

Borrelia burgdorferi

VlsE – a milestone in diagnostics?

Borrelia infections still represent a major challenge for laboratory diagnostics.

Antibodies are often not detectable in the early phase of the infection.

On the other hand, it is not uncommon that specific antibodies against borrelia are detectable in persons in the endemic regions without there being an acute infection with the associated clinical symptoms. For these reasons, serological laboratory diagnostics can only play a supporting role at present. The diagnosis of Lyme borreliosis must ultimately always be made clinically.

Faced with this dilemma, researchers worldwide are searching for new markers to improve diagnostics. One marker, intensely discussed in recent years, is the VlsE (variable major protein-like sequence, expressed). This is a recombinant-produced part of the surface protein of *Borrelia burgdorferi*. The outward-oriented part of the native protein is highly variable. It changes itself constantly and is used by borrelia to evade the immune system of the host. A protein of this nature is at first sight uninteresting for diagnostics based on stable antigens present in an identical form in as many borrelia as possible.

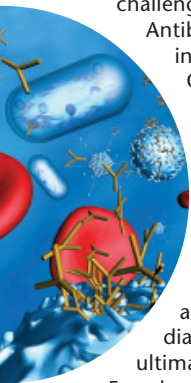
But then it was established that the part with which the protein is anchored in the membrane is very stable. Towards the outside, the stable region is superposed by the variable regions thereby evading access by the immune system. Only once antigen-presenting cells have broken down borrelia in the course of the immunoreponse and the stable regions of the VlsE have presented their surface, is it possible for the immune system to form antibodies against it.

In theory, this would mean that the VlsE would not contribute to improving the sensitivity of a screening ELISA during the early phase of the immunoreponse. On the contrary: An ELISA, which is only based on VlsE should be less sensitive, because other important antigens for diagnostics are absent. However, in the late phase of the immunoreponse, so many different antibodies are identified in a total antigen ELISA that recombinant-produced VlsE should have no effect in plate coating.

For this reason, we compared RIDASCREEN® Borrelia IgG EIA (total antigen Pko strain) with two commercially available IgG-ELISAs. One of the ELISAs is based on total antigen with additional recombinant VlsE. In the other ELISAs, the plates were only coated with VlsE. The species from which the antigen originates can be found in the instructions for use. 80 serum samples were analysed. Most of these samples were submitted to laboratories with the question "borreliosis?". The laboratories kindly provided us with the samples on completion of their diagnostics. The samples were supplemented with a number of blood donation serums.

Each serum, which reacted positively in one of the ELISAs, was checked with a Western blot. A blot was used on the basis of total antigen (Pko strain) and a blot with recombinant antigen. A serum was classed as clearly positive if one of the blots was evaluated positive. Following Western blot analyses, 32 of the 80 serums were clearly IgG positive. Four serums showed positive ELISA results, which were not clearly confirmed in the Western blot.

Of the 32 clearly positive serums, only 26 were positive in all three ELISAs. There was no serum that reacted positive in one of the VlsE tests and negative in RIDASCREEN®



IgG-positive serums	RIDASCREEN® (total antigen)	ELISA A (total antigen + VlsE)	ELISA B (VlsE)
Serum 1	positive	negative	positive
Serum 2	positive	negative	negative
Serum 3	positive	negative	negative
Serum 4	positive	positive	negative
Serum 5	positive	positive	negative
Serum 6	positive	negative	negative
Serum 7-32	positive	positive	positive

EIA. Five of the six discrepant serums were also positive in IgM detection (ELISA + WB). These serums showed a lower number of bands in the IgG blot, which implies an early phase of immunoresponse.

The results show that in an ELISA, the use of VIsE does not necessarily lead to an improvement in sensitivity. It is important for a screening test to obtain the greatest possible diversity of early epitopes. This

ensures the sensitivity necessary for screening. Furthermore, the matching of the overall system is crucial for the quality of an ELISA (selection of antigens, quality of the plate coating, specific activity of the conjugate, cut-off setting). If this is selected correctly, one more protein among a multitude of antigens, which also only has a relatively late effect on the immunoresponse, does not play a decisive role.

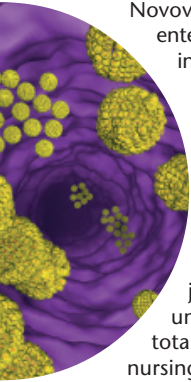
RIDASCREEN® Norovirus ELISA enters its third year!

Our intensive involvement with norovirus diagnostics going back over two years now and the experience gained since the initial launch of the RIDASCREEN® Norovirus ELISA in February 2003 has led to a better understanding of the diagnostics of this pathogen. The second generation of the RIDASCREEN® Norovirus ELISA from R-Biopharm has been successful on the market since April 2004.

According to the Robert-Koch-Institut (RKI), the awareness of this pathogen, which is subject to mandatory reporting, grew intensely in the winter 2004/2005 as a result of the number of infections reported in the middle of December 2004.

This accumulation originates from a new circulating virus variant within the genogroup II by the name of Jam(boore)-II/4. This was first discovered in the Netherlands in the summer of 2004, but not subsequently pursued.

Novovirus infections – first reports of fatalities in Japan!



Novovirus infections have not only entered the realm of public interest in Germany or Europe, but above all in Japan. The "Japan Times" reported for the first time on 28th December 2004 of several fatalities from an infection traceable to this pathogen. In several outbreaks in different nursing homes for the elderly in Hokkaido, Fuku-jama, Hiroshima (7 fatalities) and in Kanagawa (1 fatality) a total of eight residents of these nursing homes died.

This shows how serious an infection with this pathogen can be! Not without good cause did the RKI emphasise that, on the first signs of a norovirus infection, measures should be taken to prevent further infections

without awaiting microbiological results.

The preventative hygienic measures include:

- Isolating the patient (with their own WC), whereby they and their visitors must be instructed to disinfect their hands with an antiseptic agent effective against viruses
- Wearing disposable gloves and a protective coat should be mandatory for the care of the patient

- Daily cleaning of surfaces with disinfectant, including door handles with which the patient comes into contact
- Only carrying out disinfection and removal of vomit and contaminated objects with a surgical face mask
- Transporting of bed and personal laundry in a closed sack
- Washing laundry at a temperature above 60°C

Our project manager Dr. Georgios Kiourkenidis is available to provide further information and answer your questions on norovirus diagnostics under the telephone number +49 6151 8102-96 or you can contact him by e-mail (g.kiourkenidis@r-biopharm.de).



Explanatory notes for a better understanding of VTEC/ EHEC diagnostics in stool

Routine EHEC diagnostics have gained special significance, especially since 1999 when the new German Protection against Infection Act (IfSG) came into effect, in which EHEC infections were classified as infectious diseases subject to mandatory reporting. Screening of stool samples suspected of containing verotoxin takes place in routine diagnostic laboratories, i.e. a yes/no diagnosis is made and it is decided which therapeutic measures should be introduced for the patient and whether further analyses of the sample are necessary.

The highest priority for the ELISA as a screening technique must therefore be its maximum sensitivity, i.e. the avoidance of false-negative results. The enrichment step of the sample is crucial for sensitivity. The nature of the medium and the enrichment conditions have a crucial influence on the quantity of verotoxin expressed into the medium. The optimal induction of toxin formation is essential for raising sensitivity, but it also leads to an increase in the death rate of VTEC/EHEC bacteria in the enrichment broth containing mitomycin C - because the release of verotoxin is always accompanied by the lysis of induced bacteria cells.

Bearing in mind further that the ratio between the VTEC/EHEC bacteria to be enriched in a stool sample under analysis and the other coli flora is 1 : 200-300, then it soon becomes apparent that enrichment, along with the simultaneous cell lysis caused by the toxin release, leads to a drastic decline in the still potentially reproductive pathogen, which, as a result, may ultimately be lost for further cultivation techniques.

In practice, this consequently means that a true-positive verotoxin result in the first ELISA screening may not be confirmed when it is attempted to repeated pathogen enrichment from the sediment of the first enrichment, whether in TSB for repeated ELISA testing or on enterohemolysin agar for subsequent PCR measurement. It is only too easy to rush to the conclusion of a false-positive ELISA result without considering the underlying biology of the pathogen and the conditions under which verotoxin formation occurs.

The potential technical errors in the performance of ELISAs, which mainly lead to real false-positive ELISA results, will not be examined in more detail at this juncture, especially because they are completely avoidable in most labs after thorough troubleshooting. An extensive troubleshooting guide can be requested from R-Biopharm or viewed on the homepage. In very few exceptional cases the specificity of antibody used in the ELISA is the cause for the false-positive results ("unspecific adhesiveness"

of the sample used). The main cause lies in the biology of the pathogen as previously mentioned. Starting with the enrichment conditions selected, to the transport conditions (period, temperature), under which the bacteria from the enrichment culture sediment is dispatched, and finally the transport medium, whose consistency also has an influence on how many bacteria are wiped in the medium when the swab is removed and are consequently lost for the subsequent reproduction desired.

The extent to which potentially reproductive VTEC/EHEC bacteria are lost from one enrichment process to the next was impressively demonstrated by the German Federal Institute for Risk Assessment (BfR) in Dessau (unpublished data).

Here an in-house simulation (i.e. additional transportation and the associated transport temperatures were thereby excluded) showed that the proportion of potentially reproductive bacteria suitable for further verotoxin formation, after initial enrichment in mTSB with mitomycin C (primary culture), repeated inoculation in the same medium and enrichment for 18 h at 13% and inoculation on enterohemolysin agar and incubated for 18 h for PCR, had reduced by 23%.

If the same primary culture was inoculated as a sediment swab from a transport medium in such a way as is proposed by the Reference Centre at the Robert Koch-Institutes (RKI) in its official guide for the dispatch of positive pre-screened samples, and then freshly cultivated for 18 h in mTSB with mitomycin C or on enterohemolysin agar and tested in the ELISA and PCR, the loss of verotoxin forming bacteria in the ELISA was 22% and in the PCR as much as 48%.

This clearly demonstrated that a change in the biological situation may occur for a large proportion of stools as a result of the intermediary stages (enrichment culture, transport medium, enterohemolysin plate), and the subsequent PCR, as well as the ELISA can be negative as a consequence even though the original result is correctly positive.

The validity of this laboratory simulation was investigated in a field study promoted by R-Biopharm AG with both the above institutes and routine diagnostics labs across Germany.

Here samples from routine ELISA pre-screening in the laboratories were sent to the RKI as sediment swabs for validation and from there to the BfR for processing. In parallel, an original stool sample was sent to R-Biopharm AG to be analysed with three commercial ELISAs.

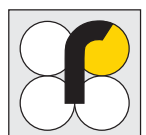


Table. 1: Correlation of screening ELISAs for verotoxin detection

	RIDASCREEN	Premier	Prospect
Samples analysed	112	89	101
of which false-positive	7	7	5
of which false-negative	1	0	3
Deviation (%)	7,1	7,9	7,8

An ELISA was considered as false-negative or false-positive only if it alone deviated from the other ELISA results (Darmstadt, Wernigerode, Dessau) and/or from the confirmation by means of PCR (Wernigerode, Dessau)

Overall the field study turned out somewhat disappointing, because of the 117 samples sent to the RKI, only 81 in total were forwarded to the BfR. And only 91 stool samples reached R-biopharm AG directly.

Therefore only 58 samples successfully passed through all three validation centres.

Of these, 39 had an ELISA value of > 0.5 in primary screening, the remaining 19 were in part significantly lower (5x under 0.2). At the RKI, of the 39 samples, 28 were confirmed with PCR (-28 %) and 30 (-23 %) with ELISA; at the BfR, 22 were confirmed with PCR (-44 %) and just 20 (-49 %) with ELISA, at R-Biopharm 24 (-38 %) were still positive with ELISA.

The BfR simulation results were therefore largely verified in the field study, as the transport and storage conditions had a negative impact on reproductive and toxin formation capability according to expectations.

Whether a stool swab has advantages over a sediment swab dispatched following enrichment in the transport medium in terms of the capability of bacteria to survive was separately tested with 21 samples. Here it emerged that the stool swab was at a slight advantage both for PCR and ELISA. This confirms that bacteria certainly experience an impairment in their further ability to reproduce during the initial enrichment.

The study population of 21 tested samples is however too small to evaluate this result as significant.

Nevertheless, a far more interesting aspect for normal routine work and one of greater importance for ELISA screening was to see the extent to which any one of the three commercial ELISAs on its own showed a different result to the other two ELISAs and/or to the validation centres at the RKI and the BfR (PCR and ELISA were performed) in detecting verotoxin. These results are presented in Table 1.

In summary, it can be ascertained that the commercial ELISAs are all of a comparable standard and the results they provide are reliable within the bounds of the anticipated sensitivity and specificity, assuming they are performed carefully and the directions specified in the instructions are closely observed. The occasional statements expressed on false-positive ELISA results do not stand up to close inspection and analysis and are only justified in a very few exceptional cases.

Because a primary ELISA result not confirmed with PCR is not a deficit of the ELISA specificity, but is essentially attributable to the biology of the pathogen, on the one hand, and probably to a little extent caused by the failure of the subsequent confirmation methods, on the other hand. In this respect, the ELISA can be considered a suitable efficient and reliable method for the detection of verotoxin in stool samples.

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R-Biopharm AG
Landwehrstraße 54, 64293 Darmstadt
Telefon: (0 61 51) 81 02 - 0
Telefax: (0 61 51) 81 02 - 40

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