by the Weil-Felix. However, in comparative studies, the IFA demonstrated the greatest diagnostic capacity, corroborating the recommendations for use by health agencies.

Despite such good results, especially when using the IFA, the scarcity of recent studies is a source of concern. In fact, only two studies were published in this century, suggesting a lack of interest in developing an improved diagnostic method, which cannot be justified by the lack of case notification, since the number of cases is multiplying in some regions. In Brazil, for example, there was an increase in case notifications from 2021 to 2022, confirming that the infection is present and the rise in cases is worrisome [71]. Furthermore, there are few studies focusing on the development of new technologies or new antigens to improve existing techniques. The scarcity of research can be explained by the socioeconomic profile of the affected populations, which often reside in low-income areas. These communities are more exposed to tick vectors due to problematic living and working conditions, in addition to precarious housing and proximity to habitats that attract the vectors, thereby elevating the risk of contact with ticks [72].

This underscores the need to develop a faster, more effective diagnosis for this neglected disease. Some measures to improve diagnostic performance can be pointed out, such as investing in faster method, easier to store and manage, in addition to presenting simplified testing, such as point-of-care platforms, and conducting more research aimed at developing new antigens for serological tests, such as recombinant proteins, multiepitope proteins, and synthetic peptides, which represent a promising strategy to boost the sensitivity and specificity of serological tests. In fact, these antigens offer the advantage of functioning without the need for specific biosafety requirements when handling microorganisms, as well as being more suitable for assay standardization [73]. Furthermore, improving the sensitivity of the tests could positively reflect on early disease diagnosis. Investing in the use of different methods is another way to go, such as electrochemical immunosensors, which provide a more efficient option for detecting reactive antibodies to *R. rickettsii* with high specificity and sensitivity, thus reducing the sample volume for analysis [74]. Finally, studies on the development of new tests could be improved, such as expanding the sample size supported by statistical programs capable of arriving at more reliable results.

In summary, RMSF and BSF serodiagnosis is a field replete with significant bottlenecks, requiring the development of a better diagnostic method. Researchers and health agencies must invest in new methods to develop a quick, effective test, aiming to facilitate early identification, monitor its spread, and assist in the creation of more efficient prevention strategies.

Author Contributions: Conceptualization, K.A.S., V.B.P., R.R.S., M.V.P.R., R.A.R.O., T.J.R.F., C.C.S., M.A.R., S.P.P., L.S.S., and A.A.M.G; writing - preparation of the original draft, K.A.S., V.B.P., R.R.S., M.V.P.R., R.A.R.O., T.J.R.F., C.C.S., M.A.R., S.P.P., L.S.S., and A.A.M.G; wrote original draft - K.A.S., V.B.P., R.R.S., M.V.P.R., R.A.R.O., T.J.R.F., C.C.S., M.A.R., S.P.P., L.S.S., and A.A.M.G; review and editing, K.A.S., V.B.P., J.M.M., R.A.M.A, R.T.F., M.A.C.F, E.A.F.C., R.C.G., M.C.P., A.A.M.G; supervised and had leadership responsibility for the planning and execution of research activity, including guidance to the core team. All authors read and agreed with the published version of the manuscript. K.A.S. and V.B.P have contributed equally to this work.

Institutional Review Board Statement: Not applicable

Data Availability Statement: Not applicable.

Acknowledgments: The authors would thanks to CNPq, CAPES (code 001), FAPEMIG (APQ-02704-23, BPD-00647-22, RED-00067-23, RED-00193-23**)** UFSJ, UFMG, and UESC. RAMA, RTF, EAFC, RCG, ASG also thanks to CNPQ for their PQ/DT Fellowship. ASG and RCG thank to the National Institute of Science and Technology on Industrial Biotechnology (INCT-BI), and National Institute of Science and Technology on Tropical Diseases (INCT-DT), respectively. Finally, ASG thanks to the discipline´s team, all students for commitment and efforts in this work and our partner, Randall Johnson for English revision of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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