Diagnostic assays for Rickettsiosis infectiveness (review article)

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SUMMARY

The laboratorial diagnosis of Rickettsiosis, using major immunological techniques (Indirect Immunofluorescence, micro-agglutination, ELISA and Immunoblotting), is a powerful tool to reduce lethality of zoonosis, helping to monitoring epidemiological programs in various species involved (hosts, vectors, reservoirs or sentinels) and the man. Several methodologies are applied but still focused in the basic research, as Immunoblotting (used for antigenic activity) and the Polymerase Chain Reaction (PCR), which allows fast and precise detection of Rickettsii. In this review, we will discuss about the major technologies, mainly the immunological and the molecular tests, used in routine and in the research labs and their interests for identification of Rickettsii species and phylogenic groups and of their vectors or reservoirs in ecoepidemiology and etiophysiopathology of such zoonosis transmitted by arthropod vectors.

Keywords: Rickettsiosis, diagnosis, indirect immunofluorescence, ELISA, PCR.

Introduction

Rickettsiosis is a group of diseases caused by Rickettsia spp., transmitted by arthropod vectors and related to the physiopathology of Rock Mountain Spotted Fever, endemic and epidemic typhus. The epidemic typhus (Rickettsia prowazekii) occurs frequently on crowded situations as in prisons, refugees’ camps and during wars, where hygienic conditions are very low, increasing the lice population. The endemic typhus (R. typhi) appears when humans live close to rodents, usually associated to garbage dumps. Infections from Spotted Fever Group (SPG) occur in human living in the countryside, habitat of ticks and other vector mites and are included the Rock Mountain Spotted Fever, Brazilian Spotted Fever (R. rickettsii), Asiatic typhus (R. tsutsugamushi), the Mediterranean Fever (R. conorii) and the African typhus (R. aethiopica) [19].

Rickettsii are divided into two groups: Typhic Group (TG) and the Spotted Fever Group (SFG), based upon clinical presentation, immunological reactivity, DNA CG amount and the intracellular position [11]. The TG has three rickettsii: R. prowazekii, R. typhi and R. canadensis, transmitted mainly by fleas and lice. The SFG includes 60 species, from which, 13 are pathogenic to humans: R. rickettsii (in Brazil, causing the Brazilian Spotted Fever), R. parkeri, R. conorii, R. africanae, R. australis, R. honei, R. sibirica, R. japonica, R. felis, R. mongolotimonae, R. slovaca, R. helvetica and R. akari [21].

Besides that, Rickettsia prowazekii (typhus bacteria) and R. Rickettsii (Rock Mountain Spotted Fever bacteria) are microorganisms with potential use as biological army to induce panic in the population as well as bioterrorism, using genetic engineering to produce antibiotic resistant to rickettsii [43].

Rickettsias are obligated intracellular bacteria at both host vertebrates and invertebrates, but the species involved in the Rickettsiosis differs according to its association to the vector, to the vector behaviour, to the infection and to the physiopatology and to the disease evolution. Comprehending the Rickettsiosis pathogenic phases is essential to develop strategies of protection against bacteriological threats. Unfortunately, pathogenic mechanisms are poorly understood and the immunological mechanisms as well, particularly because of the need for animal models to simulate human diseases [2].

Rickettsia rickettsii is the most pathogenic species with variable virulence. Its cellular wall is peptidoglycan with muramic acids and diaminopimelic acids, as Gram-negative. It multiplies itself by binary division and the generation time in cell culture varies from eight to ten hours at 34°C. It is a pleomorphic microorganism and it can be seen as coccus, short sticks, in pairs, isolated or as filaments. Among protein antigens, R. rickettsii have two superficial proteins (190 and 135 kDa) with other protein thermolabile epitopes specie-specific.
common to various organisms in the group. It tends to associate to different cellular organelles and occasionally forms masses in the cytoplasm or nucleus of the infected cell, depending on the specie involved [14].

The cellular wall of the Gram-negative bacteria is composed by a peptidoglycan layer and three other components externally: lipoprotein, external membrane and lipopolysaccharide. The peptidoglycan is responsible for the cell shape and cytoplasm osmotic balance protection. It is formed by two amino sugars, acid N-acetyl glucosamine and N-acetyl muramic acid and by a tetrapeptide, always linked to the N-acetyl muramic acid residue; peptide subunit chains are linked to polysaccharide adjacent chains, linked to each other by direct or indirect bridges [26]. The peptidoglycan is in the periplasmatic space, between membranes, where the hydrolytic enzymes (phosphatases, nucleases, proteases and others) are located. These enzymes enable the bacteria nutrition and linkage proteins to capture sugar and amino acid from the enzymes that inactivate some antibiotics [26].

The laboratorial diagnosis of Spotted Fever in humans can be done by specific antibodies, by the isolation of the pathogenic agent in blood samples or skin biopsy, followed by the cell culture and in ticks collected from humans and from animals. Serological methods as well as molecular ones (PCR) are still the major diagnosis methods for Rickettsiosis [28].

Immunoassays

Immunoassays are nowadays a great tool based upon antigen-antibody reaction, having methodologies from Agglutination to Biosensors [9]. Most used immunoassays are: ELISA, based on the enzymatic binding; RIA (radioimmunoassay), based upon radioisotope binding; Western Blot, based upon protein localisation after an electrophoresis run and a membrane transfer; Immunofluorescence (IF), using fluorescent markers for the antibodies; the strips and the immuno-localisation property of the antigen (bacteria) to its antibody marked with a wished to identify [5]. This test is based on the linkage property of the antigen (bacteria) to its antibody marked with a fluorescent dye, and the immunofluorescence observed proves the bacterial presence in the sample [16]. The Indirect Immuno-fluorescence Reaction (IFI) is the most used for human diagnosis and for identifying Rickettsia SFG (Spotted Fever Group) infections, pathogenic or not. There are commercial supplies of the immunofluorescence tests for the SFG and typhus Rickettsi group (Pan Bio Inc., Baltimore, MD; Focus Technologies, Cypress, CA and Bio-Mériéux, France). The Indirect Immunofluorescence is now considered as the simplest, economic and sensible test for precocious diagnoses of Rickettsiosis for epidemiological studies or for identification of Rickettsii isolates. Positive identification shows the antigenic group to which the pathogen belongs, and not to the Rickettsii species. With the indirect micro-immunofluorescence, it is possible to use a drop of sera, diluted with six to nine different antigens simultaneously, increasing the ability to differentiate Rickettsii strains inside a strain group of SFG [28].

RICKETTSII INDIRECT IMMUNOFLUORESCEENCE (IFI)

The indirect Immuno-fluorescence assay is the recommended test from OMS for the Rickettsii diagnosis, using the antigen wished to identify [5]. This test is based on the linkage property of the antigen (bacteria) to its antibody marked with a fluorescent dye, and the immunofluorescence observed proves the bacterial presence in the sample [16]. The Indirect Immuno-fluorescence Reaction (IFI) is the most used for human diagnosis and for identifying Rickettsia SFG (Spotted Fever Group) infections, pathogenic or not. There are commercial supplies of the immunofluorescence tests for the SFG and typhus Rickettsi group (Pan Bio Inc., Baltimore, MD; Focus Technologies, Cypress, CA and Bio-Mériéux, France). The Indirect Immunofluorescence is now considered as the simplest, economic and sensible test for precocious diagnoses of Rickettsiosis for epidemiological studies or for identification of Rickettsii isolates. Positive identification shows the antigenic group to which the pathogen belongs, and not to the Rickettsii species. With the indirect micro-immunofluorescence, it is possible to use a drop of sera, diluted with six to nine different antigens simultaneously, increasing the ability to differentiate Rickettsii strains inside a strain group of SFG [28].

An IgM title higher than 1:64 indicates recent infections but it can be a false positive result in the absence of IgG reaction [4, 33]. Positive titles for IgG higher than 1:256 usually indicates recent infections while positive titles form 1:64 to 1:128 indicate recent infections or anterior infections [4, 33]. For the Rock Mountain Spotted Fever, IFI gives a title of ≥ 64 from the second week of symptoms, but the efficient
treatment should start on the fifth day of disease to avoid complications and death [3]. The United States and Europe have another Rickettsiosis, which needs to be treated as well [3]. For the Eschar-Nodular Fever, IFI title is around 40 and it can be seen in 46% between the 2nd and the 5th days, 90% on the 20th until the 29th days and in 100% of cases after this period [24]. For the murine typhus, IFI title is present on 50% of the cases at the end of first week and for 100% after 15 days of contagion [25]. At endemic areas, a higher title is necessary, for example, the Cerrado typhus in populations living at endemic areas, IFI title for O. tsutsugamushi is ≥ 400 and 96% specific and 48% sensible, with the sensibility increasing from 29% in the first week and 56% on the second week [10]. Such considerations are not very important for sporadically visitors on the endemic areas; each laboratory must establish its own cut-line and the percentage of sensibility. Similar results were shown for other Rickettsiosis, using IgG standards [3].

Other tests can be cited as agglutination for OX-19 and OX-2 Proteus vulgaris strains for Rickettsiosis and strain OX-K from Proteus mirabilis for infections of O. tsutsugamushi. Agglutination tests, however, must be replaced by more sensible ones, as ELISA or IF. Therefore, some countries still have the agglutination tests as the major technology available, specially used for lice transmitted typhus. Today, it is known that emergent diseases as Japanese Spotted Fever and Flinders Islands Spotted Fever include Proteus antibodies as agglutination results [3].

The presence of etiological agent inside the area of transmission of Brazilian Spotted Fever is based upon confirmatory test in loco and upon serological quiz on the primary hosts, done by blood sampling and immunofluorescence using Rickettsia bellii and Rickettsia rickettsii, in accredited laboratories or research centres [33]. Aiming to evaluate capybaras (Hydrochoerus hydrochaeris) potential as sentinels for rickettsii, 73 animals were sampled at six locations in São Paulo State, by IFI using Rickettsia rickettsii, Rickettsia parkeri and Rickettsia bellii as antigens; first results were found for Rickettsia parkeri infections in capybaras and first natural infection in vertebrate hosts [32]. Human and canine sera tested in IFI against four antigens (R. rickettsii, R. parkeri, R. felis and R. bellii) showed that 16 canine sera (64%) and 1 human sera (2.8%) reacted with, at least, one antigen, with titles ≥ 64. Seven canine sera (28%) and one human serum (2.8%) showed title for R. rickettsii, at least, 4 times higher than other antigen title. Such title can be explained by the infection of R. rickettsii, stimulating the reaction. None human or canine sera were found positive for R. parkeri, R. felis, or R. bellii stimulations. Results indicated that dogs can be considered as good sentinels for the presence of R. rickettsii in Amblyoma aureolatum, a tick from infested areas [34].

Rickettsii in dogs and its ticks were evaluated for Brazilian Spotted Fever at São Paulo metropolitan endemic area, where there were dogs supposed to be the main infection hosts, with ticks as vectors. By IFI, antibodies were found in dogs sera (title higher than 256). Not only the Amblyomma species can be related to R. rickettsii, also Rhipicephalus sanguineus are involved in the transmission to humans, confirmed in the results obtained before by Mexican and American researchers [29]. Clinical disease in dogs caused by Rickettsia rickettsii has been reported only in the USA [12]. Two cases of natural Spotted Fever in dogs confirmed by sera conversion, molecular analysis and treated with doxycycline were reported in Brazil. The IFI test was performed in these dogs using antigens purified from 6 rickettsii (R. rickettsii, R. parkeri, R. amblyomii, R. felis, R. rhipicephali and R. bellii) in Brazil. Whereas the first plasma sample showed only reaction against the R. rickettsii antigen, the second plasma sample presented titles for all the antigens tested [23]. In horses, the occurrence of antibodies against Rickettsia rickettsii was studied by IFI (the reaction was considered as positive when serum title was ≥ 64) from 2003 to 2005, at São Paulo Zoonosis Center on a total of 363 horses [30]. Among them, 64 (17.6%) [6/77 (7.8%) in 2003, 16/110 (14.5%) in 2004 and 42/176 (23.9%) in 2005] were positive for R. rickettsii with title ranged from 64 to 1 024 [30]. These results demonstrated a low seroprevalence for R. rickettsii in horses although in previous reports, the Spotted Fever in endemic areas prevalence ranged from 56.5% to 77.8% in horses [30].

**Molecular assays**

The PCR was made for DNA amplification of R. rickettsii, R. conorii, R. japonica, R. typhi, R. prowazekii, R. africae, R. felis, R. helvetica, R. slovaca and O. tsutsugamushi, usually from peripheral blood, clot or plasma and, rarely from tissues, frozen or soaked in paraffin, or from arthropods collected from patients. For all pathogenic Rickettsii, the lipoprotein gene with 17 kDa is the major target, using primers as “CAT-TACTTGGTTCTCAATTCGGT” and “GTITTTATTA GTGTTACGTAACC”, which amplifies a 231 pb fragment. The 16 S rRNA and the OmpA gene were also amplified. The identification of Rickettsii was made by polymorphism pattern or by restriction fragments (RFLP), using Alu I and Xba I or sequencing PCR products. Although PCR is a great tool for diagnosing, false positives are common, due to contamination of DNA, lack of DNA or inhibitors in the reaction, as chitin [3]. PCR has been used to detect Rickettsii in tick crude grinding, followed by gene sequencing, allowing a phylogenetic analysis of the different species. Phylogenically, the establishment of orthologous groups (OGs) Open Reading Frames will distinguish nucleus genes from rickettsii and other specific gene groups (class 1 OGs or C1OGs) from those present in all rickettsii dendrograms (class 2 OGs or C2OGs). A preserved region of 14.354 ORFs is present in 10 genomes of rickettsii. Data available in PATRIC (PathoSystems Resource Integration Center) gives new information to elucidate pathogenecity of Rickettsii, increasing diagnosis, vaccines and therapeutic targets [13]. By PCR, several genes can be studied as the 16S rRNA, gltA (citrate synthase), found in all rickettsii [22] and some other genes, which codify proteins OmpA and OmpB important to the pathogenecity of SFG Rickettsii. Knowing that there is a competition in the tick from different Rickettsii [31], the presence of non-pathogenic germs decreases the transmission of the pathogenic Rickettsii, so the PCR using gene gltA evidences all Rickettsii species and positive SFG must be investigated with rOmpA gene [37].
The complete sequencing of *Rickettsia conorii* and *R. prowazekii* gave the opportunity to know and identify virulence factors in such pathogens. The role of phospholipase A2 (PLA2) on the rickettsii pathogenicity was reported, although the corresponding gene was not detected. A gene codifying the phospholipase D (PLD) was detected in 11 samples by Southern Blotting. A recombinant dimeric protein with PLD activity was found in all rickettsii and it is probably a virulence factor due to its ability to release $^3H$-choline from the phosphatidyl$^3H$ choline. Furthermore, an anti-PLD antibody reduced the virulence on Vero cell cultures, decreasing cytotoxic activity. Such study can lead to elucidate intracellular enzymes in the bacteria [35]. *Rickettsia helvetica*, from the SFG, was recently detected using PCR, followed by DNA sequencing in ticks (*Ixodes ricinus*) from European sheep and goats, mainly in Hungary [41]. SFG Rickettsii associated with ticks developed a different molecular mechanism, efficient, synchronizing its replication with the host physiology. Mechanisms involved on the SFG adaptation to unusual host conditions as the virulence mechanisms are still unknown, but the SpoT genes are cellular metabolism regulators responding to stress and strengthening the survival of bacterial cells [38].

Aiming the first molecular identification by PCR of *R. rickettsii* in naturally infected *Rhipicephalus sanguineus*, ticks were collected from dogs, in Rio de Janeiro, Brazil, where five humans were diagnosed with Brazilian Spotted Fever in 2006. All ticks were identified, separated according to its maturation stage, specie and gender. Thereafter, DNA was extracted using commercial kit QIAamp DNA (QIAGEN®, Chatsworth, CA, USA) and PCR was performed using four primers groups: Rr190.70p/Rr190.602n (OmpA, 532bp), BG1-21/BG2-20 (OmpB, 650bp), Tz15/Tz16 (17 kDa protein from gene sequencing of 246bp) and RPCs.877p/RpCS.1258n (OmpB, 318bp). The PCR products were sequenced by ABI PRISM® and compared to GenBank data using Discontiguous Mega Blast (http://www.ncbi.nlm.nih.gov). The presence of Rickettsii confirmed by sequencing (GenBank FJ356230) was obtained in only one adult tick. Molecular characterization of *R. rickettsii* in the *R. sanguineus* confirms the importance of its specie in the rickettsii transmission to humans in Brazil [6]. Later, tick samples (*Rhipicephalus sanguineus*, *Amblyoma aureolatum*, *Amblyoma longirostre* and *Amblyoma sp.*) were collected from dogs and investigated by PCR for rickettsii genes. This study reported that 3.1% of *A. aureolatum* were infected with *Rickettsia bellii* and 1.3% of *R. sanguineus* with *Rickettsia rickettsii* in Brazil [29].

Capybara (*Hydrochoerus hydrochaeris*) infection by *Rickettsia rickettsii* was evaluated as an amplifier host for horizontal transmission of *R. rickettsii* to *Amblyoma cajennense* ticks, using PCR, real time PCR and IFI with antibodies in capybaras blood samples resulting in 20 to 35% of ticks feed in capybaras infected, causing no clinical damage on capybaras, but increasing the infection in guinea pigs and in ticks, indicating *Hydrochoerus hydrochaeris* as an amplifier host of *R. rickettsii* for *Amblyoma cajennense* in Brazil [40]. In the same way, the infection with *Rickettsia rickettsii* in Opossum (*Didelphis aurita*) as amplifiers hosts for *R. rickettsii* to *Amblyoma cajennense* ticks was evaluated by PCR and IFI. Using real-time PCR with gene gltA and detections of reactive antibodies, it was demonstrated that *R. rickettsii* was able to infect opossums, without causing disease, although the ability to infect guinea pigs was low. The opossums can develop a long “rickettsimy” and they can be considered as amplifier hosts for the ticks’ infection [18].

A study to the indirect assays for rickettsii detection in humans and direct detection in ticks, mainly *Amblyoma cajennense*, were positive for IFI in 31.2% and 77.3% of dogs and horses sera, respectively [17]. To confirm natural infection, PCR was performed with *A. cajennense* and the amplification of a 230 pb fragment from the codifying 17 kDa protein gene, was evidenced only in adults (4.7%) and more rarely in larvae (1.1%). By contrast, the gene of citrate synthase, the *OmpA* and *OmpB* genes were not amplified. Two adults and four sequenced larvae showed 100% similar to *Rickettsia felis*; 99.4% to 100% for *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia parkeri* and *Rickettsia peacockii* [17]. Quantitative Real-Time PCR (qPCR) was used to quantify rickettsii from SFG in ticks from the naturally infected *Amblyoma americanum* specie and seemed to be efficient on the quantification of rickettsii in tissues such as salivary glands, intestine glands and ovarian during vertical transmission. Such technique represents a great tool for study of replication rates, pathogenecity and for the comprehension of ticks and rickettsii relations [44].

Potential diagnosis of *Rickettsia conorii* by recombinant antigens was analyzed using PCR primers site-specific to clone genes *OmpA* and *OmpB* from *R. conorii* in plasmid pMAL-c2X. Six fragments of *OmpA* and four from *OmpB* were expressed as fusion proteins, linked to maltose in *Escherichia coli*. OmpA1350-1784, OmpB801-1269 and OmpB1227-1634 regions were selected from truncated proteins as candidate antigens for ELISA diagnosis using control sera. Results were compared to an ELISA commercial kit with the antigens *OmpA* and *OmpB* from *R. conorii*. Forty samples were collected from febrile patients. Twenty sera of *R. conorii* were positive using commercial kit and eighteen using the developed ELISA test (with a sensibility of 90 %). Specificity was 100 %, with all 20 negative control samples. ELISA test using OmpB801-1269 and OmpB1227-1634 showed specificity of 99% and 95%, respectively, suggesting specific regions from *OmpA* and *OmpB* detect antibodies against *R. conorii* and truncated antigens can be used on the development of rickettsii diagnosis [7].

In 2 dogs with natural Spotted Fever in Brazil, DNA samples were analyzed using PCR tests using a 147pb GltA gene fragment and Hemi-nested PCR using the *OmpA* gene. Sequencing resulted in a 452-pb fragment identical to results obtained by Bitterroot sequence (GenBank access n. U43804) but no PCR products were found for GltA gene [23].

**Perspectives**

A century after the first description of rickettsii as human pathogen, rickettsii is still misunderstood as disease. Such bacteria are restrictedly intracellular, which it makes it difficult to study them deeply. In the last ten years, genome programs
allowed a better comprehension of the mechanisms involved in the pathogenicity of rickettsii. Nowadays, studies about interactions from rickettsii and host cells contribute for a better and careful analysis of cellular and tissue process, leading to surveillance of intracellular bacteria [1].

Sixteen new Rickettsiosis transmitted by ticks are described in the last 20 years, while in 1984, there were only four. The high increase is a result of molecular and immunological technologies to improve diagnosis in humans and in ticks. Further studies on the tick-Rickettsii interactions must give a better understanding of factors involved in the distribution, infection and its co-evolution. Vector-rickettsii relation is still not well understood neither and its impacts on horizontal transmission, time, double infection and pathogenecity need answers. The molecular base is in the beginning and studies on the extraction, detection, isolation, including electronic microscopy, immunohistochemistry and cell culture for vectors, as well as proteasome and transcriptome will elucidate the understanding of its pathogens [38].

Researches leads to recombinant proteins for detecting circulating antibodies targeted to Rickettsii in any organism and for amplifying the recognition signal several times. The use of recombinant protein can decrease the size of the sequence and ensures the target bases sequences are achieved by the antibody constructed. Another protein based test under development is the “Biosignature” of rickettsii infections, based upon the hypothesis which microbial agents produce standards for vectors, as well as proteasome and transcriptome will elucidate the understanding of its pathogens [38].

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