MINIREVIEW

Microbiological and serological diagnosis of Lyme borreliosis

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Received 6 June 2006; revised 4 July 2006; accepted 4 July 2006.
First published online 8 September 2006.

DOI:10.1111/j.1574-695X.2006.00139.x

Editor: Willem van Leeuwen

Keywords
Lyme borreliosis; *Borrelia*; serological diagnosis; microbiological diagnosis; immunoblot; PCR.

Abstract

In Europe, Lyme borreliosis is caused by *Borrelia burgdorferi* sensu stricto, *B. afzelii*, *B. garinii* and the recently described species *B. spielmanii*. For the development of diagnostic tools, the heterogeneity of the causative agents must be considered. The serological diagnosis should follow the principle of a two-step procedure: a sensitive enzyme-linked immunosorbent analysis as the first step, followed by immunoblot (IgM and IgG) if reactive. The sensitivity and standardization of immunoblots have been enhanced by the use of recombinant antigens instead of whole cell lysates. Improved sensitivity has resulted from the use of recombinant proteins primarily expressed *in vivo* (e.g. VlsE) and the combination of homologous proteins from different strains (e.g. DbpA). At present, detection rates for serum antibodies are 20–50% in localized, 70–90% in disseminated early and nearly 100% in late disease. Detection of the borreliae by culture or PCR should be confined to specific indications. The best results are obtained from skin biopsies (50–70% with culture or PCR) and synovial tissue or fluid (50–70% with PCR). Cerebrospinal fluid is positive in only 10–30%. Methods that are not recommended for diagnostic purposes include antigen tests in body fluids, PCR of urine and lymphocyte transformation tests.

Introduction

Lyme borreliosis is a multisystem disease involving many organs, such as the skin, nervous system, joints and heart (Steere, 1989; Pfister *et al.*, 1994; Stanek & Strle, 2003). This condition is the most frequent tick-borne disease in the northern hemisphere. As a result of the diversity of clinical symptoms, Lyme borreliosis is often considered as a differential diagnosis. Examinations for antibodies against *Borrelia burgdorferi sensu lato* are thus in high demand, and are among the most frequently requested serological tests in microbiological laboratories. Microbiological diagnosis in European patients must consider the heterogeneity of the agents of Lyme borreliosis in Europe.

Heterogeneity of the agents of Lyme borreliosis in Europe and *its* impact on microbiological diagnosis

In Europe, Lyme borreliosis is primarily caused by the three species *Borrelia burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*. In contrast, *B. burgdorferi* sensu stricto is the only human pathogenic species in the USA (Wang *et al.*, 1999b). These three human pathogenic species comprise at least seven OspA serotypes in Europe (Table 1) (Wilske *et al.*, 1993b). Skin isolates primarily comprise *B. afzelii* (OspA type 2), especially those from patients with acrodermatitis chronica atrophicans, a chronic skin disease not present in the USA (Canica *et al.*, 1993; Wilske *et al.*, 1993b; Ohlenbusch *et al.*, 1996). Isolates from cerebrospinal fluid (CSF) and ticks are heterogeneous with a predominance of *B. garinii* (van Dam *et al.*, 1993; Eiffert *et al.*, 1995; Wilske *et al.*, 1996a, b). Sequence analysis of PCR *ospA* amplicons from the synovial fluid of Lyme arthritis patients reveals heterogeneity (Eiffert *et al.*, 1998; Vasiliu *et al.*, 1998), whereas other studies have found mainly *B. burgdorferi* sensu stricto using PCR based on the 5S/23S rRNA gene intergenic spacer region (Lunemann *et al.*, 2001) or the flagellin gene (Jaulhac *et al.*, 1996, 2000). Very recently, a new human pathogenic species, *B. spielmanii*, has been delineated, which has been detected previously in a skin biopsy from a patient with erythema migrans from the Netherlands (Wang *et al.*, 1999a; Richter *et al.*, 2006). *Borrelia spielmanii* has been found in four patients with erythema migrans from Germany,

Table 1. Distribution of species of *Borrelia burgdorferi* sensu lato and OspA types in European isolates from ticks, cerebrospinal fluid (CSF), skin and synovial fluid specimens (Eiffert *et al.*, 1998; Vasiliu *et al.*, 1998; Wilske *et al.*, 1996b)

Species	OspA type	Ticks (%) ($n = 90$)	CSF (%) ($n = 43$)	Skin (%) ($n = 68$)*	Synovial fluid (%) ($n = 20$)†
B. burgdorferi sensu stricto	1	20	19	6	33
B. afzelii	2	9	12	84	29
B. garinii‡	3–7	71	69	10	38

*Source of skin specimens known in 46 patients (30 cases of erythema migrans, with 1, 26, 1 and 2 cases infected with OspA types 1, 2, 4 and 6, respectively; 16 cases with acrodermatitis chronica atrophicans, with one and 15 cases infected with OspA types 1 and 2, respectively.

†*Borrelia burgdorferi* sensu lato speciation from synovial fluid samples is based on *ospA* PCR results. Culture isolates from this tissue were too few to estimate species distribution.

‡Tick and CSF isolates differ in the percentages of OspA types 4 and 6. OspA type 6 was found in 53% of tick isolates, but in only 23% of CSF isolates. In contrast, OspA type 4 was found in 28% of CSF isolates, but was not isolated from ticks.

confirming the pathogenic potential of this new species (V. Fingerle and B. Wilske, unpublished results).

The most frequent genomic groups in Europe, *B. afzelii* and *B. garinii*, occur across the continent and the islands, whereas the third most frequent group, *B. burgdorferi* sensu stricto, has only been isolated rarely in eastern Europe (for a survey, see Hubalek & Halouzka, 1997). Strains may be very heterogeneous, even within small areas (Eiffert *et al.*, 1995; Rijpkema *et al.*, 1996; Gern *et al.*, 1999; Rauter *et al.*, 2002; Michel *et al.*, 2003), or a focal prevalence of certain species or subtypes may be observed (Peter *et al.*, 1995; Michel *et al.*, 2003). Mixed infections have been repeatedly observed in ixodid ticks (for a survey, see Hubalek & Halouzka, 1997) and sometimes also in specimens from patients (Demaerschalck *et al.*, 1995; Wilske *et al.*, 1996a; Vasiliu *et al.*, 1998). The heterogeneity of the causative strains (Table 1) is a challenge for the microbiological diagnosis of Lyme borreliosis in Europe, and must be kept in mind for the development of diagnostic tools, such as PCR primers and diagnostic antigens. For example, *ospA* PCR has been used widely. It is important to be sure that representatives of the three species are detected, together with the different *ospA* types of the heterogeneous *B. garinii* group (Eiffert *et al.*, 1995). In addition, PCR should detect *B. valaisiana* and the recently described new species *B. spielmanii* (Richter *et al.*, 2006), as *B. valaisiana* and *B. spielmanii* may also be pathogenic for humans, as suggested by positive PCR results (*B. valaisiana*) or cultures (*B. spielmanii*) obtained from skin biopsy specimens in a few studies (Rijpkema *et al.*, 1997; Wang *et al.*, 1999a).

An *ospA* PCR for the detection and differentiation of the various European species and OspA types has been described recently (Michel *et al.*, 2003).

Most of the proteins relevant for serodiagnosis are heterogeneous. For example, interspecies amino acid sequence identities are only 40–44% for DbpA (Osp17) and 54–68% for OspC for representative strains of *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii* (strains B31, PKo and PBi, respectively) (Wilske, 2005). DbpA, in particular, has a much higher amino acid sequence heterogeneity than DNA sequence heterogeneity, indicating immune selection. However, highly heterogeneous proteins sometimes have conserved immunogenic epitopes (e.g. the C6 peptide of VlsE) (Liang & Philipp, 1999; Liang *et al.*, 2000).

Guidelines for the microbiological diagnosis of Lyme borreliosis

The German Society for Hygiene and Microbiology (DGHM) has published guidelines for the microbiological diagnosis of Lyme borreliosis written by an expert committee (*MIQ 12 Lyme-Borreliose*) (Wilske *et al.*, 2000). The English version is accessible via the Internet (http://www.dghm.org/red/index.html?cname=MIQ). Recently, guidelines for the diagnosis of tick-borne bacterial diseases in Europe have been published, including the diagnosis of Lyme borreliosis (Brouqui *et al.*).

Except in cases with the pathognomonic clinical manifestation erythema migrans, the diagnosis of Lyme borreliosis usually requires confirmation by means of a microbiological diagnostic assay. Antibody detection methods are mainly used for this purpose, whereas detection of the causative agent by culture isolation and nucleic acid techniques is confined to special situations, such as to clarify clinically and serologically ambiguous findings. The application of these methods should be reserved to laboratories specializing in this type of examination.

Specimens for microbiological diagnosis

For culture and PCR, skin biopsy samples are the most promising specimens. In general, poor results are obtained from body fluids, with the exception of PCR of synovial fluid. The examination of urine (PCR, antigen detection) or ticks removed from patients (PCR or immunofluorescent assay) is not recommended in order to determine antibiotic prophylaxis (Brettschneider *et al.*, 1998; Kaiser, 1998; Wilske *et al.*, 2000; Klempner *et al.*, 2001). Examination of ticks should be performed only for epidemiological or other scientific studies. For antibody determination, serum or CSF can be investigated. CSF examination should always be performed together with serum antibody analysis (determination of the CSF/serum antibody index).

Direct detection methods

Culture

Borrelia burgdorferi sensu lato can be cultivated in modified Kelly's medium (Preac-Mursic *et al*., 1991; Wilske & Schriefer, 2003). However, this is a very time-consuming method (generation time of *B. burgdorferi* is about 7–20 h) characterized by a low sensitivity, especially in body fluids (Karlsson *et al*., 1990; Strle, 1999; Arnez *et al*., 2001; Zore *et al*., 2002) (Table 2). Culture may be of help in individual cases if the clinical picture suggests Lyme borreliosis despite a negative antibody assay (seronegative Lyme borreliosis), e.g. in atypical erythema migrans, suspected acute neuroborreliosis without detection of intrathecal antibodies or suspected Lyme borreliosis in patients with immune deficiencies.

PCR

There have been many reports on the methods of DNA amplification and various target sequences that have been used by specialized laboratories, e.g. plasmid-borne genes, such as *ospA* and *ospB*, chromosomal genes, such as those for the flagellar protein or p66 (clone 2H1), or gene segments of the 16S rRNA or 5S/23S rRNA gene intergenic spacer region (for surveys, see Schmidt, 1997; Dumler, 2003). However, large comparative studies to identify the best methods are still lacking. *Borrelia* PCR should allow the diagnosis of the *Borrelia* species, i.e. the medical report should contain information on which of the three species pathogenic for humans has been found. The diagnostic sensitivity of PCR is about the same as that of culture. Borreliae are detected with much more difficulty from body fluids than from tissue specimens by either PCR or culture (Karlsson *et al*., 1990; Jaulhac *et al*., 1996; Arnez *et al*., 2001). Only PCR of synovial fluid seems to surpass culture significantly with regard to sensitivity (Nocton *et al*., 1994).

Sensitivity of culture and PCR

Table 2 provides a survey of the sensitivity of direct detection methods in clinical specimens from patients with Lyme borreliosis. Culture and PCR have the highest detection rates (50–70%) in skin biopsies from patients with erythema migrans or acrodermatitis chronica atrophicans (Asbrink & Olsson, 1985; van Dam *et al*., 1993; von Stedingk *et al*., 1995; Zore *et al*., 2002). In contrast, borreliae are detected by PCR or culture in the CSF of only 10–30% of patients with neuroborreliosis (Karlsson *et al*., 1990; Wilske & Preac-Mursic, 1993; Eiffert *et al*., 1995). CSF isolates are more frequently obtained from patients with a short duration of disease than from patients with disease of long duration (Karlsson *et al*., 1990). Accordingly, CSF PCR is positive in up to 50% of patients with a disease duration of < 2 weeks, compared with only 13% of patients with a disease duration of greater than 2 weeks (Lebech *et al*., 2000). It is surprising that borreliae are detected by PCR in the synovial fluid of 50–70% of Lyme arthritis patients, but culture is rarely successful (Eiffert *et al*., 1998; Vasiliu *et al*., 1998). The best PCR results are obtained from synovial tissue, not fluid (Jaulhac *et al*., 1996).

Antibody detection

It is generally accepted that serological examination should follow the principles of a two-step approach (Centers for Disease Control and Prevention 1995; Johnson *et al*., 1996; Wilske *et al*., 2000; Wilske & Schriefer, 2003): (1) a serological screening assay; and (2) in the event of a positive or equivocal result, a confirmatory assay. A sensitive enzyme-linked immunosorbent assay (ELISA) is recommended, which, when it is reactive, should be confirmed by immunoblot.

ELISA

The ELISA tests used for screening should be at least second-generation tests (Wilske *et al*., 2000) that have been improved with respect to cross-reactivity with other bacteria (e.g. antigen extract with previous Reiter treponema adsorption) (Wilske & Preac-Mursic, 1993) or use purified intact flagella as antigen (Hansen *et al*., 1988). Strains used as antigen source should express OspC, the immunodominant antigen of the IgM response, and DbpA, an immunodominant antigen of the IgG response (Wilske *et al*., 2000). Recently, specific recombinant antigens (i.e. VlsE) or synthetic peptides (i.e. the C6 peptide derived from VlsE) have

Table 2. Sensitivity of direct pathogen detection methods in Lyme borreliosis

Specimen	Sensitivity
Skin (erythema migrans, acrodermatitis)	50–70% when using culture or PCR
Cerebrospinal fluid (acute neuroborreliosis)	10–30% when using culture or PCR*
Synovial fluid† (Lyme arthritis)	50–70% when using PCR (culture is only extremely seldom positive)

*Up to 50% of patients with a disease duration of less than 2 weeks, compared with only 13% of patients with a disease duration of greater than 2 weeks Lebech *et al*. (2000).

†Higher sensitivity of direct pathogen detection from synovial biopsy specimens.

been successfully used in the USA (Lawrenz *et al.*, 1999; Liang *et al.*, 1999; Bacon *et al.*, 2003), and in a study with European sera from patients with erythema migrans, acrodermatitis and arthritis (C6 peptide) (Liang *et al.*, 2000). However, VlsE has other immunodominant epitopes in addition to the C6 region that could improve the diagnostic sensitivity; the heterogeneity of the immunodominant epitopes must be considered, especially in Europe (Goettner *et al.*, 2005).

Immunoblot

As a confirmatory assay, the immunoblot should have high specificity (at least 95%). If a whole cell lysate is used as antigen, diagnostic bands must be defined by monoclonal antibodies. In the case of recombinant antigens, identification of diagnostic bands is much easier. For the whole cell lysate blot, strains expressing immunodominant variable antigens (OspC, DbpA = Osp17) in culture should be used (i.e. strain PKo) (Wilske *et al.*, 2000).

The immunoblot criteria recommended by the Centers for Disease Control (CDC) that are used in the USA are not applicable for European patients (Hauser *et al.*, 1997, 1998; Robertson *et al.*, 2000). Dressler *et al.* (1994) have shown, in an immunoblot study, that the immune response of European patients is restricted to a narrower spectrum of *Borrelia* proteins, compared with that shown by American patients. Using different serum panels (first serum panel from Germany, second serum panel from various European countries), Hauser *et al.* (1997, 1998) demonstrated that strain-specific interpretation rules must be defined. Immunoblot antibody binding patterns vary considerably as a function of the strain used as antigen. Thus, different interpretation rules are required in order to achieve equal sensitivity and specificity when different genospecies of *Borrelia* are used in the preparation of the blot antigen.

Interpretation criteria for the immunoblot recommended by the DGHM are published in *MIQ 12 Lyme-Borreliose* (Wilske *et al.*, 2000) and are also available via the Internet (http://www.dghm.org/red/index.html?cname=MIQ). Patients with early manifestations of acute neuroborreliosis have an immune response restricted to only a few proteins. Patients with late disease, such as acrodermatitis or arthritis, have IgG antibodies to a broad spectrum of antigens. The use of recombinant antigens for the immunoblot has several advantages over whole cell lysate antigen blots: (1) specific antigens can be selected (i.e. p83/100, BmpA); (2) homologous antigens derived from different strains can be combined [i.e. DbpA (Osp17), OspC, BmpA]; (3) truncated antigens with higher specificity can be designed (internal flagellin fragments); and (4) antigens primarily expressed *in vivo* can be used (i.e. DbpA, VlsE) (Wilske *et al.*, 1999; Heikkila *et al.*, 2002; Schulte-Spechtel *et al.*, 2003). Commercial recombinant antigen immunoblots are better standardized than conventional blots. If a broad panel of recombinant antigens (including the recently described VlsE) is used, the recombinant blot is at least as sensitive as the conventional blot. An in-house recombinant IgG immunoblot (Wilske *et al.*, 1999) has been improved significantly by the addition of recombinant VlsE and an additional DbpA homologue (Schulte-Spechtel *et al.*, 2003). By the addition of VlsE and the DbpA homologue, the sensitivity increased from 52.7% to 86.1% in 36 cases of early neuroborreliosis, and the specificity remained unchanged. Using the line blot technique, which allows the detection of antibodies against antigens with identical molecular weight (i.e. homologues of the same borrelial protein) (Fig. 1), the recombinant immunoblot becomes significantly more sensitive than the conventional sonicate immunoblot (i.e. 91.7% vs. 68.8% in patients with early neuroborreliosis for the detection of IgG antibodies) (Goettner *et al.*, 2005). The combination of different homologues of one protein is especially important for DbpA as antigen (Panelius *et al.*, 2003; Goettner *et al.*, 2005), where five major molecular groups have been identified (Schulte-Spechtel *et al.*, 2006).

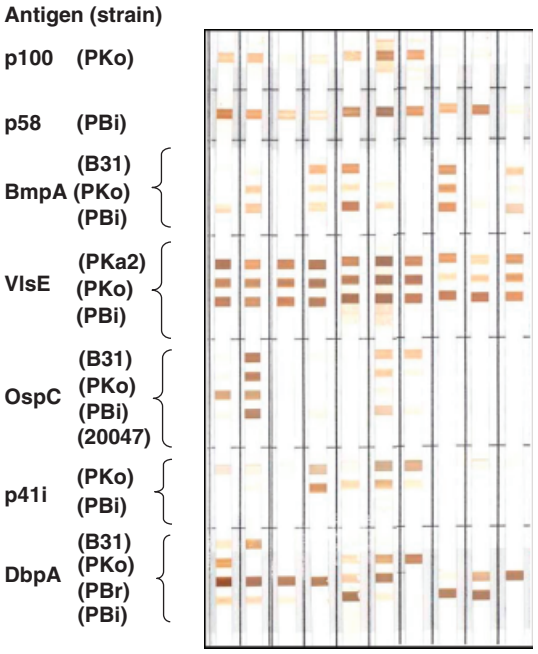

Fig. 1. Representative IgG line immunoblots of patients with neuroborreliosis. Strains belong to the following species: B31 and PKa2 to *Borrelia burgdorferi* sensu stricto; PKo to *B. afzelii*; PBr to *B. garinii* OspA type 3; PBi to *B. garinii* OspA type 4; 20047 to *B. garinii* unknown OspA type.

Determination of the CSF/serum index

Methods taking into account potential dysfunction of the blood–CSF barrier are suitable for the detection of

intrathecal antibody production (Wilske *et al*., 1986; Hansen *et al*., 1990; Hansen & Lebech, 1991). The determination of the CSF/serum index should be performed if neuroborreliosis is considered, as a positive CSF/serum index confirms present or past involvement of the central nervous system. The CSF/serum index may be positive in some cases when serum antibody tests are negative or equivocal, especially if the patient's illness has been of short duration (Wilske *et al*., 2000). Depending on the time elapsed since the first manifestation of neurological symptoms, the IgG CSF/serum index may be positive for 80–90% of patients (8–41 days after disease onset) or up to 100% of patients (more than 41 days after disease onset) (Hansen & Lebech, 1991). The detection of intrathecally produced IgM antibodies shows a high degree of sensitivity in neuroborreliosis with a short duration of symptoms, especially in children (Hansen & Lebech, 1991; Christen *et al*., 1993).

CSF/serum index determination is especially important for the diagnosis of chronic neuroborreliosis. A positive IgG CSF/serum index is essential for the diagnosis of chronic borreliosis of the central nervous system (see European Union Concerted Action on Risk Assessment in Lyme borreliosis case definitions Stanek *et al*., 1996), whereas chronic peripheral polyneuropathy is usually negative for intrathecal antibody production (Kristoferitsch, 1993).

Serological findings in various stages of the disease

The interpretation of serological test results must always be performed in context with the clinical data (Table 3). Here, case definitions are helpful (Stanek *et al*., 1996; Wilske *et al*., 2000; Brouqui *et al*., 2004). In early localized disease (erythema migrans), only 20–50% of patients are seropositive for IgM and/or IgG antibodies (Asbrink *et al*., 1985; Hansen & Asbrink, 1989). IgM antibodies usually prevail. An exception may be the immune response against the recently detected VlsE. In American patients with erythema migrans, IgG responses against VlsE are observed earlier than IgM responses (in acute erythema migrans, in 44% vs. 19%; in convalescent erythema migrans, in 59% vs. 43%) (Bacon *et al*., 2003). In European patients with erythema migrans, an early IgG response to VlsE was observed in 20 of 23 (87%) culture-confirmed cases; the IgM response has not been investigated (Liang *et al*., 2000). In early disseminated disease (acute neuroborreliosis), seropositivity (IgM and/or IgG antibodies) increases to 70–90% (Hansen *et al*., 1988; Wilske & Preac-Mursic, 1993). In principle, patients with early manifestations may be seronegative, especially in the case of a short duration of symptoms. Serological follow-up is recommended in these cases. Six weeks or more after the onset of symptoms, 100% of patients with acute neuroborreliosis are seropositive (Hansen *et al*., 1988). In cases with late disease (acrodermatis and arthritis), IgG antibodies are detectable in all patients tested (Hansen & Asbrink, 1989; Wilske *et al*., 1993a). A negative IgG test argues against late Lyme borreliosis. Thus, a positive IgM test without a positive IgG test is not diagnostic for late disease manifestations (Wilske *et al*., 2000). An exception may be a patient who receives inadequate antibiotic therapy for early disease, but sufficient drug to abrogate the IgM to IgG class switch, or a very short duration of clinical symptoms. As serological findings vary considerably and antibodies may persist for a long time in successfully treated individuals, serological follow-up is not suitable for determining whether further antibiotic therapy is warranted. The presence of specific antibodies does not prove the presence of disease; a positive antibody test may also be due to clinical or subclinical infections in the past. The more nonspecific the symptoms, the lower the predictive value of a positive serological test. Seropositivity in the normal healthy population varies with age and increased outdoor activities (e.g. in one study in Bavaria, < 5–20%) (B. Reimer, F.V. Sonnenburg, V. Fingerle & B. Wilske, unpubl. results).

Table 3. Sensitivity of antibody detection methods in the diagnosis of Lyme disease

Stage	Sensitivity (%)	Remarks
Early, localized	20–50	Predominance of IgM
Early, disseminated	70–90	In cases of short disease duration, predominance of IgM; in cases of long disease duration, predominance of IgG
Late	Nearly 100	Usually solely IgG*

*The presence of IgM antibodies without IgG antibodies is not diagnostic for late disease (for possible exceptions, see text).

Methods which are not recommended for microbiological diagnosis

Recently, various methods have been used in commercially oriented laboratories that have not been sufficiently evaluated for diagnostic purposes. These include antigen tests in body fluids, PCR of urine and lymphocyte transformation tests (Department of Health and Human Services, C.f.D.C.a.P., 2005).

T-lymphocyte proliferation assays have been used in various scientific studies performed with blood from Lyme borreliosis patients to investigate the T-cell response to *Borrelia* antigens (Dattwyler *et al*., 1988; Buechner *et al*., 1995; Kalish *et al*., 2003). However T-lymphocyte proliferation assays cannot be recommended as diagnostic tests because of their cumbersome nature and concerns about their specificity and standardization (Horowitz *et al*., 1994; Department of Health and Human Services, C.f.D.C.a.P., 2005; Wilske, 2005).

Antigen detection tests have been used for the detection of borrelial antigen in body fluids from patients with Lyme borreliosis, including CSF and urine (Hyde *et al.*, 1989; Coyle *et al.*, 1992). However, the validity of this technique is controversial and its use is no longer recommended for microbiological diagnosis (Klempner *et al.*, 2001). PCR from urine is also unreliable (Brettschneider *et al.*, 1998); borrelia DNA has also been detected from healthy seropositive individuals (Karch *et al.*, 1994).

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