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Intended category: Systematic review

Unconventional diagnostic tests for Lyme borreliosis: a systematic review

Alice Raffetin ^{1,2}, Aurélie Saunier ³, Kevin Bouiller ^{4,5}, Pauline Caraux-Paz ¹, Carole Eldin ⁶, Sébastien Gallien ⁷, Romain Jouenne ⁸, Anna Belkacem ¹, Jérôme Salomon ⁹, Olivier Patey ¹, Emilie Talagrand-Reboul ¹⁰, Benoît Jaulhac ^{2,10,11}, Antoine Grillon ^{10,*}

¹⁾ *Department of Infectious Diseases, Centre Hospitalier Lucie-et-Raymond-Aubrac, Villeneuve-Saint-Georges, France*

²⁾ *European Study Group for Lyme Borreliosis (ESGBOR)*

³⁾ *Department of Internal Medicine and Infectious Diseases, Centre Hospitalier Périgueux, Périgueux, France*

⁴⁾ *Department of Infectious Diseases, Centre Hospitalier Universitaire Besançon, Besançon, France*

⁵⁾ *UMR CNRS 6249 Chrono-Environnement, Université de Bourgogne Franche-Comté, Besançon, France*

⁶⁾ *Centre Hospitalier Universitaire Aix-Marseille, IRD, AP-HM, SSA, VITROME, IHU-Méditerranée Infection, Marseille, France*

⁷⁾ *Department of Clinical Immunology, Centre Hospitalier Universitaire Henri-Mondor, Créteil, France*

⁸⁾ *Department of Internal Medicine, Centre Hospitalier Lucie-et-Raymond-Aubrac, Villeneuve-Saint-Georges, France*

⁹⁾ *Department of Infectious Diseases, Centre Hospitalier Universitaire Raymond-Poincaré, Garches, France*

¹⁰⁾ *EA 7290 Virulence Bactérienne Précoce, Université de Strasbourg, Centre Hospitalier Régional Universitaire de Strasbourg, Fédération de Médecine Translationnelle, Groupe Borréliose de Strasbourg, Strasbourg, France*

¹¹⁾ *Centre National de Référence des Borrelia, CHRU Strasbourg, Strasbourg, France*

* Corresponding author. A. Grillon, EA 7290 Virulence Bactérienne Précoce, CHRU de Strasbourg, Fédération de Médecine Translationnelle de Strasbourg, 3, rue Koeberlé, 67000 Strasbourg, France
Strasbourg, France. Tel: +33(0)369 551 582; email: antoine.grillon@chru-strasbourg.fr

1 **ABSTRACT (316 words)**

2 *Background:* Lyme borreliosis (LB) diagnosis currently relies mainly on serological tests and
3 sometimes polymerase chain reaction or culture. However, other biological assays are being
4 developed to try to improve *Borrelia*-infection diagnosis and/or monitoring.

5 *Objectives:* To analyze available data on these unconventional LB-diagnostic assays through a
6 systematic literature review.

7 *Methods:* We searched PubMed and Cochrane Library databases according to the PRISMA-DTA
8 method and the Cochrane Handbook for Systematic Reviews of Interventions.

9 We analyzed controlled and uncontrolled studies (published 1983–2018) on biological tests for adults
10 to diagnose LB according to the European Study Group for Lyme Borreliosis or the Infectious
11 Diseases Society of America definitions, or strongly suspected LB.

12 Two independent readers evaluated study eligibility and extracted data from relevant study reports; a
13 third reader analyzed paper full-texts to resolve disagreements.

14 The quality of each included study was assessed with the QUADAS-2 evaluation scale.

15 *Results:* Forty studies were included: 2 meta-analyses, 25 prospective, controlled studies,
16 5 prospective, uncontrolled studies, 6 retrospective, controlled studies, and 2 case reports. These
17 biological tests assessed can be classified as: (i) proven to be effective at diagnosing LB and already
18 in use (CXCL-13 for neuroborreliosis), but not enough yet standardized; (ii) not yet used routinely,
19 requiring further clinical evaluation (CCL-19, OspA and interferon- α); (iii) uncertain LB-diagnostic
20 efficacy because of controversial results and/or poor methodological quality of studies evaluating them
21 (lymphocyte transformation test, interferon- γ , ELISPOT); (iv) unacceptably low sensitivity and/or
22 specificity (CD57+ NK cells and rapid diagnostic tests); and (v) possible only for research purposes
23 (microscopy and xenodiagnoses).

24 *Discussion:* QUADAS-2 quality assessment demonstrated high risk of bias in 25/40 studies and
25 uncertainty regarding applicability for 32/40, showing that in addition to polymerase chain reaction and
26 serology, several other LB-diagnostic assays have been developed but their sensitivities and
27 specificities are heterogeneous and/or under-evaluated or unassessed. More studies are warranted to
28 evaluate their performance parameters. The development of active infection biomarkers would greatly
29 advance LB diagnosis and monitoring.

30 **Key words:** Lyme borreliosis; Diagnostic tests; *Borrelia burgdorferi*; Clinical assessment

Introduction

Lyme borreliosis (LB) is the most commonly reported vector-borne disease transmitted by ticks in the United States and Western Europe [1–3]. It is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex. The greater variety of human pathogenic genospecies in Europe (mainly *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) than the United States explains the wider range of clinical pictures [4]. The most common clinical manifestations in Europe are erythema migrans (EM) and Lyme neuroborreliosis [4]. EM (early localized stage) is pathognomonic and does not require further exploration [5]. The early (<6 months) and late (>6 months) disseminated LB stages mainly affect skin, nervous system and joints, and rarely heart and eyes [4]. Some subjective symptoms (asthenia, polyalgia, cognitive complaints, etc.) may also be present at all stages and may persist after well-conducted treatment, with the latter being called post-treatment Lyme disease syndrome (PTLDS) [4,6–8]. Reliable diagnostic tests are needed to dissociate active from inactive LB, and from other diseases that share some similar clinical manifestations.

LB diagnosis relies mainly on serological tests and polymerase chain reaction (PCR). Their sensitivities and specificities at disease onset may be less reliable and depend on the anatomical site sampled [9]. A recent meta-analysis [10] showed that LB serological tests had heterogeneous sensitivities, depending on the disease stage: 50% (95% CI=40%–61%) for localized EM, 77% (95% CI=67%–85%) for Lyme neuroborreliosis, 97% (95% CI=94%–99%) for acrodermatitis chronica atrophicans (ACA), 96% (95% CI=93%–100%) for Lyme arthritis (LA), and 73% (95% CI=53%–87%) for unspecified LB. Specificity was ~95% for investigations with healthy controls and 80% for cross-sectional studies. All the recent European guidelines recommend a two-tier test strategy for serological testing [11–16]. *Borrelia* PCR sensitivity varies according to the disease stage and its anatomical location: 65%–90% for cutaneous biopsies of EM and ACA [12,17–20], 50%–85% for joint samples [20–22] and 10%–30% for cerebrospinal fluid (CSF) [20,23,24]. The usefulness of *Borrelia burgdorferi* sensu lato PCR on urine and blood has not yet been proven [4,24], even if PCR techniques are improving [25–27]. PCR is currently recommended for ambiguous LA (synovial fluid/tissue) and ACA (skin biopsies) [11–16].

Beyond those recommended tests, researchers have tried to develop alternative assays to serology, PCR and culture to improve diagnosis and monitoring of early and late LB, even for cases

with non-specific symptoms. Several studies on these alternative tests have been published and it is often difficult to make a well-thought-out decision about them.

This literature review was undertaken to analyze available data on unconventional tests to diagnose LB, and to make physicians aware of their characteristics and limitations.

Methods

This literature review was based on screening of the PubMed and Cochrane Library databases, according to the PRISMA-DTA method and the Cochrane Handbook for Systematic Reviews of Interventions [28–32]. Two independent readers (content-area experts AR and AG) assessed study eligibility by reviewing titles and abstracts first, and, if relevant, by examining the full-texts. AR and AG extracted data from retained reports. They knew the authors' names, institutions, publication journal and results when they applied the eligibility criteria. When they disagreed about a study's relevance, a third expert (BJ) was asked to analyze the full-text. The quality of each included study was assessed with the QUADAS-2 evaluation scale (Fig. 3 and 4, Suppl table 4) [33].

We included studies published from 1983 to 2018, conducted on adult humans, with control groups or not, randomized or not and in which LB cases were defined according to the European Study Group for Lyme Borreliosis (ESGBOR) or the Infectious Diseases Society of America (IDSA) criteria [4,34], or cases reported as strongly clinically suspected LB but without microbiological documentation. The latter choice was made to avoid excluding studies describing diagnostic tools for ambiguous LB cases with no microbiological documentation. Meta-analyses and case series were included; reports with only abstracts available, congress communications and letters were excluded, as were studies evaluating serological tests and PCR. When Cochrane Handbook for Systematic Reviews of Interventions were closely respected, studies already included in those meta-analyses were excluded and all subsequently published studies were included. No language restriction was applied.

First, we conducted a free search to get an overview of all the available diagnostic tests using the following terms: "Lyme borreliosis" or "Lyme disease" and "diagnostic tests" or "diagnosis". Then, the following MeSH terms were used: "Lyme borreliosis" or "Lyme disease", and "diagnostic tests", "CXCL-13", "apolipoprotein B-100", "CCL-19", "lymphocyte transformation test", "interferon", "CD57",

“xenodiagnosis”, “membrane protein OspA”, “rapid diagnostic test”, “focus-floating microscopy”, “dark-field microscopy”, “silver-stain microscopy” and “microscopy”. We could not conduct a meta-analysis for any of the tests detailed below because study quality was too heterogeneous and too few publications reported test performances (sensitivity/specificity).

Results

Forty studies were included: 2 meta-analyses, 25 prospective, controlled studies, 5 prospective, uncontrolled studies, 6 retrospective, controlled studies, and 2 case reports (Fig. 1).

Tests exploring inflammatory or autoimmune responses

CXCL-13

The C-X-C motif chemokine ligand-13 (CXCL-13), produced by antigen-presenting cells, selectively attracts B lymphocytes [35–37]. During Lyme neuroborreliosis, CXCL-13 released into the CSF by resident mononuclear cells [38] triggers B-cell migration into the CSF, resulting in the characteristic B-cell–enriched CSF pleocytosis [39].

Database searches identified 117 reports. After removing duplicates, 52 publications were screened. Nine full-text articles were assessed for eligibility. Four studies were finally retained: two meta-analyses [40,41], one retrospective, controlled study [42] and one prospective, uncontrolled study [43]. QUADAS-2 quality assessment demonstrated low risk of bias and low applicability concerns for both meta-analysis [40–41], unclear risk of bias and uncertainty regarding applicability for the retrospective controlled study [42], and high risk of bias and high concerns regarding applicability for the prospective uncontrolled study [43].

As demonstrated previously for Lyme neuroborreliosis, the CSF CXCL-13 concentration is high [40–44]. CXCL-13 is detectable from days to weeks before antibodies in CSF and its level rapidly declines after antibiotics, suggesting its potential role as a therapeutic marker [40–43,45,46]. For the

meta-analyses, including 961 Lyme neuroborreliosis patients and 3,282 controls, the pooled CSF CXCL-13 sensitivity range was 89%–97% and its pooled specificity was 96% (95% CI=92%–98%) [40,41]. The specific antibody index recommended to diagnose definite Lyme neuroborreliosis had 86% (95% CI=63%–95%) sensitivity and 94% (95% CI=85%–97%) specificity [47,48].

Optimal cut-off values were determined to be 162 pg/mL in one meta-analysis [41] and 131 pg/mL using the Luminex platform versus 259 pg/mL for enzyme-linked immunosorbent assay (ELISA) in a retrospective, controlled study [42]. Cut-off determination is a major issue for CXCL-13 use to diagnose Lyme neuroborreliosis, since no official recommendation has yet been advanced. In addition, few available kits are marked European Community-in vitro diagnosis (European conformity authorization). Each laboratory has to determine its own threshold, which might lead to different interpretations.

Moreover, the CSF CXCL-13 level is elevated in other central nervous system (CNS) disorders, like neurosyphilis, cryptococcosis, trypanosomiasis, viral meningitides, meningoencephalitides and CNS lymphoma [41,42]. Attention should also be paid to those pathologies when interpreting the results.

CXCL-13 seems to be a good add-on marker, with high sensitivity and specificity to diagnose untreated, acute Lyme neuroborreliosis. It could be useful for patients with early typical clinical symptoms, CSF pleocytosis and a negative specific antibody index, and for cases of reinfection. It remains to be evaluated for late Lyme neuroborreliosis.

CCL-19

In their recent prospective controlled study, Aucott *et al.* described the T-cell chemokine CCL-19 as a potential immunological risk factor of PTLDS [49]. Seventy-six patients with physician-documented EM were followed for 1 year post-antibiotics: 11/76 (14.5%) developed PTLDS-compatible symptoms [49]. Persistently high 1-year CCL-19 levels were only observed in PTLDS patients.

QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability in this study [49].

CCL-19 could be of potential interest to screen patients at risk of PTLDS. More studies are

warranted.

Apolipoprotein B-100 (ApoB-100)

We identified four papers through database searches. After removing duplicates, two prospective controlled studies were screened, their full-texts assessed for eligibility and, finally, included for the review [50,51]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability in these two studies [50,51]. Crowley *et al.* identified ApoB-100 as a target of T-cell responses in 14/37 (38%) LA patients [50]. Moreover, 5/37 (13%) LA patients had autoantibodies directed against ApoB-100, an autoantigen involved in refractory LA [50,52,53].

To date, no study has used ApoB-100 as a diagnostic tool for LB. It cannot be used in routine practice.

Tests exploring cellular immunity

Lymphocytes-transformation test

A lymphocyte-transformation test evaluates the lymphoproliferative response of peripheral blood mononuclear cells to *B. burgdorferi* antigens. The results are expressed as a stimulation index (SI), with SI>10 considered positive and SI<10 negative [54].

Among 38 publications identified through database searches, 24 were screened, after removing duplicates, and 14 full-texts were assessed for eligibility. Ten studies were finally retained for the review: eight prospective, controlled studies [54–61], one prospective, uncontrolled study [62] and one retrospective, controlled study [63]. QUADAS-2 quality assessment demonstrated low risk of bias and uncertainty regarding applicability for two prospective controlled studies [54,61], unclear risk of bias and uncertainty regarding applicability for six prospective studies [55-60], and high risk of bias and high applicability concerns for the two others [62,63].

Only four provided sensitivities and specificities for any stage with combined respective ranges of 45%–89.4% and 33%–98.7% [54,59–61]. Only one study reported data on late LB with 45% (95% CI=30–60) sensitivity and 95% (95% CI=87–99) specificity [54].

Among the 10 studies retained, for early LB, mean SIs were >10 in two [60,61], <10 in one [58] and not mentioned in seven [54–57,59,62–63]; while for late LB, mean SIs were >10 in five [54,55,57,60,61], <10 in two [58,63] and not mentioned in three [56,59,62]. For controls (healthy subjects or other diseases), mean SIs were <10 in five studies [54,55,57,58,63], >10 in one [61], and not mentioned in four [56,59,60,62]. Exposed healthy subjects (forest workers, *Borrelia* laboratory workers) had high seroprevalence, with mean SI>10 in one report, <10 in two [54,59] and not given in seven [55–58,61–63]. Fig. 2 summarizes these findings.

After antimicrobials, SI declined in three studies [56,59,60] and was not reported in the other seven. Zoschke *et al.* noted the late persistence of lymphocyte proliferation post-antibiotics [61].

Concordance with serological tests was found in four studies, two each performed on late LB or at all stages [55,57,60,63], and discordance in the six others conducted at all stages combined [54,56,58,59,61,62].

Those 10 studies had numerous biases. First, lymphocytes-transformation tests were standardized only in two investigations that used lymphocytes-transformation test– memory lymphocyte immunostimulation assay (MELISA) [59,62]. Second, LB was defined with criteria other than those of ESGBOR or IDSA in six [4,54–56,58,59,62]. Third, the international SI cut-off to interpret results was not respected in four [59,60,62,63] and not mentioned in five [55–58,61], meaning that nine studies do not enable any conclusion to be drawn. Finally, the numbers of patients with SI>10 were not given in six studies [55–57,59,62,63] that reported only mean SIs.

At present, no guidelines recommend using lymphocytes-transformation tests because of their lack of specificity [11,13,14,16,64,65]. Studies on lymphocytes-transformation tests yielded heterogeneous results and mixed quality, without clear sensitivity and specificity. They do not permit conclusions to be drawn about the usefulness of lymphocytes-transformation tests to diagnose LB, including for rare seronegative patients.

Interferon-γ (IFN-γ) ELISPOT

IFN-γ release into the blood is assessed to explore T-cell activation after *B. burgdorferi* sensu lato stimulation.

Among 73 publications identified by database searches, 39 were screened, after removing

duplicates, and 16 full-texts were assessed for eligibility. Eight studies were finally retained for the review: seven prospective, controlled studies [66–72] and one retrospective, controlled study [73]. All but one of them [73] respected ESGBOR or IDSA clinical and microbiological definitions of LB. QUADAS-2 quality assessment demonstrated low risk of bias and low applicability concerns for one study [67], low risk of bias and uncertainty regarding applicability for 3 studies [69,71,72], unclear risk of bias and unclear applicability concerns for one study [70], unclear risk of bias and high applicability concerns for 2 studies [66,68], and high risk of bias and high applicability concerns for one retrospective controlled study [73].

All eight studies showed that IFN- γ was associated with exposure to *Borrelia*. Circulating IFN- γ was elevated in patients with early LB, late LB or PTLDS and seropositive, asymptomatic subjects. It was significantly less elevated in healthy seronegative subjects in all studies ($p < 0.01$). Moreover, IFN- γ concentrations did not parallel *Borrelia* activity in six studies [66–68,73]. Sensitivities during early LB ranged from 36% to 69% in 2/3 studies [69,70] and only one study provided specificity (~82%) [70]. Callister *et al.* demonstrated that combining positive *Borrelia*-specific C6-peptide–ELISA and IFN- γ results increased sensitivity to 83% [69]. Only one study on late LB reported 84% sensitivity and 96% specificity for the iSpot Lyme [68]; four studies failed to mention any test performance [66,71–73].

Only one study found an IFN- γ decrease after antibiotics [69]. No study demonstrated any concordance between serological test results and circulating IFN- γ .

The main limitation of IFN- γ ELISPOT appears to be its wide variability. First, the cut-off to interpret results was not given in 6/8 studies. In the two studies indicating a threshold, only two patients with anaplasmosis served as controls [69] or a receiver operating characteristics curve with 105 controls was used [68]. Second, specificity varied markedly according to the control population chosen. Third, among the studies, different *Borrelia* species were used to assess the performance of the circulating IFN- γ level: two did not mention the species [68,69], three used a *B. afzelii* strain (ACA-I) [66,71,73], two used a *B. garinii* strain (Ip90) [70,72], and the last used the *B. burgdorferi* strain B31 combined with antigens specific to each of 4 species (*B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. bavariensis*) [67]. Pertinently, the *B. burgdorferi* B31 lysate cross-reacts with other spirochetes [67,71]. Finally, no standardized test has been validated at present: notably, three studies had demonstrated lack of reproducibility for LB [67–69].

IFN- γ secretion has been also studied in other tissues/biological fluids (skin biopsies, synovial

fluid, CSF) to assess its role in the inflammatory response to *Borrelia* but it was not considered a diagnostic tool [66,71,72,74–78].

At present, IFN- γ ELISPOT cannot be used in routine practice because its role has only been established as a witness of *Borrelia* exposure. Specificity, cut-offs and reproducibility require more investigations.

IFN- α

Among 24 articles identified through database searches, 12 were screened, after removing duplicates, and three full-texts were assessed for eligibility. Finally, only one prospective comparative study was included [79]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability for this study [79].

Jacek *et al.* [79] demonstrated higher IFN- α activity in PTLDS patients, which suggests the existence of an immune-related disease process in patients with persistent symptoms, perhaps contributing to ongoing symptoms. The authors also reported that β -lactam therapy did not modulate the activated immune response.

More studies are warranted to assess the reliability of this test.

CD57+ natural killer (NK) cells

CD57 is an NK-cell marker but is also expressed on other cells. The method is based on an immunophenotyping technique.

Among 10 articles identified through database searches, five were screened, after removing duplicates, and three full-texts were evaluated for eligibility. Two prospective controlled studies were finally retained for the review [80,81]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability in these two studies [80,81].

Stricker *et al.* reported lower CD57+ lymphocyte counts (mean=30 \pm 16 cells/ μ L) in 73 patients with late LB before starting antibiotics; the lymphocyte count increased after treatment (mean=66 \pm 39 cells/ μ L) [80]. That study had several biases: low numbers of patients included, bias of presentation of the results, no repeated test on the same patients to follow CD57-expression kinetics, no validated

control groups, and no clear case definition and treatment. Marques *et al.* found no significant difference ($p=0.68$) among nine PTLDS patients, the 12 cured of LB and nine healthy volunteers [81]. The authors did not report CD57 results before antibiotics (all were already treated or healthy), and very few patients and controls were tested. CD57-marker specificity for LB is probably low, because low CD57 levels were also found in patients with numerous autoimmune diseases, infections or cancers [82].

CD57 measurement does not seem useful, even after antibiotics or for persistent symptoms. To our knowledge, no study has provided this test's performance (sensitivity/specificity).

Microbiological tests

Xenodiagnosis

Xenodiagnosis, an experimental test used in animal models of LB, uses uninfected ticks to detect low-level infection, even after antibiotics.

Our database searches identified 22 papers, among which 11 were screened, after removing duplicates, and five full-texts were assessed for eligibility. Only one prospective, controlled study was included [83]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability in this study [83].

This study investigates the feasibility of xenodiagnosis in humans: 2/26 patients treated for LB had positive xenodiagnoses post-treatment (one control EM subject and one PTLDS). Xenodiagnosis was positive only by DNA detection after PCR of tick cultures or the tick itself. No spirochetes were detected. Bockenstedt and Radolf questioned whether *Borrelia*-DNA detection was sufficient to confirm the diagnosis [84].

Currently available xenodiagnosis performance is insufficient to assess its diagnostic usefulness.

Outer surface protein-A (OspA) detection

OspA, a *Borrelia burgdorferi* lipoprotein that binds to its receptor TROSPA in the tick midgut, is required to infect the tick.

The two identified studies were included in this review [85,86]. QUADAS-2 quality assessment demonstrated low risk of bias and uncertainty regarding applicability in one study [85] and unclear risk of bias and uncertainty regarding applicability in the other study [86].

In their prospective, uncontrolled study on only three patients, Cheung *et al.* described a liquid chromatography–tandem mass-spectroscopy method to directly detect OspA in sera from early-infected patients (EM) [85]. That technique detected low OspA levels in the three patients' sera. Although the proof-of-concept merits further examination, neither sensitivity nor specificity was reported. In a prospective controlled study on 140 patients, Magni *et al.* described a nanotrap technology-based method to detect the OspA carboxyl-terminus domain in urine samples [86]. Although the authors reported excellent sensitivity (100%) and specificity (100%) before antibiotics, those results must be confirmed by other studies.

Upcoming investigations on this technique should be of interest.

Direct microscopy of human tissues

Database searches identified 81 studies. After removing duplicates, 29 publications were identified, on “dark-field microscopy”, 18 on “silver staining” and 2 on “focus-floating microscopy”. All papers were screened. Nine full-texts were assessed for eligibility and seven were retained: two prospective, controlled studies [87,88], two prospective, uncontrolled studies [89,90], one retrospective, controlled study [91] and two case reports [92,93]. QUADAS-2 quality assessment demonstrated low risk of bias and unclear applicability concerns for one study [87], unclear risk of bias and uncertainty regarding applicability for two studies [88,91], and unclear risk of bias and high applicability concerns in four studies [89,90,92,93].

Electron microscopy, silver staining with light microscopy and focus-floating microscopy enabled spirochete detection in various samples [87,91-94]. Nevertheless, it has limited clinical utility because of the apparent *Borrelia* scarcity in mammalian tissues and its 41% sensitivity [90], and the time required for analysis [95,96]. False-positive results have also been reported [88].

Laane *et al.* published an uncontrolled study describing a modified dark-field microscopy technique, called LM-method, which identified structures claimed to be *Borrelia* in 21/32 (66%) patients' blood samples among those with non-specific symptoms [97]. Using the same methodology

and including a control group, Aase *et al.*'s findings refuted Laane *et al.*'s results, i.e., the LM-method was positive for 85% of the 41 healthy controls and was unable to detect *Borrelia* in the 21 positive controls with five *B. afzelii* per 100 red cells [98]. The LM-method should not be used for LB diagnosis.

At present, *Borrelia* detection by microscopy can only be used for research purposes. Inclusion of positive/negative controls and the microscopy reader's expertise are necessary.

Rapid diagnosis tests (RDTs)

Among the 32 articles identified by database searches, 16 were screened, after removing duplicates, and two full-texts were assessed for eligibility. Two retrospective, controlled studies were included in the review [99,100]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding applicability in these two studies [99,100].

Two RDT categories are available: immunochromatography-based methods and microfluidic-based point-of-care tests.

Immunochromatography-based methods are commercially available at pharmacies throughout Europe for patient self-use. These RDTs require a drop of blood and can be done with minimal training. Only one study assessed RDT performances and no regulations are available [99]. Smit *et al.* used two commercially available RDTs with sensitivities of 26% and 32%, and specificities of 85% and 88%, values that are much lower than those of laboratory-based diagnostic tools [99]. Their results do not support RDT use for diagnostic strategies. Neither European nor American guidelines recommend using RDTs.

A microfluidic-based point-of-care test that can be performed in 15 minutes is not yet commercially available [100]. This test's performance was comparable to that of the laboratory-based C6-peptide–ELISA with 84% sensitivity and 92% specificity. However, it was shown to be significantly less sensitive than a recombinant (VlsE+DbpA+OspC) ELISA [100].

RDTs for LB remain experimental, requiring further investigation.

Discussion

Summary of evidence

In addition to polymerase chain reaction and serology, several other LB-diagnostic assays have been

developed but their sensitivities and specificities are heterogeneous and/or under-evaluated or unassessed.

Study Strengths and weaknesses

Included studies

We included studies defining LB cases according to ESGBOR or IDSA criteria [4,34] and avoided excluding studies describing diagnostic tools for ambiguous case lacking microbiological documentation. That choice represents a strength of our study as it attempts to identify which test might perform well at diagnosing such ambiguous cases.

QUADAS-2 quality assessment demonstrated high risk of bias in 25/40 studies and uncertainty regarding applicability for 32/40 (cf. Fig. 3, Fig. 4, and Supplementary Table S4) [30,33]. Most authors failed to report the sensitivities or specificities of the evaluated diagnostic tests, indicating that the studies on alternative diagnostic tests were not methodologically well-conducted.

Review process

Two databases were screened, without search filters or language restriction. Consensus was reached for the selection of included studies. We report all the QUADAS-2 domains to evaluate each included study (Supplementary Table S4, Fig. 3 and Fig. 4). For all the included studies, Supplementary Tables S1 to S3 report: study design, patient-inclusion criteria, stage, controls, other tests; main results including reported sensitivities and/or specificities and limitations. A meta-analysis could not be undertaken because of too extensive heterogeneity among included studies and the small numbers of studies for some tests.

Conclusion

Studies on unconventional biological LB-diagnostic strategies can be classified as follows: (i) tests proven to diagnose LB accurately and already used routinely (e.g. CXCL-13 for Lyme neuroborreliosis), which, nonetheless, remain to be standardized; (ii) tests not yet used in routine practice requiring clinical evaluation (e.g. CCL-19, OspA and IFN- α); (iii) tests with uncertain abilities to diagnose LB because of controversial results and/or poor methodological quality of the studies (e.g. lymphocytes-transformation tests, IFN- γ ELISPOT); (iv) tests with unacceptably low sensitivity and/or

specificity (e.g., CD57+ NK-cells and RDTs); and (v) tests possible only for research purposes at present (e.g., microscopy and xenodiagnoses).

For early LB, CSF CXCL-13 is the only test whose performances allow its development for routine practice. For late LB, satisfactory performance has not yet been demonstrated for any test. IFN- γ was only associated with *Borrelia* exposure. CCL-19, ApoB-100 and IFN- α might be of potential interest to screen patients at risk for PTLDS.

In addition to PCR and serology, several other diagnostic assays have been developed for LB but their performances are heterogeneous, underevaluated and/or unassessed. More studies are warranted to evaluate their sensitivities, specificities and reproducibilities. Identification of biomarkers for active *Borrelia* infection would represent a major advancement in LB diagnosis and monitoring.

Conflicts of interest: The authors declare no conflict of interest related to this work.

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Figure Legends

Fig.1. Flow chart of article selection for the systematic review.

Fig.2. Pooled Lymphocyte-transformation-test results of the included studies for Lyme borreliosis (LB), according to different groups of patients. SI, stimulation index.

Fig.3. Proportions of studies with low, high or unclear risk of bias (%), according to the QUADAS-2 domains (Quality Assessment of Diagnostic Accuracy Studies-2)

Fig.4. Proportions of studies with low, high or unclear concerns regarding applicability (%), according to the QUADAS-2 domains (Quality Assessment of Diagnostic Accuracy Studies-2)

Fig. 1

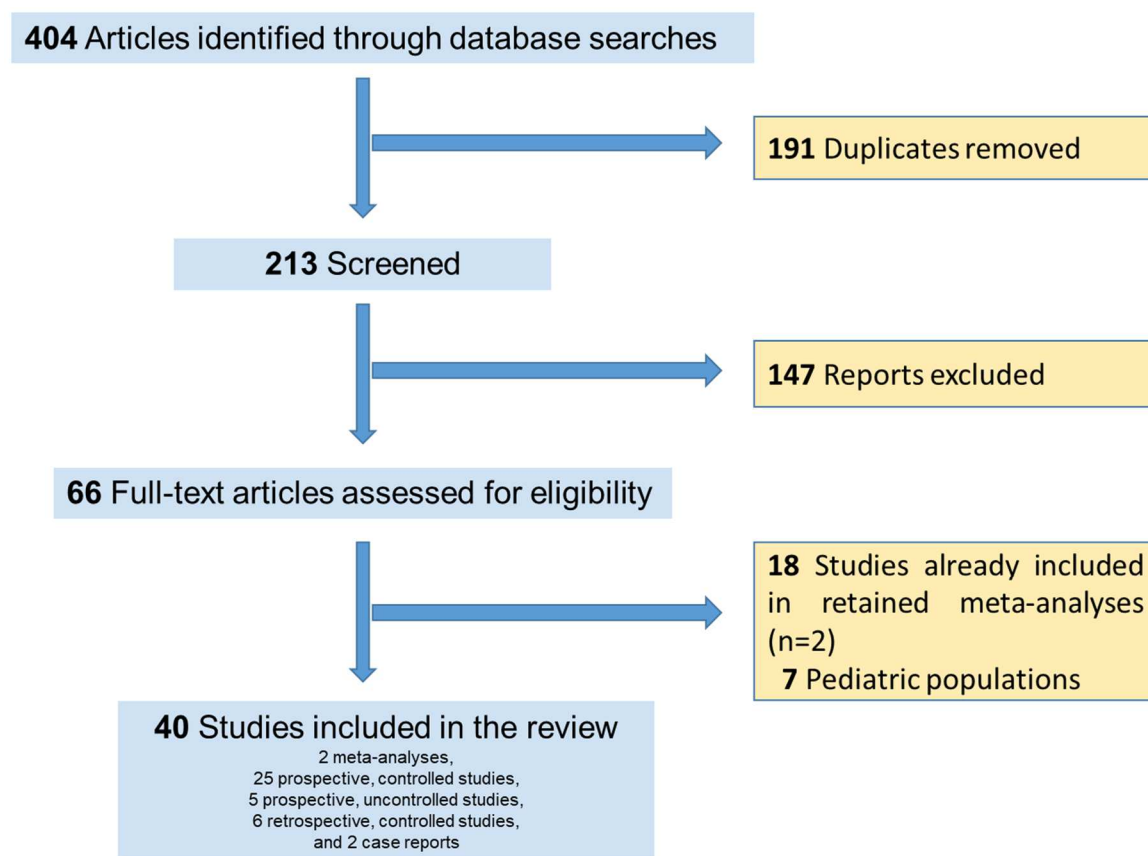


Fig. 2

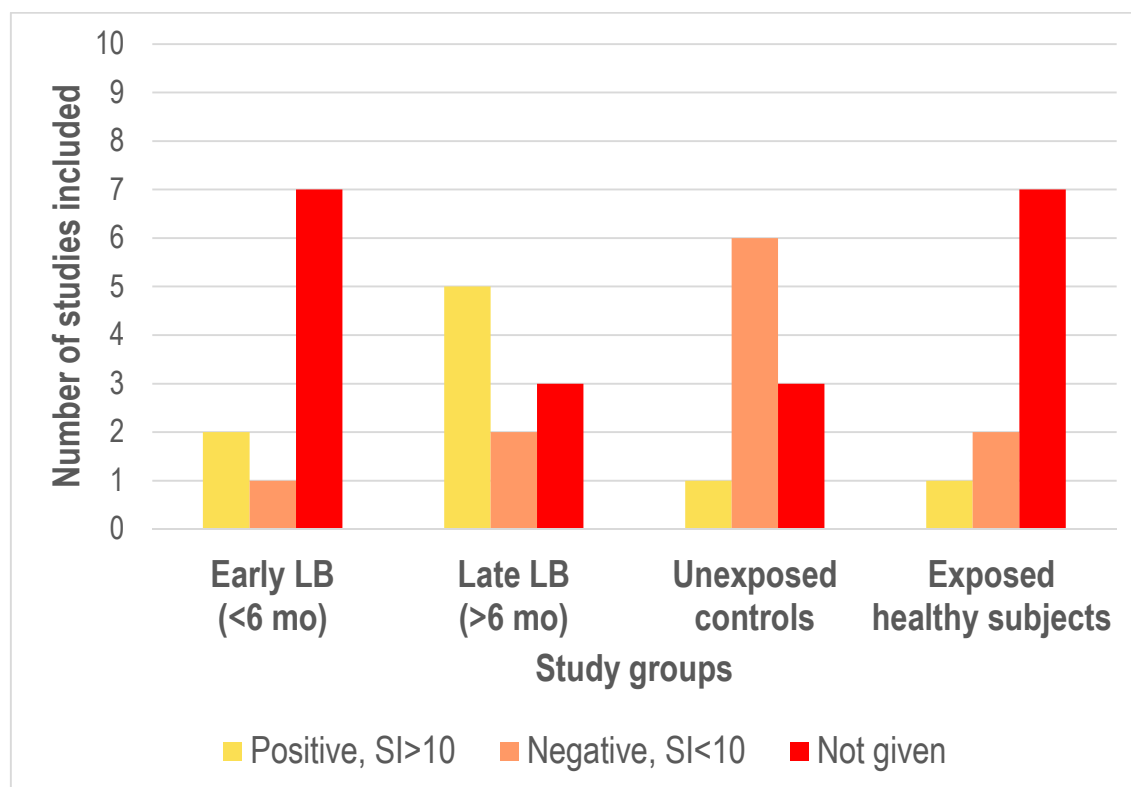


Fig. 3

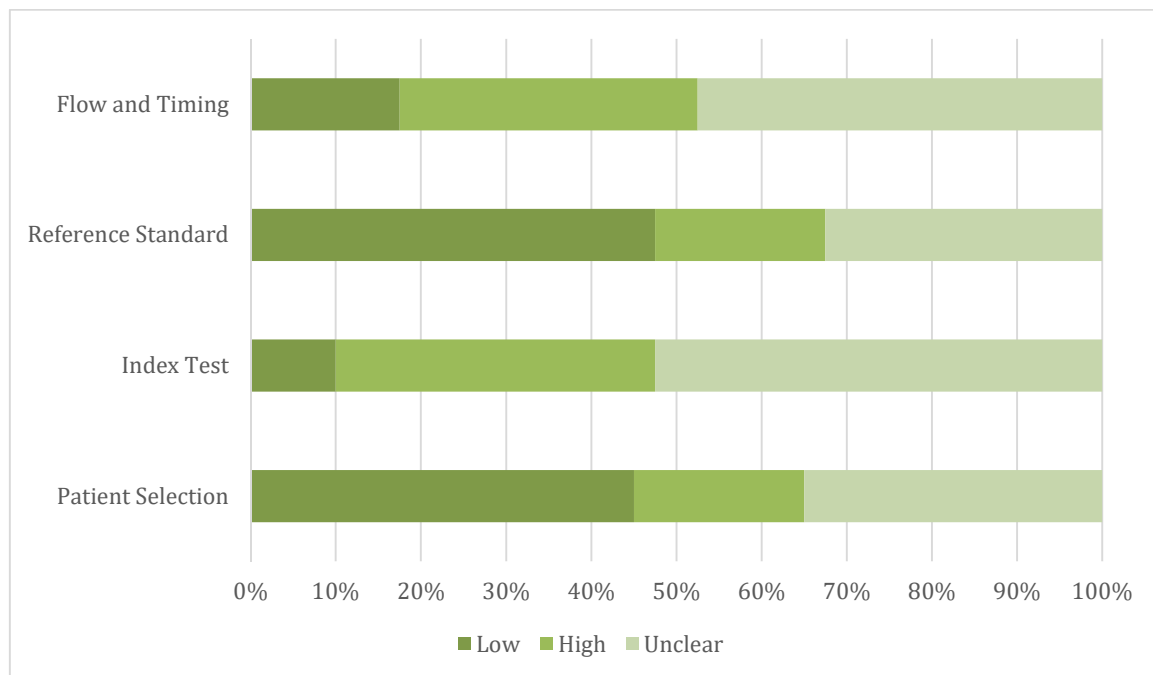


Fig. 4

