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Performances des tests diagnostiques pour les différentes espèces de spirochètes

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Résumé

Les bactéries du complexe *B. burgdorferi* sensu lato, qui causent la maladie de Lyme, appartiennent au phylum des spirochètes. Les difficultés diagnostiques rencontrées dans la maladie de Lyme sont en partie dues aux caractéristiques des spirochètes qui sont des bactéries dont la culture est fastidieuse, voire impossible pour certaines d'entre elles. Nous avons réalisé une revue de la littérature concernant les performances des différents tests diagnostiques dans les spirochétoses d'intérêt médical que sont la borréliose de Lyme, les borrélioses récurrentes, la syphilis et la leptospirose. Cette revue permet de dégager un certain nombre de points communs pour ces quatre infections. La PCR en temps réel a pris une place importante ces dernières années dans le diagnostic direct de ces infections. Cependant, le diagnostic direct reste difficile du fait d'un manque de sensibilité persistant et les sérologies gardent donc un rôle central dans le raisonnement diagnostique. Tous les outils diagnostiques actuels présentent des imperfections avec un risque éventuel de faux positifs et de faux négatifs en fonction du contexte clinique. Ceci doit inciter les cliniciens à une interprétation des tests diagnostiques en cas de suspicion de maladie de Lyme, Borréliose récurrente, syphilis ou leptospirose, toujours en relation avec le contexte clinique et épidémiologique dans lequel ils se trouvent.

Abstract

Bacteria of the *B. burgdorferi* sensu lato complex, responsible for Lyme disease, are members of the spirochetes phylum. Diagnostic difficulties of Lyme disease are partly due to the characteristics of spirochetes as their culture is tedious or even impossible for some of them. We performed a literature review to assess the value of the various diagnostic tests of spirochetes infections of medical interest such as Lyme borreliosis, relapsing fever borreliae, syphilis, and leptospirosis. We were able to draw similarities between these four infections. Real-time PCR now plays an important role in the direct diagnosis of these infections. However, direct diagnosis remains difficult because of a persistent lack of sensitivity. Serological testing is therefore crucial in the diagnostic process. All currently available diagnostic tools are imperfect, with a potential risk of false positive and false negative results depending on the clinical context. Physicians should always take into consideration the clinical and epidemiological context when Lyme disease, relapsing fever borreliae, syphilis, and leptospirosis are suspected.

Introduction

Bacteria of the *Borrelia burgdorferi* sensu lato group (responsible for Lyme borreliosis) are spirochetes just like *Borrelia* spp. responsible for relapsing fever, *Leptospira* spp. responsible for leptospirosis (worldwide zoonosis transmitted through contact with urines of rodents), and *Treponema* spp. including the causative agents of syphilis (a sexually-transmitted disease). Bacteria belonging to this phylum have specific characteristics, such as a helical form and motility due to pseudoflagella which help them move in viscous media [1]. Some spirochetes are commensal bacteria of humans, mainly of the oral flora, and others play a specific role in human pathology. Some *Borrelia* spp. transmitted by ticks or lice, are responsible for infections very different from Lyme disease, and are called relapsing fever borreliae, caused by ticks or lice. These infections all have very different clinical symptoms, and their diagnosis is based on the physical examination and physician's judgment as well as on confirmatory microbiological tests. Other specificities of bacteria belonging to this phylum are their non-Gram staining characteristics and their inability to be cultured using standard culture media. Laboratory diagnosis is therefore tedious.

Current debates are mainly sparked by patients' associations and some physicians who are questioning the reliability of Lyme borreliosis diagnostic tests. There is no such debate for other spirochetes. We assessed literature data on the value of diagnostic tests for *Borrelia* spp. responsible for Lyme disease and for other spirochetes of medical interest.

Current quality standards and French regulations for diagnostic tests in humans

Before analyzing scientific literature data, we should note that biomedical laboratories commercializing and performing diagnostic tests in humans have to comply with various regulations and standards in Europe and France: CE marking, inspections performed by the French Agency for the Safety of Health Products (French acronym ANSM), and COFRAC

accreditation for laboratories performing biological tests. Manufacturers apply the CE marking on their tests, thus guarantying the conformity of their products with legal European requirements. The ANSM (https://ansm.sante.fr/) also performs a retrospective inspection to ensure conformity of the various biological tests marketed in France with health and safety requirements. The French accreditation committee, named COFRAC (https://www.cofrac.fr/), is the only national agency responsible for the accreditation of both public and private biology laboratories. For molecular biology techniques such as PCR detection of *Borrelia*, *Treponema*, and *Leptospira,* laboratories must therefore have their methods validated. Additional tests must therefore be performed to ensure the absence of laboratory contaminations for instance. Quality of the tests performed by laboratories not complying with these requirements is not ensured. It should be noted that veterinary laboratories do not have to obtain the COFRAC accreditation, and that they are not allowed to perform human biological tests since May 30, 2013.

Lyme disease diagnosis

Except in case of typical erythema migrans, a positive biological test is required to confirm Lyme borreliosis diagnosis.

Direct diagnosis

Just like other spirochetes of medical interest, *Borrelia* spp. responsible for Lyme borreliosis cannot be detected by standard optical microscopy nor by Gram staining. They can, however, be detected by dark-field microscopy or phase-contrast microscopy [7].

However, bacteremia observed during Lyme borreliosis, especially with European clinical presentations, is moderate, of a short duration, and only occurs at the beginning of dissemination [7]. Looking for the bacterium in blood samples when patients do not have fever is therefore useless in case of Lyme borreliosis. The few pieces of data available after

so-called positive microscopy have actually been demonstrated to be artifacts and not *Borrelia* [8]. The microscopic examination does not perform well enough to be a useful tool for the biological diagnosis of Lyme borreliosis.

The *in vitro* culture of *Borrelia* from tissue biopsy (skin, synovial membrane) or from biological fluid (CSF, synovial fluid) is performed on specific liquid media: BSK-II, MKP, or BSK-H (commercialized), incubated at 32-34°C. As the mean time to *Borrelia* growth is 7-20 hours, the time to positive culture is usually more than 15 days, or even more than eight weeks [9]. Cultures should therefore be checked once a week with a dark-field microscope for at least eight weeks before being able to conclude to a negative result, because *Borrelia* growth does not cloud the culture medium [9]. As *Borrelia* are highly fragile bacteria, specimens should be inoculated directly at the patient's bedside. Considering these technical requirements, *Borrelia* culture from human specimens is only performed by a few specialized laboratories in Europe. Although cultures perform well with erythema migrans specimens, this method lacks sensitivity when it is used with other biological specimens (CSF, skin biopsy of acrodermatitis chronica atrophicans, and synovial fluid where only a few isolates are observed) [7]. Moreover, no strain has so far been isolated from blood specimens of patients presenting with chronic disorders several years after a tick bite; culture from blood specimens is therefore currently not recommended.

The PCR detection of *Borrelia* is not associated with the constraints of culture. Several PCR techniques are available and their value varies in terms of sensitivity, specificity, and detection spectrum [10]. Several PCR kits are currently commercialized, but their performance is poorly known. Direct detection of *Borrelia* by PCR test should thus only be performed by official and accredited specialized laboratories. However, sensitivity of the PCR test is much higher than that of culture in disseminated skin and articular presentations of the disease [11]. Nonetheless, a negative PCR test result cannot rule out the Lyme borreliosis

diagnosis. Physicians should always keep in mind that prescribing *Borrelia* PCR testing in case of a negative serology is not recommended, except for patients presenting with early atypical skin lesions of erythema migrans or very early Lyme neuroborreliosis [2].

Among all direct diagnostic techniques, only PCR testing and culture – despite their limitations – can be used and have been validated for the detection of *Borrelia*. Microscopy techniques are indeed too often associated with artifacts and should be avoided.

Indirect diagnosis

Because of the above-mentioned limitations of direct techniques, the biological diagnosis of Lyme borreliosis is currently mainly based on serological testing. Results should always be interpreted in light of the clinical context and the incidence of the disease as they impact pre-test probability and consequently the positive and negative predictive values of the tests. Lyme borreliosis diagnosis in France is based on two-tiered serological testing in France, just like in most European countries and in the United States. This process improves the specificity of the final result [2]. The first test is usually an ELISA assay; in case of a positive or dubious result, a second confirmatory test is performed by immunoblot technique (or Western blot) to confirm the specificity of anti-*Borrelia* antibodies [2].

A European meta-analysis recently demonstrated that immunoblot tests used on their own were not associated with better sensitivity or specificity than ELISA tests for the diagnosis of Lyme borreliosis in Europe, whether it be for Lyme arthritis or Lyme neuroborreliosis [12]. The overall sensitivity on serum in cases of Lyme neuroborreliosis was 81% (CI: 70-89%) for ELISA tests and 81% (CI: 57-96%) for immunoblot tests, with 92% specificity (CI: 88-89%) for ELISA tests and 94% (CI: 91-96%) for immunoblot tests. For Lyme arthritis, the overall sensitivity of ELISA tests was 94% (CI: 86-98%) and 95% (CI: 84-98%) for immunoblot tests, with 97% specificity (CI: 94-98%) for ELISA tests and 92% (CI: 84-96%) for immunoblot tests. This study also highlighted the lower accuracy of ELISA tests

compared with immunoblot tests [12]. The authors of this meta-analysis also reported a slight overall improvement of these tests, although statistically non-significant, when using purified antigens and/or recombinant antigens, mainly in patients presenting with Lyme neuroborreliosis [12].

This two-tiered serological testing process improves the positive predictive value of the final result compared with a single ELISA or immunoblot test (Western blot) as they may yield up to 27% of false IgM positive results [13]. As both methods have some degree of specificity, their successive use contributes to increasing the specificity of the final result [12].

However, these specificity and sensitivity values vary depending on the infection stage. No biological test is required for erythema migrans as the serological test is negative in more than 50% of cases in Europe [14]. Erythema migrans is the most frequent clinical presentation of Lyme borreliosis. It is a localized infection, associated with a low immune system response. A negative serological test result is therefore associated with a probability of wrongly ruling out the diagnosis. The biological diagnosis of Lyme neuroborreliosis is based on the joint analysis of blood and CSF [15]. CSF analysis reveals early lymphocytic cellular reaction in more than 93% of patients presenting with meningoradiculitis [16]. Lyme serology is positive in serum specimens at the acute phase of Lyme neuroborreliosis in 70-89% of cases, and sensitivity in CSF specimens at the acute phase is >90% [16]. At the stage of Lyme arthritis or acrodermatitis chronica atrophicans, the sensitivity of Lyme serology is 95% and 98%, respectively [12]. The very high negative predictive value of serology should lead to question the suggested diagnosis in case of a negative result in patients presenting with clinical signs and symptoms. One should also bear in mind that, following effective treatment, anti-*Borrelia* antibodies (including IgM) may persist for months or even years after clinical

cure [2]. Serological testing is therefore not useful for the follow-up of treated patients and the presence of IgM is not indicative of a persistent *Borrelia* infection.

Various species of the B. burgdorferi sensu lato complex

Various species have been defined as belonging to the *B. burgdorferi* sensu lato complex. Their geographical distribution is varied and they are responsible for various clinical signs and symptoms [17]. Predominant species in Europe are *B. garinii* and *B. afzelii*, while *B. burgdorferi* sensu stricto is most frequently observed in the United States [17].

Partial differentiation of species among the *B. burgdorferi* sensu lato complex by serological testing is possible because of molecular and antigen changes of surface proteins. In 1994 Wilske *et al.* developed an immunoblot technique based on the use of five recombinant antigens. This technique helped detect seroreactivity differences between patients infected with *B. afzelii, B. garinii,* or *B. burgdorferi* sensu stricto [18]. Two years later, Norman *et al.* developed an immunoblot technique using several strains of *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto [19]. The authors revealed that European sera were more reactive to Western blot prepared with strains of *B. garinii* and *B. afzelii,* while sera collected from Northern American patients were more reactive to *B. burgdorferi* sensu stricto antigens [19]. Sera of patients presenting with neurological signs were also more reactive to *B. garinii* antigens and those of patients with dermatological clinical presentations were rather reactive to *B. afzelii* antigens [19]. One should note that, in this study, the interpretation of the Western blot result (positive or negative) could vary depending on the strain used for 8% of tested specimens. Other European studies using the Western blot technique reported similar results, with a preferential association of *B. garinii* and neurological disorders, *B. afzelii* and late cutaneous signs, and a slight predominance of *B. burgdorferi* sensu stricto in joint manifestations [20–22]. Consequently, as early as 1999, standardization of Western blot techniques prepared with *B. garinii* and *B. afzelii* strains was suggested in Europe [23]. To do

so, the authors used a panel of sera from patients coming from various European regions, that had been provided by members of the European Union Concerted Action on Lyme Borreliosis (EUCALB), and compared them with those of healthy blood donors [24]. However, composition and sometimes performance differences are observed between ELISA and Western blot techniques commercialized in the various European markets [12,24]. These tests are usually prepared with a mixture of recombinant antigens from the three main pathogenic species for humans (*B. burgdorferi* sensu stricto*, B. garinii,* and *B. afzelii*) [6]. Including antigens from these various species mainly aims to improve the sensitivity of serological tests. However, accurate differentiation of *Borrelia* species can only be performed with molecular techniques; reactivity differences observed with serological tests in antigens of various species do not always result from culture or PCR, and may lead to interpretation errors.

Other diagnostic tests for Lyme borreliosis

Some tests are sometimes suggested for the diagnosis of Lyme borreliosis, although their bioclinical value has not been extensively assessed. Other tests are not validated using the methodology approved in medical biology [2]. These various techniques include lymphocyte transformation tests (LTT). LTTs currently lack validation and published studies have substantial methodological biases [3]. For instance, the diagnostic value of LTTs in Lyme neuroborreliosis is low (36% sensitivity and 82% specificity) [4]. Another test relies on CD57 marker detection. Little data is available and the only case-control study performed (NIH study) reported the complete lack of specificity of this test [5]. As for rapid diagnostic tests for self-testing, sensitivity and specificity data is currently very limited [6].

Relapsing fever borreliae

Relapsing fever borreliae are mainly observed in tropical and subtropical regions, and mainly in Africa. The causative agents are usually *B. duttonii*, *B. crocidurae, B. recurrentis,*

B. persica, and *B. hispanica* [25]. They are transmitted by soft ticks (Argasidae) and for some of them by body lice (*B. recurrentis*) [25]. An emerging species, *B. miyamotoi,* is transmitted by hard ticks [25].

Direct diagnosis

Relapsing fever borreliae, unlike Lyme borreliosis, can lead to severe bacteremia during febrile episodes. This is why the usual diagnostic method is optical microscopy after Giemsa staining of blood smear or thick blood drop. This technique may detect concentrations of 10^3 to 10^5 microorganisms/mL [26–28]. Comparative studies reported that additional methods could increase the sensitivity of optical microscopy. Differential centrifugation, acridine orange, quantitative buffy coat (QBC), and fluorescence microscopy could contribute to increasing microscopy sensitivity [29–31]. Quantitative buffy coat is supposed to be 100 times more sensitive than thick blood drop for the diagnosis of relapsing fever borreliae [31]. However, such technique requires specific laboratory equipment that is often not available in endemic regions.

Molecular biology techniques such as standard PCR and quantitative PCR have been developed. Several comparative studies reported the better sensitivity of these techniques compared with usual microscopy techniques. These findings are detailed in Table 1 [32–34].

Culture may be performed but it requires expert skills, thus limiting its routine use. It is thus very rarely used with clinical samples. BSK-H culture medium is the same as the one for *Borrelia* spp. responsible for Lyme disease. All *Borrelia* species responsible for relapsing fever do not grow on this medium though. Growth is detected by dark-field microscopy.

Antigen detection in blood or other tissue specimens is being developed, but it is currently not available in clinical practice. Specific monoclonal antibodies have for instance been developed to detect *B. crocidurae* and *B. hermsii* [35,36]. Another innovative technique

is the MALDI-TOF test. It has been used in the detection of *Borrelia crocidurae* in *Ornithodoros sonrai* ticks [37].

Indirect diagnosis

Specific serological tests for the causative agents of relapsing fever borreliae have been developed. They are based on the use of GlpQ antigen or BipA antigen, that are supposed to be absent from species of the *burgdorferi* group [7,38,39]. However, several studies reported cross reactions with bacteria of the *burgdorferi* sensu lato complex responsible for Lyme disease. This has for instance been observed between *B. miyamotoi*, *B. burgdorferi*, and *B. hermsii* in the United States [40]. The serological test is also often negative during the first fever episodes of relapsing fever and should therefore rather be used as a retrospective diagnostic tool.

Syphilis: *Treponema pallidum*

Syphilis is a sexually-transmitted infection caused by *T. pallidum.* It is a public health problem as its incidence has been on the rise in France since the years 2000 [41]. The main obstacle to diagnosis and to the study of *T. pallidum* is the inability to culture the bacterium in an axenic medium.

Direct diagnosis

Direct examination by dark-field microscopy of chancre or skin lesion samples is highly contributory and immediate. Performances vary according to laboratories and staff. Specific material and qualified staff are required. False positive results (commensal spirochetes, especially on the mouth or anus) and false negative results (a negative direct examination should not rule out syphilis) may be observed [42,43]. The sensitivity of the examination is higher at the primary and secondary phases and in early congenital syphilis (chancres, condyloma latum, mucous patches, adenopathy) [44].

Direct immunofluorescence for the detection of *Treponema* has been associated with satisfactory results at the mucous, skin lesion, or tissue levels. The sensitivity of these techniques is close to 80% [45–47]. However, these techniques have become obsolete since the commercialization of PCR tests. Various methods such as standard PCR, nested PCR, RT-PCR, or quantitative PCR, have been used by targeting several genes such as *bmp*, *tpp47*, *tmpA* encoding for surface lipoproteins, or *pol A* involved in genome replication [48]. Recent studies reported no performance differences between PCR testing targeting the gene encoding for the membrane protein of 47 Kd and that targeting the gene encoding for the "*pol A*" polymerase DNA gene [49]. Sensitivity is approximately 80% and specificity 95%; and 95% and 89% negative and positive predictive values have been reported, respectively [49,50]. Just like for other spirochetes of medical interest, the values of PCR tests depend on biological samples tested and infection stages. For illustrative purposes, patients presenting with secondary syphilis or patients co-infected with HIV with a low level of CD4 lymphocytes, have higher rates of spirochetemia than patients presenting with primary syphilis or patients at the early latency phase. Blood PCR testing is therefore associated with better sensitivity in these patients [51]. PCR techniques have been assessed in the detection of *T. pallidum* DNA in swabs of chancre, cutaneous biopsy of secondary syphilis, bones, various biological fluids such as serum, CSF, urines, placenta, gastric lesions [52–60]. PCR testing is thus recommended as a diagnostic tool for primary and secondary syphilis. However, because of an insufficient sensitivity, its negativity should not rule out the diagnosis and an indirect diagnostic test should also be performed.

Indirect diagnosis

No serological test for syphilis is able to differentiate venereal syphilis caused by *Treponema pallidum pallidum*, and non-venereal *Treponema* infections caused by *T. pallidum* subsp. *endemicum* (responsible for Bejel), *T. pallidum* subsp. *pertenue (*responsible for

Yaws), and *T. pallidum carateum* (responsible for Pinta). Two types of tests are available: nontreponemal tests (NTT) and treponemal tests (TT).

Nontreponemal tests (NTT)

These tests are based on the use of a complex antigen, made of cardiolipin, lecithin, and cholesterol [44]. The venereal disease research laboratory test (VDRL) and rapid plasma reagin test (RPR) are the most frequently used. The RPR test is made of charcoal particles coated with a mixture of lipid antigens [44]. Consequently, anti-lipid antibodies detected by these tests are not only produced during *Treponema* infection, but also in case of any other disease responsible for tissue lesions. As many cross reactions are observed, these tests alone cannot confirm the syphilis diagnosis. In case of infection, these tests are positive 10 to 15 days after primary chancre, i.e. six weeks following contact. In the absence of treatment, the highest level is reached between one and two years and remains positive at lower levels during late phases of syphilis [44]. Complete seronegativation during tertiary syphilis is extremely rare. NTT titers are also correlated with the infection activity, and are thus used to control treatment effectiveness. Sensitivity values of these tests thus vary depending on the disease stage [44]. During the primary phase (at the chancre stage), sensitivity of the RPR and VDRL tests is low, respectively 86% and 78% [44,61]. Sensitivity is 100% for both NTTs at the secondary phase, and 71% for VDRL and 71% for RPR at the late phase [44,61]. Specificity is 98% at all infection stages [44]. The risk of prozone reaction triggering false negative results is another problem observed with NTTs. This reaction is observed in up to 2% of patients presenting with secondary syphilis, in serum specimens with a high number of antibodies. The normal antigen-antibody reaction is therefore inhibited [44]. Weakly positive results, dubious results, or even negative results are in that case observed. It is thus recommended to dilute samples to first obtain increased titers and then a progressive decrease [44].

These tests are also associated with a risk of false positive results, mainly because of the type of lipid antigens used: they yield a positive result in cases such as acute hepatitis, infectious mononucleosis, pneumonia, chickenpox, measles, pregnancy, or malaria [44]. Chronic diseases such as autoimmune diseases (mainly lupus), cancers, leprosy, or intravenous drug use may lead to positive results with NTTs.

Treponemal tests (TT)

TTs are based on the detection of antibodies targeted against antigens that are part of *Treponema*. All TTs use *T. pallidum* as antigen. Their specificity is better than that of NTTs, but they always yield positive results after treatment. Active syphilis thus cannot be distinguished from a serological scar [44]. These tests are *T. pallidum* hemagglutination assay (TPHA), *T. pallidum* particle agglutination assay (TPPA), and fluorescent treponemal antibody absorbed test (FTA) [62]. More recent immunoenzyme techniques are available, such as the ELISA assay or CMIA (chemiluminescent microparticles immunoassay). Agglutination and fluorescence tests are usually manually performed (TPHA, TPPA, FTA), whereas immunoenzyme tests are automated and multiplex tests have even been recently developed [62]. Sensitivity and specificity values of the various TTs are reported in Table 2. Automated techniques (ELISA, CMIA BIOPLEX) are usually more sensitive and specific than manually performed tests (FTA, TPPA, and TPHA) [62,63].

Western blot tests may also be used to confirm results, with up to 99.9% sensitivity and specificity [41,64–66]. These tests are able to detect antibodies targeted against antigen proteins such as TpN15, TpN17, TpN45, or TmpA. They are considered positive with at least two strips of IgG or one strip of IgM. They are particularly useful when other serological tests yield discordant results, or for congenital syphilis diagnosis because pregnancy is a cause of false positive results with NTTs and possibly with the more usual TTs [41]. Looking for IgM

by Western blot technique in children enables the distinction between passive transmission of IgG mother antibodies and active synthesis of IgM by the newborn.

Strategy for serological diagnosis

The French National Authority for Health (French acronym HAS) suggested a new screening strategy in 2015. This strategy is based on two-tiered serological testing: an automated TT (ELISA, EIA, or CMIA) to detect IgG and IgM [67], followed by confirmatory quantitative NTT if the first test result is positive. A recent study performed by the centers for disease control and prevention (CDC) evaluated five laboratories using this strategy in the United States. The authors revealed that up to 56% of patients with a positive automated TT result had a negative NTT result (RPR) and that 36% of them also had a negative TPPA or FTA result [68]. This proportion of false positive results was higher among populations known to be associated with a low prevalence of syphilis [68]. Such evaluation has so far not been conducted in France, where the use of this strategy is more recent.

Leptospirosis

Leptospirosis is a zoonosis caused by pathogenic spirochetes of the *Leptospira* genus. It is transmitted to humans by direct or indirect contact (through contaminated water mainly) with the urine of infected animals [69]. The clinical presentation of the disease is divided into two phases: a febrile, bacteremia phase of variable duration and an immune phase characterized by antibody production and excretion of *Leptospira* in urines, that may be associated with fever [69]. Most symptoms associated with disease severity (jaundice, lung involvement, renal involvement, meningitis) are observed during the immune phase. Just like other spirochetes mentioned in the present article, the value and interpretation of biological tests depend on the infection stage.

Direct diagnosis

The detection of *Leptospira* requires a dark-field microscope or a phase-contrast microscope [69]. *Leptospira* are spiral-shaped bacteria (6 to 12 µm long and 0.1 µm in diameter), with mobile hooked ends [69]. The direct examination with a dark-field microscope is weakly sensitive and poorly specific, with a detection threshold of 10^4 *Leptospira*/mL [69]. This method only yields positive results during the bacteremia phase of the infection, but has to be performed by a laboratory technician. The risk of false positive results is also high due to the presence of fibrin filaments or protein fragments which are moved by Brownian motion process [69]. *Leptospira* may be cultured from biological fluids such as blood, CSF, or urine. Blood samples should be collected in heparin tubes during the febrile period and inoculated on the specific EMJH culture medium [70]. Samples should be incubated in a dark room at 28-30°C for at least eight weeks, and analyzed weekly with a dark-field microscope [69]. CSF may be cultured in case of meningitis signs and symptoms, with the same modalities. Urines are alkalized and inoculated within four hours (the acidity of urines lyses *Leptospira*) with serial dilutions at 1/10 and 1/100 [69]. Isolated strains are identified by agglutination tests on specific antiserum. Genotypic identification techniques are also more frequently used [71,72].

PCR is currently the most frequently used direct diagnostic method and allows for the early diagnosis of the infection before antibody detection. *secY* (flagellin), *rrs*, *flaB*, and *rrl* genes and the LA 3521 locus are used in the various methods currently available [70]. PCR detection has been used on blood, urine, CSF, and aqueous humor samples with different performances [70]. The sensitivity and specificity values on blood samples are unfortunately highly variable and are even lower with late PCR testing than early PCR testing at the start of the infection (from 9% to 73% and 52% to 100%, respectively) [73, 74]. The detection threshold is set at 10 to 50 *Leptospira*/mL of blood. PCR testing on urine samples is associated with better performance and specificity and sensitivity values of 100%, although

based on only one study [70, 74]. Quantitative PCR has now replaced standard PCR, with increasing sensitivity and specificity values (96% and 100%, respectively) [75].

Indirect diagnosis

The historical reference serological technique is the microscopic agglutination test (MAT) [69]. Developed in 1918 by Martin and Pettit, this method relies on serum incubation with *Leptospira* antigen suspension of various serovars and on looking for agglutination with a dark-field microscope. Serological titer can thus be determined, corresponding to the highest dilution at which a 50% agglutination is observed [69]. This method requires particular expertise to maintain representative strains of each serovar in culture. It is thus only performed by reference centers. It also helps identify the causative serogroup, which is useful for epidemiological purposes. Although considered a reference method, sensitivity values are not optimal at all stages of the infection (82% at Week 2 of the infection, 96% after Week 4) [69]. Significant titers are 100 in endemic regions and 400 in non-endemic regions [76].

Various ELISA serological tests enabling the detection of anti-*Leptospira* IgM, have been commercialized over the past few years. These tests are more easily used and are better standardized. The value of these commercialized tests is highly variable depending on studies (sensitivity ranging from 4% to 97%), mainly because some studies performed the serological test during the first week of the infection. Nonetheless, studies performing the serological test after Day 7 reported sensitivity and specificity values between 75% and 100% and 78% and 97%, respectively [77–81]. These tests are therefore currently recommended as first-line strategies during the immune phase of the infection, with secondary confirmation by MAT [81].

Another type of technique based on unit tests with visual interpretation on strips has been developed [81]. It is based on the same principle as the ELISA assay, but the antigen is

fixed on a strip. The added value of this test relies on its ease of use, although limited by its expensive price. This method has never been fully assessed in metropolitan France, but its sensitivity is believed to be lower than the usual ELISA techniques. Its use is therefore not currently recommended, except in isolated regions of French overseas territories [81]. The slide microagglutination test with a thermoresistant antigen has long been used as a screening technique before MAT confirmation. Total antibodies can thus be detected during the second week of the infection [81]. Tests used in France are associated with sensitivity values ranging from 45% to 63% and specificity values from 84% to 88%. The HAS thus decided in 2011 to no longer use this test as part of the diagnostic strategy of leptospirosis [81].

Diagnostic scores

Considering the limitations of the various diagnostic tests of leptospirosis and their poor availability in resource-limited countries where the infection prevalence is high, the WHO developed specific diagnostic criteria in 1982: Faine criteria (Table 3) [82]. These criteria are divided into a clinical category, an epidemiological category, and a biological diagnosis category (Table 3). The latter category was modified in 2012 to include more recent diagnostic methods than MAT, as well as the risk factor of "rainfall before symptom onset" [83]. The leptospirosis diagnosis is highly suspected when the score is >20, with high probability when >24 (Table 3) [84]. This modified score has newly been evaluated in a recent study conducted in Sri Lanka, and sensitivity and specificity values of 95% and 56%, respectively, have been reported [85]. Several predictive diagnostic scores have since been developed. A Sri Lankan team thus suggested using a score based on a derivation cohort of 450 patients suspected of having leptospirosis and on a validation cohort of 142 patients with a confirmed diagnosis of leptospirosis [86]. This score includes five items, each with values ranging from 4 to 6: bilirubinemia >30 mmol/L, >80% of neutrophils, exposure to

contaminated water or soil, creatininemia >150 µmol/L, platelet count <85 Giga/L [86]. A threshold score of 14 was associated with an 80% sensitivity, a 62% specificity, a positive predictive value of 54%, and a negative predictive value of 84% [86].

Conclusion

This literature review on biological diagnostic tests for spirochetes responsible for infections in humans, highlighted several similarities between these bacteria. For all studied bacteria, the direct diagnostic methods are not optimal. Dark-field microscopy has to be performed by a laboratory technician. Culture is always tedious or even impossible on an axenic medium such as for syphilis. The commercialization of real-time PCR techniques represented major progress for the four infections studied in the present article, as they are more sensitive than culture and as they can be more easily standardized. However, the likelihood of a positive PCR result depends on the infection stage (bacteremia stage for *Leptospira* or relapsing fever borreliae) and on the type of sample collected (erythema migrans biopsy for Lyme disease, genital chancre for syphilis).

As for indirect diagnostic methods, false negative results are mainly observed at the early stage of the four infections when no or not enough specific antibodies are produced. Serological tests are also all associated with cross reactions, with a risk of false positive results, and consequently with a risk of excessive diagnoses and treatments.

This risk of false positive results is known and can be measured thanks to Bayes' theorem, developed in the 18th century. False positive results are indeed an inherent difficulty to all medical diagnostic tests as no test is perfect. This risk is higher if the probability of having the disease in the studied population is low. Thus, to reduce the risk of false positive results with a given test, one should select a population associated with an "a priori" higher disease prevalence than in the general population. Several strategies have been developed.

The first strategy consists in performing the test in a population that has been selected for its risk factors for the infection. For instance, when performing an HIV serological test in a group of intravenous drug users sharing syringes, the infection prevalence will be much higher than if we were testing random people among the general population. Such strategy thus leads to decreasing the number of false positive results. Diagnostic scores such as those developed for leptospirosis are also based on that same principle. Another strategy consists in performing two tests: a screening test, more sensitive than specific, which will yield many false positive results although enabling the selection of a population subgroup where the disease prevalence is a priori higher. The first test should then be confirmed with a more specific test to improve the overall relevance of the test result. For Lyme borreliosis, this strategy is applied with a sensitive but weakly specific ELISA serological test – and thus associated with false positive results – followed by a more specific confirmatory Western blot test.

Disclosure of interests

Didier Raoult and Oleg Mediannikov applied for a patent for a monoclonal antibody enabling the detection of *Borrelia crocidurae*.

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Tableau 1. Sensibilité des différentes méthodes de diagnostic direct des borrélioses récurrentes

Table 1. Sensitivity of the various direct diagnostic methods for relapsing fever borreliae

Method	**Characteristics**	**Reference**
Multiplex qPCR for *B.crocidurae,* *B.duttoni/recurrentis*, and *B. hispanica*	Cut-off 36 Ct=100 copies of plasmids 5 µl	[32]
B. recurrentis qPCR	3 copies 40.94 32 copies 36.92	[87]
Thick blood drop	10^4-10^5	[31]
Quantitative buffy coat	10^3	[31]
Inoculation to mice	*Borrelia vivantes* in culture	[88]

qPCR: quantitative polymerase chain reaction

Tableau 2. Résumé des valeurs de sensibilité et spécificité des différents tests tréponémiques

Table 2. Summary of sensitivity and specificity values of the various treponemal tests

	Primary phase	Secondary phase	Latency phase	Late phase	Specificity
FTA-ABS	84 (70-100)	100	100	96	97 (94-100)
TPHA	76 (69-90)	100	97 (97-100)	94	99 (98-100)
TPPA	84-100				99.5
CMIA	99.38				99
ELISA	98.12				99

Tableau 3. Score diagnostique de la leptospirose

Table 3. Diagnostic score for leptospirosis

Part A: clinical data	**Score**
Headaches	2
Fever	2
Temperature >39°C	2
Bilateral conjunctivitis	4
Meningitis	4
Muscle pain, mainly on the calves	4
Conjunctivitis + meningism + muscle pain	10
Clinical jaundice	1
Albuminuria	2
Part B: epidemiological factors	**Score**
Rainfall	5
Contact with a polluted environment	4
Contact with an animal	1
Part C: bacteriology	
Leptospira isolated from culture	**Confirmed diagnosis**
Positive serology	
Positive IgM ELISA test; positive serum agglutination test; high titer microscopic agglutination test	15
Increased serological microscopic agglutination test titer	25