PRODUCT DESCRIPTION

DualDur In Vitro System for the Diagnosis of Lyme Borreliosis

Manufactured and distributed by: *Lyme Diagnostics Ltd.*
2011 Budakalász, Zöldfa utca 16.
Contact Person: *Bózsik András Pál, Managing director*
Website: *http://lymediagnostics.com/*
Phone: *+36 20 9388 408*
E-mail: *apbozsik@lymediagnostics.com*

CONTENTS

Executive Summary 4

Historical background of Lyme borreliosis 4

DualDur© 5

Artificial Intelligence Microscopy 6

Results 7

- In vitro sensitivity and specificity – manual microscopy and PCR 7
- Level of detection 8
- In vitro Conclusions 9
- Clinical examination 9
- Sensitivity and specificity, PPV 10

Other Results 12

CE Registration 12

References 14

EXECUTIVE SUMMARY

Lyme borreliosis has been probably around for thousands of years and has been of increasing medical and economic significance since the turn of the millennium. The disease is caused by spirochetes belonging to the Borrelia burgdorferi group.

Dark-field microscopy has been a well-established scientific method for studying spirochetes since 1909. It is, however, highly dependent on the concentration of the pathogenic bacteria in the sample. In the case of blood samples, the vulnerability of the Borrelia and the formation of artifacts (pseudospirochetes) are also a problem. The DualDur (DD) medium and preparation method provides a solution to the above problems and allows the presence of the pathogen to be confirmed by direct testing methods before serological reactions become positive and/or symptoms can be seen. Manual dark field microscopy and PCR have been validated using the DD technology. Samples stored in the DD medium in a temperature range of 2 to 36 °C remain stable for at least 172 hours.

To validate the DualDur medium and method, we conducted a prospective, multicenter clinical study in eight Central European study sites involving 400 subjects.

The study results of using the DD medium and method coupled with Artificial Intelligence Microscopy (AIM) showed a sensitivity of 69%, a specificity of 80%, and a positive predictive value (PPV) of 96%. The combination of the DD medium and AIM is the ideal screening tool for the typical patient seen in Lyme clinics. The currently used and widely accepted Western Blot method has a sensitivity of 43% and a specificity of 100%.

HISTORICAL BACKGROUND OF LYME BORRELIOSIS

Borrelias causing Lyme disease have certainly been around for thousands of years, as they have been isolated from the body of Ötzi, a 5,300-year-old ice man who also suffered from the symptoms of the disease.

Reverend John Walker, visiting Deer Island (Jura) in Scotland, known for its flocks of deer, described as early as 1764 how a tick bite can cause

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

the symptoms we attribute to Lyme disease today. (3)

It took several centuries for science to identify the single cause behind the otherwise seemingly independent symptoms. In 1873, Otto Obermeier published his observation that spirochetes, morphologically similar to the causative agent of Lyme disease, could be identified in the patient's blood by dark-field microscopy during recurrent febrile periods, but not in relatively asymptomatic periods.

As technology progressed, more and more features of spirochetes were described. Since 1909, examinations of unstained and unfixed preparations by dark-field microscopy have been able to show with complete certainty the presence of infection caused by Spirochetes, even before the appearance of antibodies (1).

However, there are mild cases where the clinical suspicion is well-founded but the usual tests fail to identify the pathogen due to the low germ count. To solve this problem, the microhematocrit **concentration method**, i.e. the thickening of the blood sample by double centrifugation, has been used since 1972. (2)

In summary, there have always been clinical cases which are not accompanied by elevated antibody levels.

After the exclusion of infections with a different clinical presentation from those of Lyme borreliosis, the morphological detection of the pathogen is diagnostic in itself.

Direct detection of the pathogen Borrelia burgdorferi sensu lato clearly confirms the presenceof Lyme borreliosis.

DUALDUR ©

The DualDur medium (hereinafter: DD medium) developed by Dr. Béla Pál Bózsik for the laboratory diagnosis of Lyme disease can be used for the preparation of blood samples for the detection of pathogenic bacteria using dark-field microscopy. Based on the expertise of Dr. Béla Pál Bózsik, which he gathered during decades working on Lyme

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

disease, we can safely conclude the following: Infected blood samples containing Borrelias stored in the DualDur medium and concentrated using the DualDur method are free from blood cells and fragments, and Borrelia, as well as other spirochetes, can be easily recognized using dark-field microscopy, even if they are only present in extremely low concentrations. At all stages of the infection, Borrelia count is at least two magnitudes higher than the level of detection of DualDur.

The DualDur medium provides a high sensitivity and specificity in microscopic and PCR examinations. This was also confirmed by examining artificially inoculated serum samples, in which the presence of Borrelias could be detected reliably. The limit of detection is orders of magnitude lower than that of conventional methods.

ARTIFICIAL INTELLIGENCE MICROSCOPY

Medical image processing is an interdisciplinary field encompassing computer science, electrical engineering, physics and medicine. Its fields of application are extremely wide and can be customized for a specific purpose, from the detection of malignancies on CT images to the detection of bacteria under a microscope. Using computerized data processing and evaluation, images and videos recorded with medical imaging devices can be analyzed faster, more accurately, and in a more consistent and more reproducible way than with traditional methods. The results of the automated analysis would normally be confirmed by an experienced investigator, thus leaving the final decision in human hands.

The automated image processing algorithm developed in the framework of the DualDur project also fits into this trend. It aims to significantly reduce the time spent performing the microscopic examination of the samples, to enable examinations 24 hours a day if necessary, and to reduce the cost of the examination by reducing the time skilled human resources are required. In addition, by eliminating test errors, you can increase the diagnostic effectiveness of examination for the detection of Lyme disease by using the DualDur Blood Sample Preparation Method in conjunction with dark-field microscopy. Samples stored in the DD medium undergo a special preparation. The

LYME DIAGNOSTICS

automatic microscope of Lyme Diagnostics Ltd. scans the slide along a predefined route and uses a proprietary software to search for spirochetes characteristic for the causative agent of Lyme disease in each field of view. The microscope uses readily available electronic devices and their software for its operation.

The results produced by the microscope and the software need to be validated by a skilled laboratory specialist and interpreted by a clinical specialist.

RESULTS

In vitro sensitivity and specificity – manual microscopy and PCR

Sensitivity	93%
Specificity	87%
Positive Predictive Value (PPV)	88%
Negative Predictive Value (NPV)	92%

CONCLUSIONS:

DualDur microscopy can identify a Borrelia infection with great certainty (sensitivity 93%).

DualDur microscopy can differentiate Borrelia species from Treponema species visually, with 88% specificity.

DualDur microscopy has a high positive and negative predictive value.

DualDur with PCR has a sensitivity and specificity of 100%. The combination of the DualDur method with the PCR direct assay resulted in a sensitivity and specificity of 100%.

LEVEL OF DETECTION

Borrelia concentration in the original sample	Cells / ml
DualDur with dark field microscopy	1 900
EDTA [1] PCR method	53
DualDur PCR method	Less than 5*

** The lowest dilution level in the experiment was 5 cells / ml*

[1] *Ethylene-diamine-tetraacetic acid*

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

CONCLUSIONS

The DualDur method may be able to reach the lowest level of detection possible by a microscope (1 spirochete in 50 fields of view)

The DualDur + PCR method reduces the limit of detection by at least one order of magnitude vs. conventional blood collection using EDTA.

STABILITY

Conclusions: Blood samples stored in DualDur remain stable for at least 172 hours at room temperature so the Borrelia can be analyzed by PCR and microscopy.

IN VITRO CONCLUSIONS

Using the DualDur medium and method, known bacterial strains inoculated into blood plasma can be tested and distinguished both morphologically and genetically. Spirochetes inoculated into a blood sample are stable and can be tested for 172 hours, no artifacts are formed. Detectability can reach the theoretical limit of the respective test methods. Both direct studies demonstrated the potential of this technology, so the launch of a clinical trial was warranted. It should be noted that Borrelias are known to show a constant morphology and a high genetic variability, so for culture-grown Borrelias of known genetic constitution, the 100% performance of the PCR test is not surprising. This does not contradict the low (below 10%) sensitivity seen in clinical practice.

CLINICAL EXAMINATION

A multicenter, prospective clinical study representing the patient population of Lyme clinics and including negative controls was conducted in 8 Central European study sites involving 400 subjects. The aim of the study was to investigate the agreement of the tests results with the clinical diagnosis. In accordance with the EU guidelines, sensitivity, specificity and comparison with standard laboratory methods were examined. Of the laboratory procedures examined, Artificial Intelligence Dark-Field Microscopy (AIM) after application of the DualDur medium and method was the most accurate.

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

SENSITIVITY AND SPECIFICITY, PPV

In our clinical study, we compared the standard, validated methods currently used in Europe with less common and experimental methods. For PCR, we used the standard kit tested in in vitro experiments (examining the sequence encoding the 16S rRNA region). Of the available ELISA and Western Blot tests, we used the German kits which gave the best results in our previous experience. The modified Western Blot procedure is a modification developed by Dr. Béla Pál Bózsik. The method to be examined was Artificial Intelligence Dark-Field-Microscopy (AIM) on samples treated with the DualDur medium and method.

Test methods	ELISA	Western Blot	Western Blot (Bózsik)	PCR	DualDur AIM
Sensitivity	60%	43%	62%	1%	69%
Specificity	83%	100%	94%	98%	80%

Also, **the positive predictive value (PPV) of the DualDur method was 96%, making it an excellent screening test** in addition to achieving the highest sensitivity in this sample. The sensitivity-specificity relationship is significantly influenced by the fact that pathogens can be detected in the blood at the beginning of the infection, while the serological response only develops several weeks later, and the symptoms characteristic of the disease become apparent only years later. Using contextual modelling, the DualDur artificial intelligence system was able to differentiate between cases that were asymptomatic but presumably already recommended for treatment by Lyme experts. Thus, the system was able to provide the clinician with more than just a yes or no answer in both the positive and the negative arm of the study, e.g. equivocal cases where a further observation is recommended.

Based on the conclusions reached above, DualDur compares favorably to other tests available internationally.

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

LYME DIAGNOSTICS

	Method	Principle of operation	Benefits	Disadvantages	Sensitivity
INDIRECT METHODS	Elisa (enzyme-liked immunosorbent assay)	Serology, Binding of antibodies to antigens	Higher sensitivity	Low specificity False negative in Seronegative cases	45-60%
	Western-blot, immunoblot	Serology, Staining by specific antibodies, gel electrophoresis	High sensitivity	Low specificity False negative in Seronegative cases	25-40%
	MELISA (Memory Lymphocyte Immunostimulation Assay)	Type IV hypersensitivity reaction mediated by T cells	High sensitivity	Complex equipment and complicated process, many possibilities for errors, the sample must be tested within 48 hours	89% in late cases, not known for early cases
	Modified LTT (Lymphocyte Transformation Test)	Type IV hypersensitivity reaction mediated by T cells	Shows changes in immune reaction. Can also be used during treatment	Complex equipment and it is complicated, many possibilities for errors Only for known antigens	40-80% in late cases, not known for early cases
	Detection of CXCL 13 from cerebrospinal fluid	Measuring amount of CXC ligand 13 by ELISA kit	High sensitivity and specificity	Invasive and risky intervention Can be used only in cases of neuroborreliosis	91-100%
DIRECT METHODS	Morphological test from 1-2 drops of blood dark-field, grey-field microscopy	Microscopy, from native blood	Can see all spirochetes, including new species, and those with new genetic stock	Specificity issues Non-concentrated sample, ow sensitivity	<30%
	Culturing	Culturing biopsy or blood samples on culture medium	High specificity	Test too long, 1-2 weeks. Biopsy samples are rarely available Success rate is low from blood	13-17%
	Real-time PCR, Nested PCR	Bacterial DNA/ RNA multiple copying, division, separation of specific sections by gel electrophoresis improved methods	High Specificity Ideal for detection of pathogens from ticks	Strongly depends on the primer, New strains cannot be tested, Human DNA masks bacterial DNA, Presence of PCR enzyme inhibiting substances	15-40%
	Next generation sequencing, "OMICS" approach	Detection of the total gene stock of the collected sample	Can also be used in general cases suspicion of Lyme borreliosis is not required.	Not demonstrated in case of Lyme b., suitable DNA database is required, the low cell count of borrelia is problematic	n.a.
	T2MR magnetic resonance assay	Amplification of intact borrelia DNA with the help of T2MR	Testing small concentration bacteria	Experimental procedure	31%

1. table Currently available diagnostic methods for Lyme disease

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

OTHER RESULTS

Our clinical trial, which was the largest multicenter diagnostic study of Lyme borreliosis in Europe, yielded results that clarified assumptions in a number of additional areas. One such important fact was that a correlation was found between groups of symptoms and the different Borrelia strains (serological results) and the morphological statistics. This greatly enhances the chances of successful treatment, as different strains have differing antibiotic susceptibility.

The special native immunofluorescent staining technology, also developed by Dr. Béla Pál Bózsik, tested on a small number of samples on residual blood proved to be a promising procedure and will be studied further.

The next step will be the routine use of our test method in patients presenting at different Lyme clinics, thereby providing further proof of the applicability and relevance of these tests in routine clinical use.

CE REGISTRATION

The DualDur in vitro diagnostic system complies with all applicable laws, directives, regulations and harmonized standards. It is an officially registered tool with certified performance and quality conformity marking (CE marking).

WRITINGS ABOUT US

1. European Parliament resolution of 15 November 2018 on Lyme disease (Borreliosis) https://www.europarl.europa.eu/doceo/document/TA-8-2018-0465_EN.html

2. InvestEU Project overview, 22/07/2020
https://europa.eu/investeu/projects/fast-diagnosis-lyme-disease_de

3. Law Proposition, to study the recognition of the chronicity of the disease of Lyme
National Assembly
http://www.assemblee-nationale.fr/dyn/15/textes/l15b2258_proposition-loi

4. Detection of the cystic forms of Borrelia burgdorferi s.l. to the experimental units BALB/c, NEKOLOVÁ Martina. 2014
https://www.muni.cz/en/research/publications/1184888

Lyme Diagnostics Ltd. | apbozsik@lymediagnostics.com
+36 20 938 8408 | www.lymediagnostics.com

REFERENCES

1. Spirochaeta pallida: Methods of examination and detection, especially by means of the dark-ground illumination. 1909., Brit. med. J., , pages: 1117-20.

2. The use of Microhematrocit Technic for the Recovery of Borrelia duttonii from the Blood. Goldsmid, JM. Mohamed. (1972)., Am. J. Clin. Pathol., pages: 58:165–169 .

3. Lyme Disease – a tick-borne spirochetosis? Burgdorfer, W, Barbour, A. G., Hayes, S., F., Benach, J. L, Grunwaldt, E., Davis, J. P. 1982. June 18., Science, pages: 216 (4552): 1317–9.

Lyme Diagnostics Ltd. | *apbozsik@lymediagnostics.com*
+36 20 938 8408 | *www.lymediagnostics.com*

Lyme Diagnostics Ltd. | *apbozsik@lymediagnostics.com*
+36 20 938 8408 | *www.lymediagnostics.com*