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DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to a biochip comprising an antigen bouquet, to be inserted into an optical reader and relates to a method for detecting and monitoring antibodies associated with the presence of diseases in biological samples, using such biochip and such optical reader.

Description of Prior Art

1 - Biochip and Antigen Bouquet

[0002] There are some documents disclosing the use of antigens and also biochips to perform a more sensitive diagnosis of some diseases, comparatively to ELISA and WB. The present invention was first directed to solve the technical problem of how to use and improve the performance of such technology and simultaneously detect autoimmunity related Lyme disease and coinfection of *B. burgdorferi* with other pathogenic bacteria, such as *Babesia microti* and *Ehrlichia*.

[0003] Some tests were already performed for detecting other diseases and the results were significantly better than with those obtained with the conventional methods or even with other biochips, antigens, optical readers and methods previously disclosed.

Lyme Disease

[0004] Lyme disease is an endemic disease, transmitted by ticks and caused by the bacterium *Borrelia burgdorferi f.l.*, affecting 63 countries around the world, including the 27 member States of the European Union and also the United States of America.

[0005] The incidence in children is higher than in adults. The Regional Office for Europe of the World Health Organization (WHO) estimates that, currently, every year 85.000 new cases of Lyme disease arise in Europe (evaluating national data), but there is a general concern about this number being much higher, because in Europe the reported cases are highly inconsistent and many patients actually affected by this disease are not diagnosed properly in due time.

[0006] The number of reported cases of Lyme disease has increased since the early 1990s, and the geographical distribution has expanded due to climate change (*Lindgren, E. and Jaenson, Thomas G. T.*, 2006). In 1993, it was estimated that this disease had a cost of EUR 660 million for the community, being the cost per patient of approximately 40,750 euros (*Irwin T. Vanderhoof, PhD, CLU et al.*, 1993). Since then, the number of cases more than doubled, resulting in a cost increase to society in more than 1300 million euros.

[0007] The first symptoms of Lyme disease include fever, headache, fatigue, depression and a characteristic skin rash called *erythema migrans*. When detected in due time, the infection and the symptoms can be eliminated with the use of antibiotics. However, if this disease is not treated, the disease progresses and symptoms may affect the central nervous system, heart and joints, the symptoms becoming much more difficult to deal with and the disease more difficult to treat.

[0008] Occasionally, the symptoms such as arthritis persist after the infection has been eliminated, which suggests that the *Borrelia burgdorferi f.l.* may promote chronic and autoimmune induced infections (*Steer et al.*, 2004).

***Borrelia burgdorferi f.l.*, the bacterium causing Lyme disease**

[0009] *Borrelia burgdorferi f.l.* is a pleomorphic bacterium with a complex life cycle that encompasses a multitude of forms, including the form of corkscrew (called parental, because it is the form that this bacterium has when infects the host), the form of cysts (without cell wall), among other forms. Although known for more than a century, all the bacteria with deficient cell wall (CWDB), including *Borrelia*, were only linked to clinically relevant diseases during the last few decades.

The lack of wall in this type of bacteria makes them very difficult to detect by the usual screening methods, such as the light microscope and serological pattern test. This is because this type of bacteria triggers an immune response different from the normal pattern. As a result, the CWDB creates severe difficulties in diagnosing and treating certain infections, remaining in the body for a long period of time, frequently causing chronic diseases.

[0010] The existence of pleomorphic variants of the spirochete *Borrelia* explains the ability that this bacterium has to remain in the host as dormant, during prolonged periods of latency, clinically asymptomatic. According to the symptoms and duration of the disease, there are 3 stages for Lyme disease:

1. 1) Early stage with localized infection of the skin ("*erythema migrans*");
2. 2) Acute or intermediate stage wherein the infection is characterized by inflammation of various organs, such as skin, joints, heart and nervous system, weeks or even months after the transmission of *Borrelia*;
3. 3) Chronic stage with persistent infection with chronic inflammation and decreased

function of different organs and systems for more than 1 year.

[0011] The bacterium *Borrelia burgdorferi f.l.* was implicated in more severe clinical manifestations, such as borreliosis neocortical (neuroborreliosis), which has been associated with Alzheimer's disease.

Available diagnostic methods for detecting the presence of Lyme disease

[0012] Common symptoms caused by Lyme disease symptoms are similar to a flu, headaches, pains in the joints, gastrointestinal problems, sensitivity to light/sound and a general malaise, but the variety of symptoms differs from patient to patient: some patients present only infections on the skin while others only develop late symptoms, as arthritis.

As only 60% of patients develop characteristics of "*erythema migrans*" and approximately 25-30% of patients remember to have been bitten by ticks, the remaining 10-15% of the patients are only confirmed by laboratory tests. The late stage symptoms of this disease are a result, in part, of the lack of sensitivity and efficiency of diagnostic tools that are able to detect the disease in its early stages, when it is easily treated.

The Center for Disease Control (CDC) of the United States of America proposes a criterion of diagnosis in two levels, for the serological diagnosis of Lyme disease:

A first level where it performs a positive immunological test (ELISA), followed by a second level where a Western Blot (WB) is performed to confirm the results of the immunological assay (ELISA). Both tests have lack of sensitivity and the interpretation of WB varies from laboratory to laboratory.

The main limitations of ELISA are the false positive results due to interference with an IgM rheumatoid factor and false negative IgM results due to competition with specific IgG (Hansen, 1994). Another problem with this method is to distinguish active from past infection. WB assays appears to be as sensitive and specific as ELISA but is in fact more laborious to perform, needs more sample volume and is more expensive (Carlsson et al., 1998; Magnarelli et al., 2004). The advantage of WB is that antibodies can be detected, which may recognize different antigens of a given infectious agent, and the disadvantage is the possible identification of nonspecific antigens or cross-reactive epitopes. In addition, conformational epitopes may be lost (Hansen, 1994) and WB results are also dependent on the *B. burgdorferi* strains. Therefore WB is often used to just corroborate or support results from other diagnostic methods.

[0013] This strategy therefore failed the diagnostics for the first phase of the disease in 80% of cases (Wilske, Bettina (2005), and does not distinguish between acute or chronic infection. The same problems of sensitivity are found in the evaluation of cerebrospinal fluid (CSF), where the serological test detects only 10-30% of patients with *neuroborreliosis*.

Up to date, there is no diagnostic tool able to effectively detect with sensitivity and specificity the immune response triggered by cell wall deficient forms of *Borrelia burgdorferi f.l.*, which are

responsible for severe chronic symptoms of *borreliosis*. These forms have a pattern of protein expression changed, as well as differences in antigen compared with the form "parental". As a result, these forms are not detected with commercial methods based on antigens contained in total cell lysates of the "parental" form of *Borrelia burgdorferi f.l.*

Methods for detecting and monitoring the stage of Lyme disease

[0014] In recent years, new peptides and antigenic proteins for detection of infections by *Borrelia burgdorferi f.l.* have been described in scientific and medical articles, both in the first stage of the disease and in more advanced stages. The latest research in this area points to a combination of antigens in a single test, as being the best way to overcome the problems occurring with available tests.

[0015] Two concerns arise from the available diagnosis tools and methods:

1. 1) Detecting Lyme disease on an early stage, when it is still treatable; and
2. 2) Distinguishing between chronic (late-stage) and acute (intermediate) infection.

[0016] Document US 2013/0273572 A1 discloses a method for diagnosing Lyme disease status in biological samples obtained or derived from a mammal, determining antibodies to *Borrelia burgdorferi* outer surface proteins (Osp) and also discloses means to identify mammals having early, intermediate or chronic *B. burgdorferi* infection. One embodiment discloses a lateral flow device comprising OspA, OspC and OspF antigens in physical association with a solid matrix. Another embodiment discloses a multiplex assay comprising the OspA, OspC and OspF antigens provided in association with fluorescent beads. The OspA, OspC and OspF antigens are contacted with a biological sample derived from an equine in a manner that antibodies to such antigens, if present, will bind to the antigen and will thereby be immobilized on the antigen/fluorescent bead complexes. A detectably labelled anti-equine specific antibody is added and a multiplex analyser is used to detect the fluorescent beads and therefore determining the status of Lyme disease.

[0017] Based on the state of the art and the available products and methods in the market, two main further requirements are clearly identifiable:

1. 1- Providing a more effective and fast alternative to Western Blot (WB) and ELISA, each one of which require extensive laboratory work, is time consuming, difficult to standardize and provides a large amount of false negative results, wherein the provided alternative should be robust, easy to use and allow unambiguous interpretations of test results.
2. 2- Avoiding the need for performing two tests, accomplishing a specific and reliable diagnosis in a single platform, by a single process, and allowing the unequivocal detection of early, acute and chronic infections, including new and more effective

antigens.

[0018] Document Du W et al., Antigen biochips verify and extend the scope of antibody detection in Lyme borreliosis, Diagnostic Microbiology and Infectious Disease, 2007, discloses a streptavidin modified biochip-based immunodiagnosis and compares it to conventional methods such as enzyme-linked immunosorbent assay (ELISA) and WB. The diagnostics sensitivity of such coated biochips was demonstrated to be identical, and the results of ELISA and WB were confirmed. This document teaches that flagellar antigens lead to a better diagnosis and describes OspC antigen as being less sensitive to identify immunity to Lyme borreliosis (LB). Binding of antibodies to flagellar antigen or OspC8 peptide was detected by fluorescein isothiocyanate-conjugated rabbit antihuman IgM antibody and IgG antibody. Fluorescence values higher than two times over the mean value of healthy controls were interpreted as being positive for antigen-specific IgG or IgM antibodies. This document teaches that, taken together, a protein biochip, as a potential substitution of ELISA, and WB method offer the opportunity to study serum immunity in a multiplicity of patients simultaneously. It also mentions that the disclosed data stimulate the extended application of biochips by using more antigens and more specific and longer peptide epitopes.

[0019] US 2006/0194267 A1 and US 2012/0142023 A1 disclose methods for detection of Lyme disease. Tran, Kohl, Finn, Chimento and Ascoli disclose a multiplex test for Lyme disease (www.rockland-inc.com).

[0020] In response to a clear need of improving the existing diagnostic methods and with the above mentioned premises in mind, a consortium of SMEs B-C-BORRELIOSIS CENTRUM AUGSBURG BETRIEBS GMBH & CO KG (BCA), microLIQUID sl (MLD), STAB VIDA, RESEARCH AND SERVICES IN BIOLOGICAL LDA. (STAB VIDA), MICRO BIO DEVICES S. R. L (MICRO BIO), aimed to develop a novel integrated biochip technology system and method to improve clinical diagnostic, disease monitoring, and treatment of Lyme disease, by enabling specific and sensitive detection of the human serological response against Borrelia infections and ultimately in any given biological samples for detecting and monitoring the stage of other diseases.

2 - Biochip Optical Reader

[0021] To complement and optimize the results obtained with the biochip and the method according to the present invention, the following state of the art was identified:

Document EP1239279B1 discloses a reader for biochips, such as DNA chips and protein chips, comprising a laser for emitting an excitation light, a lens for collimating such excitation light, an optical detector for detecting fluorescent light produced by such excitation light at samples deposited on said biochip. This reader further comprises means adapted for condensing said excitation light; means for scanning the biochip with the condensed laser beam so as to reduce

the influence of speckle noise.

[0022] Document "Microfluidic biochip based on SPR optical reading for the detection of *Legionella pneumophila*, E. De Lorenzis et al." discloses an immunosensor for highly sensitive detection of *L. pneumophila* using Surface Plasmon Resonance (SPR) as transducing technique. To minimize the impact of this microorganism on environmental water, an accurate procedure was required to determine the presence and its enumeration. Although there are many methods known for detection of *L. pneumophila* in water, such as culture method or PCR techniques, they are labour-intensive and time-consuming systems. To overcome these problems, label-free biosensors for this pathogen were considered highly desirable.

[0023] With the purpose of developing the biochip optical reader of the present invention, some requirements were enumerated as follows:

- reader needs to position accurately and in a repeatable way the reaction chambers of the biochip that holds fluorescence with respect to the optical detector,
- both biochip mechanical properties and fluorescence properties are not completely known during research about reader,
- distance between detector's optic and biochip's reaction chambers has to be very accurate (in the micrometer range),
- signal to noise ratio of fluorescence may be small and some strong fluorescence signals have to be considered as noise,
- reader must implement a robust algorithm to extract fluorescence signal from images taken by the optical detector,
- enough parameters have to be available to allow sufficient tuning of the reader during the preclinical tests,
- the user needs no specific knowledge to put the biochip in the reader and to retrieve the result of the diagnostic tests, results have to be exploitable both by an external automated system and a user,
- whole equipment has to be integrated in a "small volume".

[0024] And none of the above mentioned state of the art documents or optical readers available in the market fulfilled at the time all the desired requirements.

SUMMARY OF THE INVENTION

[0025] The work leading to this invention has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement number 262411.

[0026] The object of the present invention regards an integrated system and method for detecting and monitoring the presence of antibodies associated to different stages of diseases

in the presence of a given antigen bouquet comprised within a biochip to be read in an optical detector according to a specific revealing and detection method.

[0027] The present invention comprises a biochip with specifically designed configuration and microfluidic properties (Figure 1) that increases the speed and effectiveness of the whole process of detection and monitoring. This biochip is to be read in a specifically adapted optical reader for which an algorithm was developed and implemented to provide faster and more selective results, with a revealing method using quantum dots (qDots) which are intrinsically bright, have very narrow emission spectra, have photo-stability and are easy to use. The use of these qDots in biomedical applications is just emerging and gaining popularity. Due to their spectral characteristics the qDots have fundamental high performance in a variety of applications.

[0028] In a preferred embodiment of the present invention, it comprises an antigen bouquet with at least 16 antigen coatings (Figure 3) for primary linking to antibodies present in biological samples bearing Lyme disease and providing complexes suitable for linking specifically to secondary antibodies wherein it provides the detection of early, acute and chronic Lyme disease stages and also provides the detection of autoimmune related Lyme disease and coinfection with other antigens, other than *B. burdorferi*, such as *Babesia microti* and *Ehrlichia*.

DESCRIPTION OF THE INVENTION

[0029] The object of the present invention was to develop a novel integrated biochip technology system and method to improve clinical diagnostic, disease monitoring, and treatment of Lyme disease, by enabling specific and sensitive detection of the human serological response against *Borrelia* infections and ultimately in any given biological samples for detecting and monitoring the stage of other diseases.

[0030] For that purpose, the selection of the antigenic *Borrelial* peptides and proteins that configure the biochip technology was a crucial step: the chronic nature of Lyme disease and the antigenic diversity of the spirochetes suggested that antigenic variation plays an important role in immune invasion.

For this reason, peptides from different components of *Borrelia* during different cycles were included for a best screening of *Borrelia* infection and other diseases.

In addition, another fundamental selection criteria was to choose peptides and proteins that have been proved to be antigens able to elicit specific serological responses in acute and chronic infections in Lyme patients. In this regard, outer surface proteins, autoimmune-related antigens, immunodominant antigens, variable major protein antigenic peptides, and proteins from decorin-binding proteins of *Borrelia* were tested in the present study, to evaluate their potential immunogenicity and their incorporation into the chip device. The chosen antigens and its selection reason are described as follows.

1. Antigen Bouquet

Early and Acute Infection Antigens

[0031] Diagnostic laboratory tests are needed to detect active Borrelial infection, particularly early, when the hallmark clinical sign of infection, Erythema Migrans, is not seen. However, existing antibody tests require a large disease phase, often of several weeks, to become positive.

Some studies have pointed that prominent early immune responses in Lyme disease could include reactivity with the 41-kDa flagellar antigen (FlaB, p41), outer surface protein C (OspC), and **variable major protein** of the spirochete. Thus, these acute-related antigens have been chosen for testing.

[0032] It has been reported, that patients with Lyme disease usually develop IgM antibodies against the 41 kDa **flagellar antigen (p41)** of the spirochete during the early infection, followed by the development of IgG antibodies against p41 and various outer surface proteins (Osp).

Regarding Osp proteins, the 23 kDa **Outer surface protein C (OspC)** is thought to be an important virulence factor involved in the transmission and establishment of early infection. It has been shown that *Borrelia burgdorferi* reduces the expression of OspC in response to the development of antibodies, indicating the importance of repressing OspC expression to avoid clearance and to maintain infection. Since humans produce highly specific antibodies against OspC, measurement of IgG and IgM antibodies against this highly conserved lipoprotein indicate recent infection with *B. burgdorferi*.

[0033] Other important antigens in early *Borrelia* infection are **C2** and **C6 peptides**. It is well-known that the *Borrelia* spirochete expresses a surface lipoprotein called Variable Major protein (VMP) or Variable Major Protein Like Sequence (VlsE). VlsE plays a key role in the survival strategy of *Borrelia*. After penetration into the host organism, *Borrelia* bacteria constantly change their surface-expressed VlsE and, in this way, try to escape recognition and elimination by immune system. The VlsE protein is divided in several sections: conserved regions, which serve as transmembrane domains and anchor VlsE in the bacterial membrane, as well as variable and invariable regions. The variable regions of VlsE facing outwards are constantly changed by recombination; thus, the attacking immune system consistently encounters new altered antigen epitopes. The invariable regions are masked by the variable regions and, in living *Borrelia* bacteria, are protected from direct attack by the immune system. When deceased *Borrelia* bacteria are processed by antigen-presenting cells, the complete VlsE protein is presented to the immune system and the host also forms antibodies against the invariable and conserve regions of VlsE. These antibodies are highly suitable for diagnosis of borreliosis because of the high level of conservation of their target antigens. Among the VlsE sequence, a 19-mer (C2 peptide) and 25-mer peptide (C6 peptide) are clearly conserved

across genospecies and strains of *B. burgdorferi*, *Sensu lato: Sensu stricto* and *B. garinii*. Antigenicity of this peptide was confirmed in humans, monkeys, and mice. Sera from all these hosts reacted with C2 and C6 peptide early and persistently in the course of infection, indicating that these peptides contain one or more epitopes that are broadly antigenic against *Borrelia* infection.

Chronic Infection Antigens

[0034] Most of the discovered antigens to detect *Borrelia* infections fit into the group of the chronic related proteins and peptides. Among them, outer surface proteins E (OspE) and A (OspA), in addition with decorin binding protein A (DbpA) have to be considered.

The ability of the Lyme spirochete to maintain chronic infection indicates that the bacteria is capable of immune evasion. The immune evasion of Lyme disease spirochetes is achieved by either antigenic variation or through the binding of Complement Regulatory Protein Factor H, avoiding complement recognition. In this mechanism of immune evasion **OspE** plays a key role:

this surface-exposed 19.2 kDa lipoprotein, expressed in both ticks and mammals, can elicit a strong antibody response. Analysis of the specificity of the antibody response to different OspE variants suggests that there are hypervariable regions that are targeted by antibody response during infection. Otherwise, there are conserved regions of OspE that are involved in Complement Regulatory Protein Factor H, facilitating complement evasion. Therefore, it is important to assess the specificity of the antibody response to OspE epitopes that are exposed during infection. For that reason, two different exposed OspE peptides will be tested in the present project: **OspE1** and **OspE2**.

[0035] Another outer surface lipoprotein that could serve as a biomarker for chronic Lyme disease is the 31 kDa **Osp A**. However, its selection criteria will be explained below (see Autoimmune Antigens section), because of its close relation with autoimmune disorders.

Regarding the decorin binding protein A (DbpA), this 19 kDa membrane protein plays a key role in the mammal colonization of the bacteria²⁷. It has been reported to be a sensitive and a specific antigen for the serodiagnosis of Lyme arthritis or neuroborreliosis²⁸; for this reason, this protein could be an excellent biomarker for *Borrelia* infection. Moreover, it has been suggested as a potential vaccine protein, due to the strong antibody response elicited during experimental murine Borreliosis. Since at the time of developing the present study, DbpA was not commercially available, the protein will be cloned, expressed and purified during the present invention's development.

Autoimmune antigens

[0036] Long-term chronic manifestations of Lyme disease are increasingly thought to be due to induced autoimmune conditions, rather than acute pro-inflammatory action by the infection.

[0037] Antigenic mimicry of infectious agents and autoantigens is a proposed pathomechanism for autoimmune diseases. In this regard, *Borrelia* spirochete could be another trigger of this process where amino acid sequence homology with self- antigens may trigger multi-organ system disorder in genetically predisposed individuals³¹. In order to assess the presence of autoimmune disorder associated with *Borrelia* infection the leukocyte function-associated antigen 1 (LFA 1), the human collagen type I, the myelin basic protein (MBP), and the arthritis-related proteins GST-like self-antigen (rArp) will be include in the serological test.

[0038] A prominent late manifestation of *B. burgdorferi* infection is Lyme arthritis. Development of antibody reactivity to outer surface protein A (OspA) occurs towards the beginning of prolonged arthritic episodes. The progression to an autoimmune state begins with a crossreactive response between OspA and a self-antigen, the leukocyte function- associated antigen 1 (LFA 1). The LFA-1 is found on all T-cells and also on B-cells, macrophages and neutrophils and is involved in cell recruitment to the site of infection. During *B. burgdorferi* infection, OspA stimulates a particularly strong TH1 response in genetically susceptible individuals, which may be one of the several factors that can help to set the stage for a putative autoimmune response in affected joints³³. The Identification of this cross-reactivity between OspA and LFAI may provide a model for development of autoimmune disease induced by *B. Burgdorferi*, providing then, an important antigen to incorporate into diagnostic tools.

[0039] The cross-reactivity between a *Borrelia* antigen and a self-antigen has been also observed in other human proteins. In Lyme disease patients, immune response against human collagen type I has been reported. It has been proposed, that this autoimmune response could be caused by cross-reactivity between an unknown *Borrelia* antigen (probably CRASP proteins) and human collagen type I, resulting in rheumatoid arthritis. Other example of cross-reactivity is the myelin basic protein (MBP) . *Borrelia* encephalomyelitis, a rare manifestation of Lyme borreliosis, may present as a multiple sclerosis (MS)-like disease. It is postulated that in MS, inflammation of the white matter is caused by a T-cell response directed to myelin antigens. In patients affected with Lyme disease, this autoimmune response could be due to a cross-reactivity between the self-antigen MBP and a *Borrelia* antigen, however, several investigations refused this hypothesis.

[0040] Nevertheless no matter how, both human collagen type I and MBP could be potential biomarkers for autoimmune disorders elicited by *Borrelia* infection.

[0041] Other potential biomarker for autoimmune disorders associated with Lyme disease is the arthritis-related proteins GST-like self-antigen (rArp). This 37 kDa arthritis-related protein of *B. burgdorferi* outer-surface protein has been shown to prevent or reduce the severity of arthritis. Antibody responses to this single protein resembling glutathione-S-Transference were tested in 124 antibiotic-treated patients with early or late manifestations of Lyme disease.

[0042] Among the 124 antibiotic-treated patients, 53% with culture-proven Erythema Migrans had IgG responses to recombinant glutathione S-transferase (GST)-Arp, as did 59% of the patients with facial palsy and 68% of those with Lyme arthritis. Therefore, measurement of

antibody against recombinant GST may be an additional tool for the detection of *B. burgdorferi* involvement in patients with accompanying arthritis during initial episodes of joint pain or during the maximal period of arthritis. Similar to DbpA, Arp is not commercially available.

[0043] Thus, the protein was cloned, expressed and purified during the present development.

Coinfection antigens

[0044] There are other organisms besides *Borrelia* that live in ticks and can be transmitted to humans through bites, causing other tick-borne illnesses. The most common ones include ***Babesia*** (babesiosis) and ***Ehrlichia*** (ehrlichiosis). *Babesia* and *Ehrlichia* are also spirochetes. The symptoms of babesiosis and ehrlichiosis often overlap those of Lyme disease and, like the last one, are just as difficult to diagnose and treat. Very often, *Babesia* and *Ehrlichia* are transmitted along with *Borrelia* by the same tick. Thus, peptides from these pathogens will be included in the study to exclude cross-reactivity with Borrelian antigens and to detect antibodies against all three spirochetes simultaneously.

[0045] *Babesia* species represent some of the most common infectious parasites among wild and domestic animals and are gaining increasing interest as emerging causes of zoonoses in humans. They require competent non-vertebrate and vertebrate hosts to maintain transmission cycles, infecting ixodid ticks and vertebrate erythrocytes. Several species have been shown to infect humans, although ***Babesia microti*** is the specie most frequently identified. Although the *Babesia* was originally identified as endemic in the Northeastern United States and parts of the Midwest, the parasite has expanded the distribution to many parts of Europe and Japan. Clinical signs in animals and in humans are not specific diagnostic measures for babesiosis, especially in asymptomatic or mixed infection in areas of endemicity.

[0046] Therefore, serological distinction of these infections is very important for choice of prophylactic treatment.

[0047] Members of the order *Rickettsiales* constitute a diverse group of intracellular bacteria of eukaryotic cells, including *Rickettsia*, *Orientia*, *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Bartonella* and *Coxiella*. ***Ehrlichia*** has been shown to be agent of human monocytic ehrlichiosis (HME) and human granulocytic ehrlichiosis (HGE). Genogroup II *Ehrlichia* express a markedly immunodominant outer membrane protein designated major surface protein 2 (MSP2). MSP2 is encoded by a multigene family, resulting in the expression of variant B cell epitopes. However, MSP2 includes conserved regions that enhances the antibody response against *Ehrlichia* pathogens^{40,41}. Therefore, measurements of IgG and IgM antibody against these highly conserved regions of MSP2 are the best method for assessing humoral immune response against *Ehrlichia* pathogens.

Bacterial Lysates

[0048] When using tests which are only based on recombinant antigens, it must be accepted that 25% of control sera usually show a false positive IgM result. For this reason, several *Borrelia* lysates will be included in the serological test to avoid this issue. In this regard, different genospecies of *Borrelia burgdorferi* will be evaluated for a serological response, including *sensu stricto* B31, *sensu stricto* 297, *garinii* Fuji P1, and *afzelii* P12 strains.

[0049] In addition to spirochete lysates, one of the novelties of the present invention is to include cyst forms of *Borrelia burgdorferi* in the biochip test. Cystic forms (also called spheroplasts or starvation forms) represent a low metabolic activity state or phase of *B. burgdorferi* bacterial cells that allows the spirochete to survive in a hostile environment. When the *Borrelia* spirochete finds itself in a hostile environment it appears to change into a cyst form, covering itself with a transparent mucoid capsule; one cause of a hostile environment is antibiotics in the body serum. However, when the environment becomes favorable to its growth, the cyst opens and the spirochete is released. When in the cystic form, conventional antibiotic therapy will not destroy the Lyme spirochete. It has been reported that cyst inoculation in mice induces a specific antibody response as a sign of vigorous humoral immune response, thus indicating probable active infection. Considering this, cyst lysates was included to improve Lyme disease diagnosis.

[0050] Finally, since all the recombinant proteins commercially available or produced in the present development will be generated in *Escherichia coli*, full lysates of the **E. coli DH5α** lab strain were also used, in order to discard possible detection of cross-reactivity antibodies.

Table 1 - Peptides and proteins used for Lyme disease detection

Infection	Peptide/Protein
Acute	P41
	OspC
	C2 19-mer
	C6 25-mer
Chronic	OspE1
	OspE2
	OspA
	DbpA
Autoimmune	LFA-1
	Collagen I
	MBP
	Arp
Coinfections	<i>Babesia microti</i>
	<i>Ehrlichia N-t</i>

Table 2 - Positive Serum Samples

Immunoglobulin	IgG bands	IgM bands
Acute infection, IgG and IgM positive	P41, VlsE	OspC, p41
IgG positive	OspC, p39, p41, p83, VlsE	Negative
Chronic infection, IgG positive	P58, OspC, p39, p41, p83, LBb, VlsE	Negative
IgG positive	P41, p83, Lba, VlsE	Negative
Chronic infection, IgG positive	P58, OspC, p39, p41, p83, LBb, VlsE	Negative
Chronic infection, IgG and IgM positive	P18, p20, OspC, p41, p83, VlsE	OspC
Chronic infection, IgG and IgM positive	P21, p39, p41, p83, VlsE	OspC, p41
IgG positive bands and high Chlamydia pneumonia activity (IgA and LTT)	P58, p39, p41, p83, LBb, VlsE	Negative
IgG positive	P21, p39, p41, p83, LBb, VlsE	Negative
Chronic infection, IgG positive	P58, p39, p41, p83, VlsE	Negative
Chronic infection, IgG and IgM positive	P19, p58, OspC, p39, p41, p83, LBb, VlsE	OspC
Chronic infection, IgG and IgM positive	P21, OspC, p41, VlsE	OspC, p41
Chronic infection, IgG positive	P39, p41, p83, LBb, VlsE	Negative
Chronic infection, IgG and IgM positive	OspC, p41, op83, VlsE	OspC, p41

[0051] Serum samples from patients confirmed with Lyme disease were tested by ELISA against the antigens previously described, in order to select the best immunogenic response antigens for the biochip.

[0052] Considering the results, all tested samples were IgG and/or IgM positive for at least one antigen, confirming the Lyme disease diagnosis obtained by commercial kits. These results showed that the inclusion of the pre-selected antigens grants a sensitivity of 100% to detect samples infected with Borrelia. However, not all the ELISA tested samples reacted with the "classical" antigens included in commercial kits (p41, OspC and VlsE), achieving lower sensitivity and specificity than expected (Table 3).

Table 3 - Sensitivity and specificity of "classical antigens in ELISA test

Antigens	IgG sensitivity (%)	IgG specificity (%)	IgM sensitivity (%)	IgM specificity (%)
P41	21,4	21,4	14,3	0
OspC	35,7	28,6	50,0	50,0
C2 19-mer	42,8	42,8	-	100,0
C6 25-mer	57,1	57,1	-	100,0

[0053] Autoimmune conditions are thought to be responsible of long-term chronic manifestations of Lyme disease. Thus, in a serological assay the positive autoimmune antigens should be detected nearly together with chronic ones. In the results obtained with autoimmune antigens, all the IgG positive samples for LFA 1 were positive for the chronic markers OspA, OspE1-2, and/or DbpA, suggesting that this antigen is highly specific to detect Lyme disease patients in chronic stages with arthritic episodes. Specially, samples in which OspA and LFA 1 were both detected could correspond to patients at the beginning of these arthritic episodes, when OspA was supposed to cross-react with LFA 1. The same results were obtained with the MBP antigen in which the IgM positive serum sample was also positive for IgM OspA and DbpA. In the same way, Arp positive samples also coincided with the ones showing OspA and DbpA IgM response. Considering the novelty of this antigen and since more samples were detected with this protein when compared to IgM MBP results, the above mentioned negative sera were also used to better characterized the Arp serological sensitivity. Once included, the results confirmed the previous Arp positive samples obtained. Thus, rArp inclusion in the antigen bouquet of the present invention was considered due to the higher number of IgM positive samples obtained, when comparing with the other IgM autoimmunoreactive antigen (MBP). Regarding collagen, a well-known characterized antigen, all the positive IgG samples coincided with positive OspA, OspE1-2, and DbpA. In this case, when comparing this classical antigen with the other autoimmune antigen IgG positive (LFA 1), collagen seemed to be less specific.

[0054] Regarding coinfections, some samples seemed to be infected with *Babesia* and *Ehrlichia*, respectively, representing probable cases of babesiosis and ehrlichiosis. These positive results reinforce the fact that these antigens should be included in a Lyme diagnosis test, in the antigen bouquet of the present invention, in order to detect possible coinfections by other spirochete pathogens.

When analyzing cyst results, a vigorous IgG response was surprisingly observed, suggesting that the inclusion of this antigen in a new test could represent a real and novelty improvement in the sensitivity and specificity of Lyme disease diagnosis.

Finally, the fact that all the samples were negative for *E. coli* lysates indicated that any potential cross-reactivity of all the recombinant proteins synthesized in this cellular system (including rDbpA and rArp) can be discarded.

[0055] A preferred embodiment of the antigen bouquet of the present invention is Figure 3.

2 - Biochip

[0056] The biochip of the present invention (Figure 1) comprises the bouquet of antigens (described above and with a preferred embodiment in Figure 3) in microfluidic chambers, and was built as follows:

1. a) all functions are integrated, preferably without external connections (input reservoirs, valves, mixers, area detection and fluid pumps);
2. b) the biochip has the desired properties of smoothness and moisture content of the wall structure of channels of fluidics and a high ratio of appearance to allow strong capillary forces.
3. c) the biochip has pumps, preferably capillary pumps, that allow the transport of the sample and reagents up to the detection area. This structure has a flow rate in the order of about 0,4 $\mu\text{L}/\text{min}$, allowing a total assay time under 20 minutes, and is capable of pumping the entire volume of the test sample through the detection chamber.
4. d) has a camera detection, in which the immunoglobulins of human serum, against the antigens of *Borrelia*, are captured by the immobilized antigen, with a capacity of more than 1000 immobilization of protein molecules. The cameras are designed to be compatible with a chemo-luminescence specific Reader and include the structures required for routing of wave lengths.
5. e) the approach to the production of a new low cost Biochip, highly reproducible and reliable.
6. f) Procedures for immobilisation of the antigen in the camera detection area and the conjugation of quantum Dots (qDots) with secondary antibodies against biological samples, preferably fluid samples and more preferably serum or cerebrospinal fluid, to be incorporated as reagents for stocking inside the biochip. The quality of the coating on the surface of the camera, and the qDots were evaluated using state -of- art techniques.
7. g) integration of low cost pumps and valves in the biochip not requiring external actuators.
8. h) open architecture design of the biochip, which can easily be adapted to other applications and diseases.

3 - Optical reader

[0057] The main objective of this deliverable is to present the development of a complete user-friendly automated reader specifically adapted for highly sensitive fluorescent read-out technology with Quantum Dots (QD) fluorescent markers performed in the biochip of the present invention. The Quantum Dots are a marker added to the system in order to measure how biochemical markers interact with patient serum or other biological, preferably fluid, samples.

[0058] This research also takes into account the cost over efficiency ratio. The aim was to use techniques that are possible to industrialize at a reasonable production cost for small to medium volumes.

The biochip optical reader of the present invention is for highly sensitive fluorescent read-out technology comprising a fluorescence detector containing an inner structure comprising a light source, preferably LED (16), a camera (17), a lens tube (18), Kohler illumination system (19), motorized translation stage for auto-focus (20), a mechanical frame (21), and a fixed objective positioner (22), and such fluorescence detector containing an intermediate structure comprising light shields (23), a housing frame (24), a plug panel (25), a stiffener (26), a computer compartment, an outer structure comprising the main casing (27), a touch screen (28), ventilation holes (29) and a trap door (30) wherein the fluorescence detector is specifically designed to use quantum dot fluorescent markers and is specifically adapted to be optically coupled and aligned with a biochip insertion and removal system (31) through a clamping system (32) and an auto-focus system (20), having a detection field of view up to 1 mm in length, preferably 480 μm , and up to 1 mm in width, preferably 480 μm , such detection field of view comprising the biochip field of view (A4) with about 65 μm tolerance in both X and Y-axis for positioning accuracy. The clamping system (32) comprises three contact points of reference with mechanical stoppers (33) with adjustable position and two pushers (34) which push the biochip against these stoppers wherein in addition to the X, Y clamps, two elastic clamps hold the biochip in position along the Z-axis and the return springs (35) pull back the pushers to allow the insertion and removal of the biochip. The insertion and removal system (31) consists of a drawer system comprising a piece of magnetic stainless steel material and a guiding mechanism with two guideway chariots (36) per rail, where in the closed position the drawer is blocked by an electromagnet (37) wherein the contact between the drawer and the electromagnet ensures a perfect X-axis alignment between the detector and the biochip, keeping the detector isolated from light, and in the opened position the drawer is blocked with two permanent magnets (38) to maintain it in position during the biochip loading.

4 - Method of the present invention

[0059] The Biochip is loaded with at least 16 specific antigens for antibodies developed for Lyme disease. The biological sample, preferably serum or cerebrospinal fluid, supposedly infected is collected and loaded into the chambers of biochip, waits a few seconds so that the sample flows inside the biochip. Then, the excess of sample is removed and a mixture of reagents and chemical buffers is loaded into the chamber, a process that is repeated twice. After this procedure, the biochip is inserted into the specifically adapted optical reader to be analysed with the help of an easy to use software, the algorithm of which was developed in the present invention (Figure 7).

[0060] The antibodies for secondary linkage (known as "secondary antibodies"), who recognize the IgM and IgG antibodies attached to the antigens, are conjugated with Quantum Dots. When an antibody in the sample binds to an antigen and afterwards with a secondary

antibody, the following connection is expected to occur: antigen - sample antibody - secondary antibody - Quantum dot. Thus, when this connection occurs in the biochip of the present invention, the Biochip Reader of the present invention will excite these Quantum Dots which in turn transmit fluorescence, reading this signal as a positive indication of the presence of infection in the sample and further interpretation parameters apply regarding the stage (early, acute or chronic) and type (autoimmune or coinfection) for the case of Lyme disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0061]

Figure 1 - Design and configuration of the biochip:

1. 1 - Reservoir 1 (sample)
2. 2 - Reservoir 2 (PBS1 and PBS2 washings)
3. 3 - Reservoir 3 (Reagent)
4. 4 - Working area (field of view and capillary valves)
5. 5 - Pump 1
6. 6 - Pump 2
7. 7 - Microfluidic channel 1
8. 8 - Microfluidic channel 2
9. 9 - Microfluidic channel 3
10. 10 - Microfluidic channel 4
11. 11 - Microfluidic channel 5
12. 12 - Microfluidic channel 6
13. 13 - Microfluidic channel 7
14. 14 - Microfluidic channel 8
15. 15 - Microfluidic channel 9

Figure 2 - Optical micrograph of the field of view (4) comprising analysis chambers with up to 50 μm length, up to 50 μm width and up to 15 μm deep.

Figure 3 - Antigen bouquet for differential detection of Lyme disease.

Figure 4 -Clamping system of the biochip optical reader

16 - mechanical stoppers

17 - two pushers

Figure 5 - Insertion and removal system of the biochip optical reader

18 - two guideway chariots

19 - electromagnet

20 - two permanent magnets

Figure 6 - Software algorithm architecture as implemented on the biochip optical reader.

DETAILED DESCRIPTION OF THE INVENTION

1. *Bouquet* of 16 antigens

[0062] An innovative set of antigens was designed, which we call a new *bouquet* of 16 specific antigens, to be used in whole or in part for the detection of Lyme disease, they being described as: IgG and IgM, both used as positive control for the immune reaction; Cell Lysates of *E. Coli*, used as negative control for infection of *Borrelia burgdorferi f.l.*; OspC (Outer Surface Protein C), C2 19-mer (DAASVNGIAKGIGIVDAA) and C6 25-mer (MKKDDQIAAAMVLRGMAKDGGQFALK). All together, these are antigens of acute infection caused by *Borrelia burgdorferi f.l.*;

OspE1/2 (Outer Surface Protein AND1/2) OspA (Outer Surface Protein A) and DbpA (Decorin binding protein A). Together, these are antigens of chronic infection caused by *Borrelia burgdorferi f.l.*;

LFA1 (Lymphocyte Function Antigen-1) and Arp (Arthritis-related proteins GST-like self-antigen), that are autoimmune antigens of infection caused by *Borrelia burgdorferi*;

Peptides of *Babesia microti* (with the sequence IVEFNAIFSNIDLNNSSTVKNEIHK) and *Ehrlichia* (with the sequence SAVSNRKLPLGGVLMALVAAPVPIHSALLA), for detecting the occurrence of co-infections;

Borrelia burgdorferi B31 spirochete, *B. burgdorferi* B31 cysts, *Borrelia garinii* and *Borrelia azfeli*, used as cell lysates as positive control for infection by *Borrelia* and for their nearest related species.

[0063] In this *bouquet*, 14 of antigens had already been discovered and 2 antigens were discovered and produced specifically for this *bouquet* for detection of Lyme disease, being here identified as the antigens **DbpA** and **Arp**.

1.1 Cloning of the DNA coding for antigen Decorin binding protein A (DbpA)

[0064] To express the antigen DbpA (rDbpA), we selected the plasmid pBAD/His for inducible expression in *E. Coli*. The protein will be expressed as a fusion protein with a 6-His-Tag in position N-terminal. The 6-His-tag is small, not immunogenic and will allow the purification through connection matrices of ionized metal.

[0065] Initially the expression plasmid was amplified and purified (Midiprep QIAfilter Plasmid

Purification Kit # 12243). Subsequently, 2 µg of the purified plasmid and the entire amplicon DbpA were digested with the restriction enzymes *HindIII* *XhoI* during the night at 37°C, following the conditions of Table 4 described below:

Table 4

Digestion with <i>XhoI</i> and <i>HindIII</i>	Plasmid (µl)	DbpA (µl)
Deionized Water and free from nucleases	25,5	35
DNA template	15	50
NEB 2 buffer (10x)	5	10
BSA (100x)	0,5	1
<i>XhoI</i> (20 u/ul)	2	2
<i>HindIII</i> (20 u/ul)	2	2
Final Volume	50	100

[0066] The plasmid and amplicon from DbpA protein were seen on a 0,8% agarose gel (330 V for 70 minutes) and purified with QIAQuick Gel Extraction. The cleavaged DbpA was then connected to the digested plasmid, in a ratio of 1:3, for 3 hours at room temperature, and 5 µL transformed into cells *E. coli* NEB 5-alpha (25 µL). The mixture of transformation (200 µL) was placed on plates *Luria-Bertani* (LB), containing these plates 50 µg of ampicillin per mL, and grown during the night at 37 °C.

Table 5

Reagents used in the reaction of ligation	Ratio 1:3 (µL)	Without the plasmid insertion (µl)	Without plasmid insertion and DNA ligase (µl)
Deionized water and free from nucleases	5	7	8
T4 Ligase Buffer (10x)	2	2	2
Plasmid DNA (~10 ng/ µl)	10	10	10
Plasmid insertion (~25 ng/µl)	2	-	-
T4 DNA ligase (3U/µl, Promega, #M1801)	1	1	-
Final Volume	20	20	20

[0067] Several positive colonies are amplified in 2 mL of LB (165 µg of ampicillin/mL), purified (QIAfilter Plasmid Miniprep Purification Kit # 37104), digested with *XhoI*/*HindIII* at 37°C for 2 hours, run in agarose gel (1%), to confirm the presence of the gene DbpA. 5 Minipreps are obtained and digested confirmed as positive, of which 2 are selected for sequencing. After confirmed that the sequence exactly matches the gene DbpA (included the His-tag), 200 µl of

one of the positive cultures of minipreps processed with TOP10, is grown in 200 mL of LB (100 µg of ampicillin/mL) at 37 °C during the night. The selection of TOP10 cell is because it is a strain of *recA/endA*, capable of transporting L- arabinose, but not capable of its metabolization. The DNA of DbpA construct of these 200 mL of culture is purified with Qiafilter Plasmid Midi kit (Qiagen #12243), transformed (2,8 µl, ~100 ng) in cells TOP10 (15 µl), and grown in plates of LB (100 µg/mL ampicillin) at 37°C during the night. Finally, one of the colonies was again replaced to generate stocks in glycerin and for expressing rDbpA.

1.2. Cloning of antigen Arthritis-related proteins GST-like self-antigen (Arp)

[0068] For expression of the recombinant protein Arp (rArp), the same plasmid used in the expression of the DbpA, plasmid pBAD/His (Invitrogen) is used to express the protein 6-His-tag. The recombinant plasmid is amplified and purified as described in the previous section to the DbpA, but instead of *HindIII*, *EcoRI* was used. 1 µg of plasmid and the entire gene amplified and purified Arp were digested with *XhoI* and *EcoRI* for 3 hours at 37 °C, following the conditions of the following table:

Table 6

Reagents for digestion with <i>XhoI</i> and <i>EcoRI</i>	Plasmid (µl)	Arp (µl)
Deionized Water and free from nucleases	40	11,5
DNA template	1,5	30
NEB 4 buffer (10x)	5	5
BSA (100x)	0,5	0,5
<i>XhoI</i> (20 u/ul)	1,5	1,5
<i>EcoRI</i> (20 u/u)	1,5	1,5
Final Volume	50	50

The plasmid and protein were separated on a 0,8% agarose gel (330 V for 70 minutes) and purified with QiAQuick Gel Extraction. The cleaved Arp was then connected to the plasmid of digestion, in a ratio of 1:3, during 75 minutes at ambient temperature, and 5 µL transformed into cells *E. coli* NEB 5-alpha (25 µL) . The mixture of transformation (200 µL) was placed on plates *Luria-Bertani* (LB), containing these plates 50 µg of ampicillin per mL, and grow during the night at 37 °C.

Table 7

Reagents for reaction of connection	Ratio 1:3 (µL)	Without the plasmid insertion (µL)	Without plasmid insertion and DNA ligase (µL)
Deionized Water and free from nucleases	12,7	14,5	15,5
T4 Ligase Buffer (10x)	2	2	2
Plasmid DNA (~10 ng/ µl)	2,5	2,5	2,5

Reagents for reaction of connection	Ratio 1:3 (μL)	Without the plasmid insertion (μL)	Without plasmid insertion and DNA ligase (μL)
Plasmid insertion (~25 ng/μl)	1,8	-	-
T4 DNA ligase (3U/μl, Promega, #M1801)	1	1	-
Final Volume	20	20	20

[0069] Several colonies positive were amplified in 2 mL of LB (165 μg of ampicillin/mL), purified (QIAfilter Plasmid Miniprep Purification Kit # 37104), digested with *XhoI/EcoRI* at 37°C during the night, and separated in agarose gel (1%), to confirm the presence of the gene Arp. Two colonies of positive minipreps were selected for sequencing. After confirmed that the sequence exactly matches the gene Arp (included the His-tag), 200 μL of one of the positive cultures of minipreps processed with TOP10, grow in 200 mL of LB (100 μg of ampicillin/mL) at 37 °C during the night. From this 200 mL of culture, the DNA of Arp construct was purified with Qiafilter Plasmid Midi kit (Qiagen #12243, transformed (1 μL, ~100 ng) in cells TOP10 (15 μL), and grows in plaques of LB (100 μg/mL ampicillin) at 37 °C during the night. Finally, one of the colonies was again replicated to generate stocks in glycerin and for expressing rArpn.

2. Interpretation of results the Biochip

[0070] A sample of the patient, which is possibly infected with the Lyme disease is initially collected. This sample can be a fluid (urine, whole blood, serum, plasma, cerebrospinal fluid, sweat, saliva) or tissue lysates (of different tissues). Add about 3 μL of the filtered and diluted solution (1/200) of the sample in 0,1% Tween 20/PBS and leave to the appropriate entry of the Biochip during 9 minutes. Then remove the excess of sample and wash the respective entry with about 3 μL of a filtered solution of 0,1% Tween 20/PBS. Goes back to remove the excess of the previous solution and adds about 3 μL of a solution of 1/10 and 1/100 of qDot525 and qDot625 functionalized secondary antibodies and leave it at that entry during 9 minutes. Again, removes the excess of the previous solution and goes back to perform a washing operation with about 3 μL of a solution of 0,1% Tween 20/PBS. After this procedure is performed, the biochip is ready to be read in the optical biochip reader of the present invention.

[0071] The qDots listed above are fundamental, because they are revealing agents that will allow the identification of the linkage between the first agent of connection of the reaction (one of 16 antigens fixed in Biochip) with the analyte (sample) and then with the second agent of connection to the trapped antibody (IgG or IgM). If there is a linkage between the first agent of connection of, the analyte, and then with the second agent of ligation of the reaction, when the biochip is scanned in the optical reader of the present invention, fluorescence will be emitted, indicating that the test was positive for Lyme disease and the patient is infected with the

bacterium *Borrelia*. If a connection does not occur, this means that the sample of the patient does not have antibodies against the bacterium *Borrelia burgdorferi f.l.* and the patient is not infected with Lyme disease in any extent.

3. Quantum Dots, revealing agents

[0072] QuantumDots (qDots) are a distinct group of fluorescent compounds that differ from conventional methods in that they have unique spectral characteristics. Their cores of heavy metals are responsible by these original spectral characteristics and are composed of potentiometer material: seleno to cadmium (CdSe) or indium phosphide And gallium (InGaP). The cores usually possess technological properties of coating that allows the combination with various biomolecules, including antibodies, proteins and peptides. This coating is also a polymer which provides a surface soluble in water that can modify the combination with other biomolecules such as antibodies and proteins. These qDots are adjustable in size (2-50 nm), usually of 10-20 nm, have spherical shape, that allows qDots to be adjusted to a specific wavelength emission. The qDots are intrinsically bright, have very narrow emission spectra, have photo-stability, are easy to use, and can be used in a multitude of applications. The use of these Dots in biomedical applications is just emerging and gaining popularity. Due to their spectral characteristics the qDots have fundamental high performance in a variety of applications, such as flow cytometry, immuno-histochemistry or in our case, as molecules of detection for human sera and other biological samples.

[0073] In the case of this invention, qDots 525 and 625 were chosen particularly for the case of the antigen bouquet used for detecting Lyme disease. The number mentioned after qDots corresponds to the emission peak of the said qDot, in our case the qDots chosen for this invention will have peaks of emission at 525 nm (qDots 525) and 625 nm (qDots 625). Both qDots will be combined with secondary antibodies that are anti-human IgG or anti-human IgM forming the second complex "Legend-Qdot". This complex will recognise and connect to the first complex ligand-analyte (human serum), in case that serum comprises antibodies against *Borrelia burgdorferi f.l.* If the Ligand immobilized (antigen) - analyte (serum antibodies) - Second Ligand (secondary antibody) - qDot complex is present when the Biochip is placed on the Reader of chemo-luminescence, the qDots will be excited to the wavelengths referred above, and will transmit fluorescence, accusing a positive diagnosis for Lyme disease. Preferably, for Lyme disease, IgG will be revealed and quantified by qDot 525 and IgM will be revealed and quantified by qDot 625, in order to differentiate not only the different stages (early, acute and chronic) or absence of the disease but also to detect antibodies associated to autoimmunity related to Lyme disease and coinfection between *B. burgdorferi* and other antigens.

REFERENCES CITED IN THE DESCRIPTION

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P A T E N T K R A V

1. System til at detektere og overvåge tilstedeværelsen af antistoffer, der er forbundet med forskellige stadier af borreliose, som er til stede i biologiske prøver, omfattende
- en antigenbuket
 - 5 - en biochip
 - en optisk læser til biochippen; og
- k e n d e t e g n e t v e d det faktum, at antistofferne, der er til stede i de biologiske prøver, detekteres specifikt for akut, kronisk, autoimmunitet og co-infektion relateret til borreliose, hvor antigenbuketten omfatter
- 10 seksten antigener, især IgG og IgM, cellelysater af *E. coli*, OspC, OspA, DbpA og Arp, OspE1/2, LFA1, Arp, peptider fra *Babesia microti* og *Ehrlichia*, *Borrelia burgdorferi* B31 spirochete, *Borrelia burgdorferi* B31 cyster, *Borrelia garinii* og *Borrelia azfeli*; og ved at nævnte biochip omfatter et første reservoir (1) egnet til at indeholde biologiske prøver, et
- 15 andet reservoir (2) til vaskebufferen, et tredje reservoir (3) til kvanteprikker, et arbejdsområde og synsfelt (4) omfattende ventiler, reaktionskamre og analysekamre, hvor reaktions- og analysekamrene har antigenbelægninger, med mindst en første pumpe (5), en anden pumpe (6) og ni mikrofluidiske kanaler (7, 8, 9, 10, 11, 12, 13, 14 og 15).
2. System ifølge krav 1, k e n d e t e g n e t v e d det faktum, at de biologiske
- 20 prøver er serum eller cerebrospinalvæske.
3. System ifølge krav 1, k e n d e t e g n e t v e d det faktum, at vaskebufferen er 0,1% Tween 20/PBS-opløsning.
4. System ifølge krav 1, k e n d e t e g n e t v e d det faktum, at kvantepunkterne fluorescerer mellem 400 og 900 nm, fortrinsvis mellem 500 og 700 nm, og mere fortrinsvis
- 25 ved 525 nm og 625 nm, og er konjugeret til anti-humant IgG eller anti-humant IgM antistoffer.
5. System ifølge de foregående krav, k e n d e t e g n e t v e d det faktum, at biochip-arbejdsområdet og synsfeltet (42) måler op til 1 mm i længden og op til 1 mm i bredden, fortrinsvis 350 µm i bredden, og omfatter mindst seksten reaktions- og analysekamre.
- 30 re.
6. System ifølge de foregående krav, k e n d e t e g n e t v e d det faktum, at hvert reaktions- og analysekammer, der er omfattet inden i arbejdsområdet og synsfeltet (42), har en længde op til 50 µm, op til 50 µm i bredden og er op til 15 µm dyb.
7. System ifølge de foregående krav, k e n d e t e g n e t v e d det faktum, at biochippen omfatter kapillærvæskepumper (43) og (44) med en strømningshastighed på
- 35 mindst 0,2 µL/minut, fortrinsvis med en strømningshastighed på 0,4 µL/minut.
8. System ifølge de foregående krav, k e n d e t e g n e t v e d det faktum, at den optiske læser til biochippen omfatter en fluorescensdetektor, der indeholder en indre struktur omfattende en lyskilde, fortrinsvis LED, et kamera, et linserør, Köhler-

belysningssystem, et autofokussystem, en mekanisk ramme og en positioneringsindretning til fast objektiv.

9. System ifølge krav 8, k e n d e t e g n e t v e d det faktum, at den optiske læser til biochippen yderligere indeholder en mellemstruktur omfattende lysafskærmninger, en
 5 kabinetramme, et stikpanel, en afstivning, et computerrum og en ydre struktur omfattende hovedkabinettet, en berøringsskærm, ventilationshuller og en faldlem, hvor fluorescensdetektoren er specielt designet til at anvende fluorescerende kvanteprikmarkører og er specielt tilpasset til at være optisk koblet og justeret til et biochip indsættelses- og fjernelsessystem gennem et spændesystem og autofokussystemet har et detekteringsfelt op til 1 mm i
 10 længden, fortrinsvis 480 μm og op til 1 mm i bredden, fortrinsvis 480 μm , og at detekteringsfeltet omfatter biochip-synsfeltet (4) med ca. 65 μm tolerance i både X- og Y-akse til positioneringsnøjagtighed.

10. System ifølge krav 9, k e n d e t e g n e t v e d det faktum, at spændesystemet til den optiske læser til biochippen omfatter tre kontaktpunkter med mekaniske propper
 15 (16) med en justerbar position og to skubbere (34), der skubber biochippen mod disse propper, hvor X-, Y-spænderne og to elastiske spænder holder biochippen på plads langs Z-aksen, og retur fjedrene (17) trækker skubberne tilbage for at tillade indsættelse og fjernelse af biochippen.

11. System ifølge krav 9, k e n d e t e g n e t v e d det faktum, at indsættelses- og fjernelsessystem til den optiske læser til biochippen består af et skuffesystem omfattende
 20 et stykke magnetisk rustfrit stålmaterial og en føringsmekanisme med to føringsvogne (18) pr. skinne, hvor skuffen blokeres af en elektromagnet (19) i den lukkede position, hvor kontakten mellem skuffen og elektromagneten sikrer en perfekt X-akse tilpasning mellem detektoren og biochippen, hvilket holder detektoren isoleret fra lys, og hvor skuffen er blokeret med to permanente magneter (20) i den åbne position for at holde den på
 25 plads under biochip påsættelsen.

12. Fremgangsmåde til anvendelse af systemet til at detektere og overvåge tilstedeværelsen af antistoffer associeret med borreliose i biologiske prøver ifølge krav 1 til 11, k e n d e t e g n e t v e d følgende trin:

- 30 a) at køre en algoritme i den optiske læser;
- b) at tilsætte 3 μL af en filtreret 1:200 fortynding af den biologiske prøve i 0,1% Tween 20/PBS i op til 9 minutter til det respektive kammer;
- c) en vaskeoperation med 3 μL filtreret 0,1% Tween 20/PBS;
- d) at tilsætte 3 μL af en 1:10 fortynding af kvantepunkt 525 og en 1:100 fortynding af
 35 kvantepunkt 625 i op til 9 minutter;
- e) en anden vaskeoperation med 3 μL filtreret 0,1% Tween 20/PBS;
- f) at indsætte biochippen i den optiske læsers skuffe;
- g) at indtaste information tilgængelig fra webbrugerfladen; og
- h) at trykke på starttestknappen,

hvor enheden placerer biochippen i et autofokussystem i op til 2 minutter, eventuelt venter på afslutningen af migreringen, udfører billedanalyse i op til 2 minutter, gemmer og viser resultater på websiden på op til 5 sekunder.

13. Fremgangsmåde ifølge krav 12, k e n d e t e g n e t v e d det faktum, at de biologiske prøver fortrinsvis er serum eller cerebrospinalvæske.

14. Fremgangsmåde til anvendelse af systemet ifølge krav 1-11, ifølge krav 12 til 13, k e n d e t e g n e t v e d det faktum, at biochippen indsættes og fjernes gennem et spændesystem, der omfatter tre referencekontaktpunkter med mekaniske propper (16) med justerbar position og to skubbere (34), der skubber biochippen mod disse propper, hvor X- og Y-spænderne og to elastiske spænder holder biochippen i stilling langs Z-aksen og returfjedrene (17) trækker skubberne tilbage således at indsættelse og fjernelse af biochippen er muligt.

15. Fremgangsmåde til anvendelse af systemet ifølge krav 1-11, ifølge krav 12-14, k e n d e t e g n e t v e d det faktum, at spændesystemet er en del af et skuffesystem omfattende et stykke magnetisk rustfrit stålmaterial og en føringsmekanisme med to føringsvogne (18) pr. skinne, hvor skuffens lukkede position er blokeret af en elektromagnet (19), og kontakten mellem skuffen og elektromagneten sikrer en perfekt X-akse tilpasning mellem fluorescensdetektoren og biochippen, hvilket holder fluorescensdetektoren isoleret fra lyset, og hvor skuffen blokeres med to permanente magneter (20) i den åbne position for at holde den på plads ved biochip påførelse.

16. Fremgangsmåde til anvendelse af systemet ifølge krav 1-11, ifølge krav 12-15, k e n d e t e g n e t v e d det faktum, at autofokussystemet omfatter en Z-akse-oversættelsesplatform med et bord der føres med et kugleleje, som er motoriseret med en børste-DC-servomotor og som virker gennem tre trin:

- a) den scanner et stort interval af afstande mellem biochippen og objektivet i størrelsesordenen 1 mm, med store bevægelsestrin i størrelsesordenen 50 μ m mellem hver billedoptagelse, hvor bevægelsen i Z-aksen er kontinuerlig og billeder tages under bevægelsen, og afstanden som billedet er skarpest ved registreres;
- b) den scanner nøjagtigt med små bevægelser i en størrelsesorden på nogle få mikrometer, fortrinsvis mellem 5 og 50 μ m, hvor afstandsintervallet er bestemt i det første trin, for at finde fokus-afstanden, Z-aksen bevæger sig og derefter tages et billede når Z-aksen stoppes og, så snart hele rækkevidden af det andet trin er scannet, bevæger Z-aksen sig tilbage til den position, der svarer til det skarpeste billede; og
- c) den bevæger sig ned ad Z-aksen med en afstand svarende til dybden af mikrofluidkanalerne i biochippen, fortrinsvis mellem 15 og 20 μ m og bevæger sig derefter ned langs Z-aksen op til 10 μ m for at kompensere for den optiske forskel mellem lysfeltfilter og fluorescensfiltre.

17. Fremgangsmåde til anvendelse af systemet ifølge krav 1-11, ifølge krav 12-16, k e n d e t e g n e t v e d det faktum, at algoritmen udtrækker den nøjagtige placering af

de forskellige reaktionskamre på det skarpe billede indsamlet i krav 16, udfører billedanalyse og fremstiller en rapport, der omfatter:

- a) at nedskalere af et billede til en lavere opløsning
- b) at transformere billede fra tidsdomænet til frekvensdomænet under anvendelse af
- 5 Fourier-transformation;
- c) at korrelerer det frekvensmæssige billede med sig selv gennem autokorrelation;
- d) at omvendt Fourier-transformere det autokorrelerede billede;
- e) at kortlægge det "omvendte Fourier-billede" til pixeldomænet;
- f) at beregne middel- og standardafvigelsen for pixelintensiteter af det originale billede
- 10 med lavere opløsning;
- g) at beregne korrelationsfaktoren mellem det originale billede, såsom middel- og standardafvigelse, og pixels i det "omvendte Fourier-billede";
- h) at beregne scoren baseret på i det mindste korrelationsfaktorne fra forrige trin;
- i) at finde synsfeltet, dets placering og orientering gennem mønstermatchning, hvor
- 15 mønstre dannes ved at tegne omkring 16 tilsigtede regioner, der er på linje med mønsteret, hvor disse omkring 16 regioner svarer til de områder, hvor data bruges til at tilvejebringe diagnosetestresultater; og
- j) at anvende en mønstermatchende algoritme på mønsteret gemt fra det forrige trin; hvor den nye billedanalysealgoritme fungerer ved 4 på hinanden følgende trin, nemlig at
- 20 bestemme den bedste kameraeksponeringstid, at bestemme rester og fjernelse deraf, at bestemme baggrundsfluorescens og at analysere reaktionskamrene uden rester og baggrundsfluorescens.

18. Fremgangsmåde til anvendelse af systemet ifølge krav 1-11, ifølge krav 12-17, k e n d e t e g n e t v e d d e t f a k t u m , a t d e t o m f a t t e r k ø r s l e n a f e n t i l p a s s e t a l g o r i t m e i

25 den optiske læser og:

- a) at tilsætte 3 μ L af en filtreret 1:200 fortynding af den biologiske prøve i 0,1% Tween 20/PBS i op til 9 minutter til det respektive kammer;
- b) en vaskeoperation med 3 μ L filtreret 0,1% Tween 20/PBS;
- c) at tilsætte 3 μ L af en 1:10 fortynding af kvantepunkt 525 og en 1:100 fortynding af
- 30 kvantepunkt 625 i op til 9 minutter;
- d) en anden vaskeoperation med 3 μ L filtreret 0,1% Tween 20/PBS;
- e) at indsætte biochippen i den optiske læsers skuffe;
- f) at indtaste information tilgængelig fra en webbrugerflade; og
- g) at trykke på starttestknappen;
- 35 hvor enheden placerer biochippen i et autofokussystem i op til 2 minutter, eventuelt venter på afslutningen af migreringen, udfører billedanalyse i op til 2 minutter, gemmer og viser resultater på websiden på op til 5 sekunder.

DRAWINGS

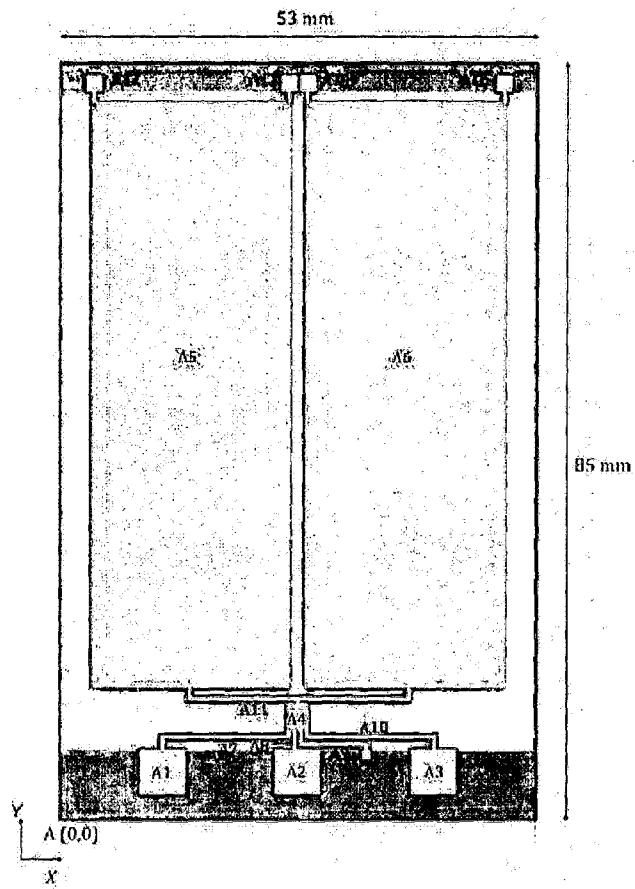


Figure 1

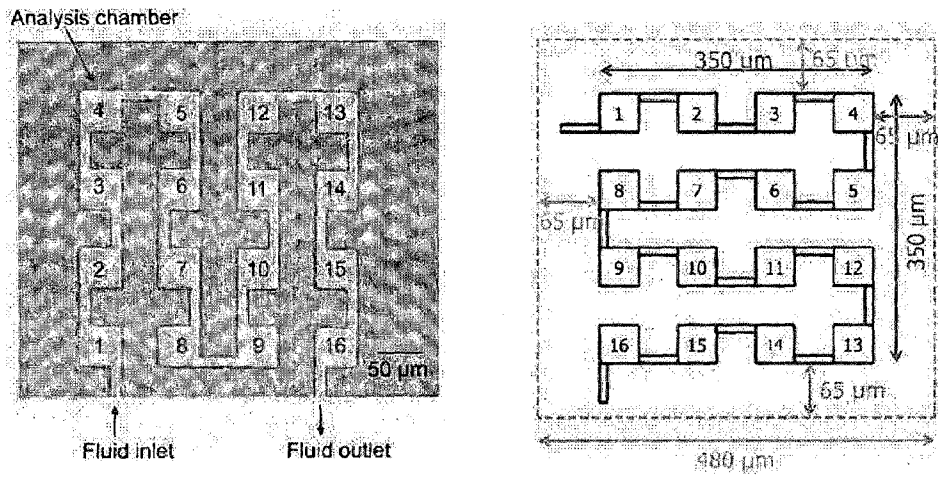


Figura 2

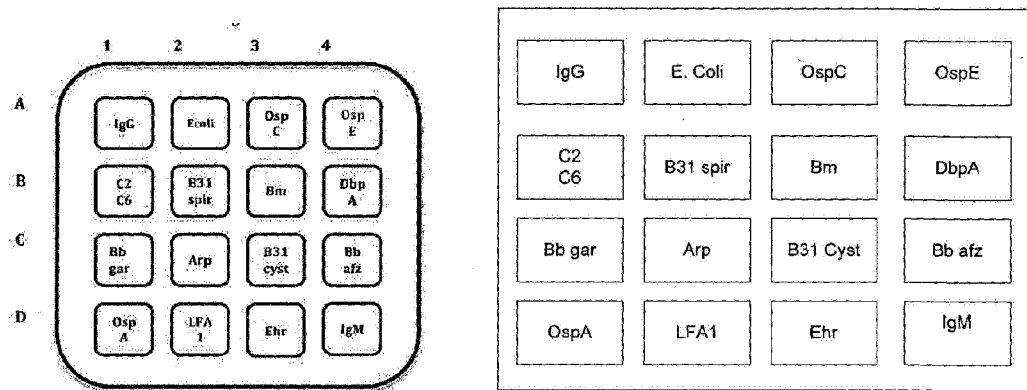


Figura 3

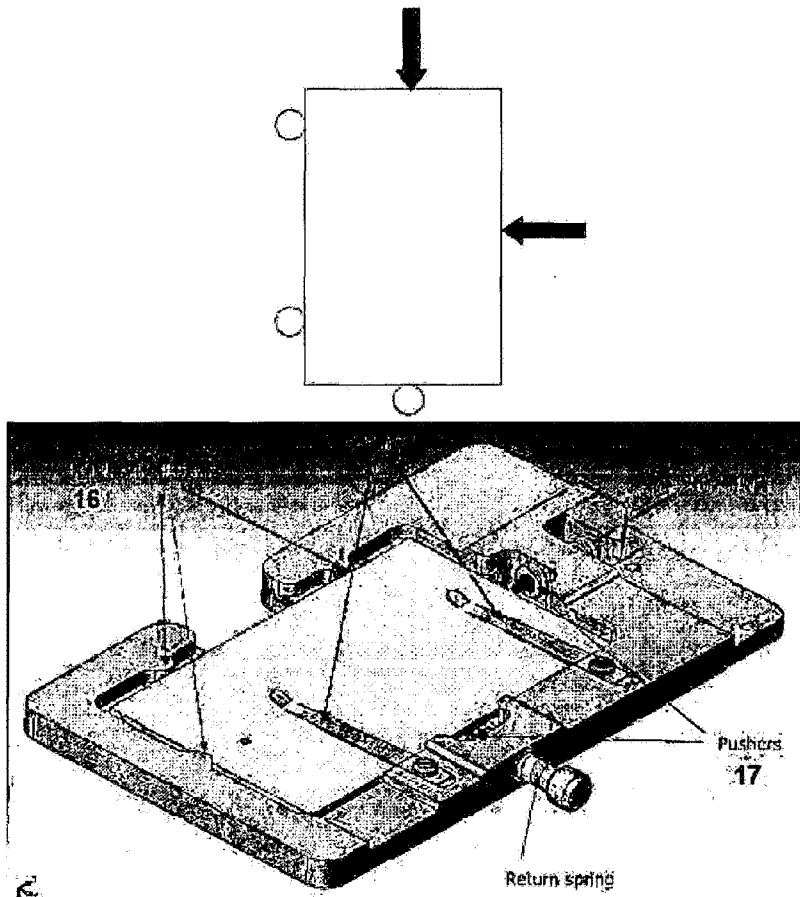


Figure 4

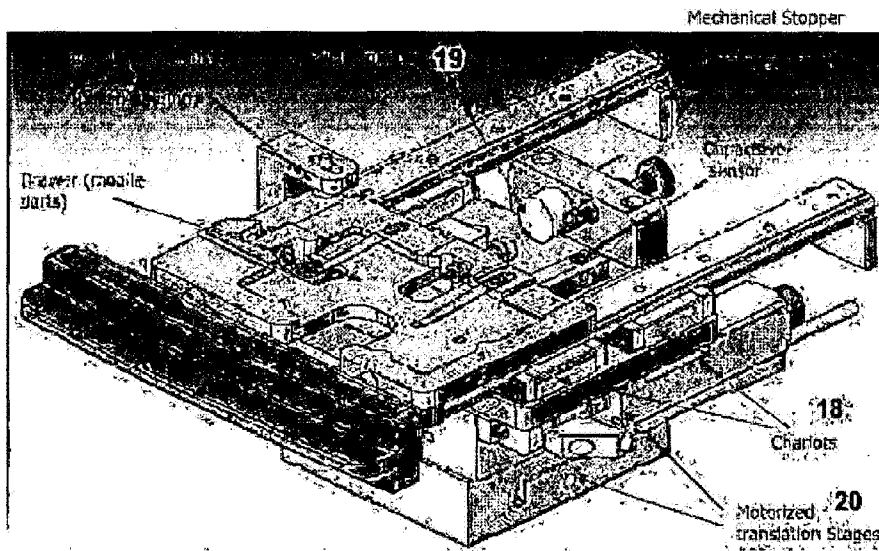


Figura 5

