# Implementation and analysis of *Babesia* immunoassay testing

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***Implementation and analysis of Babesia immunoassay testing***

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*Lifelong withdrawal from the donor population of those who have been diagnosed with babesiosis must be used for transmission prevention.*
***The aim*** *of the study was a detection of Babesia antibodies level with the usage of experimental Babesia divergens whole-cell slide antigen and commercial B. microti immunofluorescence assay substrate slide (Fuller Laboratories, USA).*
***Methods****. Experimental B. divergens whole-cell slide antigen in addition to commercial B. microti IFA substrate slide was used to create a diagnostic kit for serum Babesia antibodies level detecting, as well as for a babesiosis serodiagnosis clinical trial of different origins blood samples (patients with Lyme disease, rheumatoid arthritis and toxoplasmosis; human blood donors; cattle).*
***Results****. Antibodies to B. divergens (5.4%) and B. microti (2.3%) were detected with higher (p <0.05) frequency at Lyme disease patients (16.7%) than at blood donors (1.7%). Diagnostically significant IgG titres (= 1:128) were found in 13.3% of blood samples from Lyme disease patients and 1.7% from blood donors. Specific IgM were also found in 13.3% blood samples from Lyme disease patients. Among blood samples from Lyme disease patients, in which diagnostically significant titres of Babesia antibodies were detected (16.7%), 60% of them were represented by IgG and IgM ($r_A$= 0.63), and in 40% only one of them reached diagnostically significant titre.*
***Conclusions****. Advantages of babesiosis IFA diagnostics are combined with its significant disadvantages (principle of evaluation, low sensitivity in the initial period of the disease, probability of false positives, absence of validated test systems and research protocols for B. divergens and B. divergens-like species).*

**Key words:** *Babesia; IgG; IgM; immunofluorescence assay; Lyme disease*

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***Wykonanie i analiza wyników testu immunologicznego w kierunku Babesia spp.***

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*Stała dyskwalifikacja dawców, u których w przeszłości rozpoznano babeszjozę, jest metodą z wyboru w zapobieganiu rozprzestrzeniania się zakażenia.*
***Celem*** *pracy było półilościowe oznaczenie miana przeciwciał przeciwko Babesia spp. z wykorzystaniem komercyjnego zestawu do testów metodą immunofluorescencji B. microti substrate slide (Fuller Laboratories, USA) oraz pełnokomórkowego składnika B. divergens jako antygenu.*
***Metoda.*** *Dla stworzenia zestawu diagnostycznego do półilościowej oceny miana swoistych przeciwciał anty-Babesia w surowicy oraz w celu przeprowadzenia serodiagnostyki w kierunku babeszjozy, badano próbki krwi różnego pochodzenia (od chorych z boreliozą, RZS, toksoplazmozą, dawców krwi, bydła hodowlanego) z wykorzystaniem jako antygenu komórek B. divergens przyklejonych na szkle w komplecie z komercyjnym zestawem do badań w kierunku B. microti metodą immunofluorescencji.*
***Wyniki.*** *Przeciwciała przeciwko B. divergens (5,4%) i B. microti (2,3%) z większą częstotliwością (p<0,05) stwierdzano u chorych na boreliozę (16,7%) niż u dawców krwi (1,7%). Istotne diagnostycznie miana IgG (= 1:128) stwierdzano w 13,3% próbkach krwi od chorych na boreliozę i w 1,7% próbek pochodzących od dawców krwi. Swoiste IgM wykryto również w 13,3% próbkach krwi pochodzących od chorych na boreliozę. Wśród próbek krwi od chorych na boreliozę (16,7%), u których wykryto istotne diagnostycznie miana przeciwciał przeciwko Babesia, w 60% przypadków znaczące miana przeciwciał było w obu klasach – IgG i IgM ($r_A$= 0.63), a w 40% próbek tylko w jednej z klas.*
***Wnioski.*** *Zalety diagnostyki babeszjozy metodą immunofluorescencji łączą się również z jej istotnymi niedoskonałościami, wynikającymi ze sposobu oceny, niskiej czułości w początkowym okresie choroby, ryzyka otrzymania wyników fałszywie ujemnych oraz z brakiem walidowanych zestawów testowych oraz protokołów diagnostycznych w kierunku zakażeń B. divergens i podobnych jej typów.*

**Słowa kluczowe***: Babesia, IgG, IgM, test immunofluorescencyjny, borelioza*

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Babesiosis is gaining an increasing attention as an emerging tick-borne zoonosis of humans. The main causative agents of human babesiosis are *Babesia divergens* and *B. microti* [10,25] that need more intensive study as other infection condition [4,18]. Considering wide distribution of the vector in temperate latitudes, the prevalence of human babesiosis can be underestimated [5,16].

Although, tick-bite related route of human infection is the main, the risk of infection by blood transfusion from donors with an asymptomatic or chronic disease is considered high [13]. Donors of blood and plasma may be unknowingly infected with *Babesia* [20,21]. Serological prevalence in donor population in the regions in which the pathogen is endemic can be as high as 2%, with 12 to 20% of antibody-positive donors also being positive by PCR [19,29].

New principles for prevention of babesiosis in humans that may occur as a result of the transfusion of donated blood or its components infected by parasites are based on lifelong withdrawal from the donor population of those who have been diagnosed with babesiosis [7,26]. In 2018, the U.S. FDA approved the Imugen *B. microti* Arrayed Fluorescent Immunoassay, for detection of antibodies to *B. microti* in human plasma samples,

and the Imugen *B. microti* Nucleic Acid Test, for detection of *B. microti* DNA in human whole blood samples [28].

The aim of this study was detection of *Babesia* antibodies level in humans and cattle in Ukraine with the usage of experimental *B. divergens* whole-cell slide antigen and commercial *B. microti* IFA substrate slide.

## MATERIALS AND METHODS

A retrospective analysis was performed in patients observed in Regional Hospital of Infectious Diseases, Kharkiv, Ukraine (clinical base of the Department of Infectious Diseases, National Medical University, Kharkiv) from January 2016 to December 2016. Cattle blood samples were obtained from the Department of Parasitology and Tropical Veterinary Medicine (National University of Life and Environmental Sciences, Kiev, Ukraine).

The inclusion criteria for cases were blood donors (clinically healthy people) and patients with potential presence of *Babesia* antibodies (Lyme disease patients). Controls were patients (with seropositive rheumatoid arthritis and toxoplasmosis) that may have false-positive reactions in indirect immunofluorescence assay (IFA).

Experimental *B. divergens* whole-cell slide antigen in addition to commercial *Babesia microti* IFA substrate slide was used to create a diagnostic kit for detecting level of human serum *Babesia* antibodies in IFA, as well as for a clinical trial of babesiosis immune diagnosis by testing 130 blood samples of different origins: 30 – *Lyme disease* patients, 10 – seropositive rheumatoid arthritis patients, 15 – toxoplasmosis patients, 60 – blood donors, 15 – cattle from babesiosis enzootic regions of Ukraine.

Experimental *Babesia divergens* whole-cell slide antigen has been created in Mechnikov Institute of Microbiology and Immunology, Ukraine. This diagnostic procedure is fixed on the surface of slide plates RBC's of a sheep (infected by merozoites of *Babesia divergens* with parasitemia level of about 10%) that can enter into an immunological reaction with *Babesia* antibodies (specific total Ig, IgG and IgM) and adsorb them.

Commercial *Babesia microti* (item number: BM-12, item description: *Babesia microti* 12-well IFA Substrate Slides "Fuller Laboratories", USA) is fixed on the surface of slide plates RBC's of a mice (infected by merozoites of *Babesia microti* with parasitemia level of about 50%).

Completed diagnostic kit consists of whole-cell slide antigens of *Babesia divergens* and *Babesia microti*; commercial fluorescent Anti-Human Ig and Anti-Bull Ig, fluorescent Anti-Human IgG and IgM (Medgamal, Russian Federation); *Babesia* IFA-positive human sera (Fuller Laboratories, USA); liquids for mounting specimens (9:1 mixture of glycerol and phosphate buffer of pH 8.0). Visual evaluation of the IFA results was carried out in four cross-referencing systems by luminescent microscopy using 450-480 nm excitation and 515 nm emission filters.

Statistical analysis using *Pearson's* tetrachoric correlation coefficient was performed. Data were described using standard descriptive statistics, i.e. counts, percentages, means, and standard deviations. Statistical significance was set at $p<0.05$ and all analyses were performed using Statsoft Statistica v10.0.

The study was approved by Mechnikov Institute of Microbiology and Immunology institutional review board.

The article complies with the requirements of the Declaration of Helsinki. The study was approved by the Bioethics Committee of Mechnikov Institute of Microbiology and Immunology.

## RESULTS

Testing of samples of different origins to detect *Babesia* antibodies in IFA was conducted in two stages. Results of the first stage, when all the examined samples were tested in dilution 1:8 with detection of *Babesia* total Ig, by commercial fluorescent Anti-Human Ig and Anti-Bull Ig was given in tab 1. At the second stage of the study, we tested only samples of IFA positive sera. At this stage, sera were tested in dilutions from 1:8 to 1:1024 with detection of *Babesia* IgG and IgM by fluorescent Anti-Human IgG and IgM.

**Table 1.** Results of *Babesia* antibodies IFA detection (sera dilution of 1:8)
**Tabela 1.** Wyniki wykrywania IFA przeciwciał *Babesia* (rozcieńczenie surowicy 1:8)

| Blood samples | n | Positive IFA | |
|---|---|---|---|
| | | ***Babesia microti*** | ***Babesia divergens*** |
| Lyme disease | 30 | 2 (6,7) | 3 (10,0) |
| Rheumatoid arthritis | 10 | 0 | 0 |
| Toxoplasmosis | 15 | 0 | 1 (6,7) |
| Blood donors | 60 | 0 | 1 (1,7) |
| Cattle | 15 | 1 (6,7) | 2 (13,3) |
| Total | 130 | 3 (2,3) | 7 (5,4) |

Ten (2.7%) positive IFA samples was obtained. *Babesia* antibodies were found more frequently at patients with *Lyme disease* (16.7%) and cattle from babesiosis enzootic regions (20.0%). The frequency of *Babesia* antibodies detection at patients with *Lyme disease* exceeded ($p<0.05$) the corresponding figure (1.7%) in a clinically healthy group of blood donors. Accordingly, antibodies were detected against both of the major human species – *Babesia microti* and *Babesia divergens* that confirms circulation of not only dominant in Europe species *Babesia divergens* but also *Babesia microti* – a dominant pathogen of human babesiosis in America [11].

Frequency of diagnostically significant *Babesia* antibodies detection at *Lyme disease* patients is most often detected within (9.5 – 11.5) %, except for the US regions that are endemic for babesiosis, where the frequency reached 37.0% [5,11,17]. Frequency of *Babesia* antibodies detection at blood donors in Germany, France, and Italy, as well as at clinically healthy people, with no exposure of ticks, ranged from 1.1 to 5.4% [11,17,30,9].

Currently, universal criteria for evaluating the diagnostically significant titre of *Babesia* antibodies have not been developed [23,27,12]. We detected *Babesia* antibodies IgG and IgM titres in IFA (in three parallel reproductions) taking into account such diagnostic immunological criteria for positive tests: at humans – IgG in the titre ≥ 1: 128 and Ig M in the titre ≥ 1:32, at cattle – total Ig in the titre ≥ 1:64 [12].

According to the results of our study, diagnostical titres of anti-*Babesia* IgG were detected at four (13.3%) blood samples of Lyme disease patients and in one (1.7%) sample of blood donors. IgG to *Babesia divergens* and *Babesia microti* were detected in 60% and 40% of the cases, respectively. Diagnostic titres of anti-*Babesia* IgM were detected at four (13.3%) blood samples of Lyme disease patients. IgM to *Babesia divergens* and *Babesia microti* were detected in 75% and 25% of the cases, respectively. An important fact is that at 5 (16.7%) blood samples from *Lyme disease* patients, at which diagnostically significant anti-*Babesia* Ig were detected, 3 (60%) blood samples contained both fractions (IgG and IgM, $r_A$0.63), and 2 (40%) blood samples contained a diagnostically significant titre of only one fraction. This confirms the necessity of simultaneously determining the titres of specific IgG and IgM or total Ig in carrying out the immune diagnosis of babesiosis.

In addition, the relevance of this methodological approach is confirmed by the fact of IgG diagnostically significant titres detection (with the absence of IgM) in two blood samples from blood donors, and at one blood sample from a toxoplasmosis patient. In these cases, IgG indicates their anamnestic origin (previously asymptomatic or clinically expressed but not diagnosed babesiosis). The suitability of IFA by using of whole-cell slide antigens of *B. microti* and *Babesia divergens* to detect

the level of total Ig is demonstrated by positive results of detecting diagnostically significant titres in two (13.3%) blood samples from cattle.

## DISCUSSION

The use of IFA for immune diagnosis of babesiosis has both positive sides and significant disadvantages. Positive should be the simplicity of the method reproducibility, its high sensitivity (91 and 89%) and specificity (99 and 99%) in detection of diagnostically significant titres (1:32 and 1:64) of specific IgM in the acute phase of babesiosis caused by *Babesia microti*. Further to this fact, the high level of sensitivity and specificity of IFA the diagnosis of babesiosis caused by *Babesia microti* is based on the long practical experience of validated test systems usage [17,15]. It is expedient to apply IFA for diagnosis of an acute phase of babesiosis only in cases when preliminary negative results of microscopic and PCR tests (due to low levels of parasitemia <0.01%) were obtained, but persistent suspicion of babesiosis probability (presence of epidemiological data). Scientists' conclusions on immune diagnosis methods limitation in babesiosis are becoming even more equitable in diagnosing of an acute phase of *Babesia divergens* infection, due to the fact of a more prolonged period of seroconvergency at immunocompromised persons who are at a high risk category for babesiosis with relatively lower levels of sensitivity (62-87%) and specificity (54-85%) of IFA method [17,9,23,15,14].

In consideration of the fact that specific IgG in the blood of patients with babesiosis appear two to three weeks after the onset of the disease, and indicators of their diagnostically significant level are achieved only at the beginning of convalescence, IFA method for determining the titres of this class Ig is used by the vast majority of researchers for retrospective diagnosis of babesiosis, detection of its chronic forms (including asymptomatic and subclinical) and for conducting epidemiological studies of immune structure of population to determine the objective level of prevalence of babesiosis [17,30,9].

The results of our study do not contradict with the previous studies regarding the significance and tactics of IFA usage in babesiosis diagnostics. We did not record the results of nonspecific cross-linked immunological reactions when using different types of whole-cell slide antigens (*Babesia microti* and *Babesia divergens*) with the same blood samples, as well as false-positive reactions between these antigens and blood samples from patients with seropositive rheumatoid arthritis and toxoplasmosis, which were indicated by other researchers [15,14,8]. It should be noticed that the manifestation of the infectious process depends on many factors, such as the morphological and functional state of the immune system [3,6,2], genetic predisposition [24], the state of the target organ [22,1], and the aggressiveness of the pathogen [10,18,22].

According to the results of our study, the level of reproducibility of positive and negative IFA results was 96.9% for total Ig in serum dilution of 1:8; 76.2% for IgG, IgM and total Ig in dilutions from 1:8 to 1:1024.

## CONCLUSION

A preliminary laboratory-clinical trial of IFA performed by using a commercial (*Babesia microti*) and experimental whole-cell slide antigen (*Babesia divergens*) revealed *Babesia* antibodies to *Babesia divergens* (5.4%) and *Babesia microti* (2.3%) that were detected with higher ($p < 0.05$) frequency at Lyme disease patients (16.7%) than at blood donors (1.7%). We proposed criteria for laboratory diagnosis of babesiosis in humans which is considered to be for IgG in the titre ≥1: 128, and for IgM in the titre ≥1:32.

Nevertheless, despite the fact that IFA method remains the most accessible and popular in the practice of immuno-diagnosis of babesiosis and in conducting research on the study of immune structure of population, the influence of a subjective factor in the assessment of IFA results, as well as relatively low sensitivity of the method in the initial period of the disease, and the existing probability of cross-immunological reactions; the absence of standardized test systems and relevant research protocols (for *Babesia divergens* and close organisms) [16,18,19,22] substantiate the need to develop more sensitive, specific and more objective methods.

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