Seroprevalence of babesiosis in immunocompetent and immunocompromised individuals

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Interest in Babesia species is gaining an increasing attention as an emerging tick-borne pathogen. Infection is primarily transmitted through Ixodes ticks, and alternatively by blood transfusions from asymptomatic donors.
The aim *of the study was detection of Babesia seroprevalence in different groups of population with the usage of experimental B. divergens whole-cell slide antigen and commercial B. microti immunofluorescence assay substrate slide.*
Materials and methods. *Indirect immunofluorescence assay trial was performed by testing of 145 blood samples of different origins: healthy individuals (60 – blood donors), risk groups (30 – HIV-infected individuals, 30 – Lyme disease patients) and false-positive IFA controls (10 – seropositive rheumatoid arthritis patients, 15 – patients with toxoplasmosis).*
Results. *The study revealed Babesia antibodies to B. divergens (6.9%) and B. microti (3.4%) that were detected with higher (p <0.05) frequency in HIV-infected individuals (26.7%) and in Lyme disease patients (16.7%) than at blood donors (1.7%). Diagnostically significant IgG titres were detected at 23.3% HIV-infected individuals, 13.3% Lyme disease patients and by 1.7% of blood donors and patients with seropositive latent toxoplasmosis. Specific IgM were detected at 20.0% HIV-infected individuals and 13.3% Lyme disease patients. 57.1% of diagnostically significant titres in HIV-infected and Lyme disease patients were represented by IgG and IgM.*
Conclusion. *Immunofluorescence assay has a limited use in babesiosis: in acute form with negative microscopy or PCR; in chronic, asymptomatic and subclinical form with low level of parasitemia; and in retrospective and epidemiological studies of the population immune structure. Clinicians need to have increased awareness of babesiosis, and further studies are needed to clarify the optimal management of this infection in risk groups (including HIV-infected patients and blood donors).*

Seroprewalencja babeszjozy u osób immunokompetentnych i z deficytem odporności

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Zainteresowanie rodzajem Babesia jako nowym patogenem przenoszonym przez kleszcze stale rośnie. Za przenoszenie infekcji w pierwszej kolejności odpowiedzialne są kleszcze z rodzaju Ixodes oraz transfuzje zakażonej krwi, pochodzącej od bezobjawowych dawców.
Celem *badania była ocena seroprewalencji babeszjozy w różnych grupach populacyjnych przy zastosowaniu doświadczalnego pełno-komórkowego antygenu B. divergens w połączeniu z komercyjnym zestawem do badań w kierunku B. microti metodą immunofluorescencji.*
Materiał i metody. *Łącznie przebadano 145 próbek krwi różnego pochodzenia, wykorzystując metodę immunofluorescencji pośredniej (IIFT), w tym próbek: od zdrowych (60 honorowych krwiodawców), od osób z grup ryzyka (30 chorych HIV-pozytywnych, 30 chorych z rozpoznaną chorobą z Lyme) oraz grupy kontrolnej składającej się z próbek krwi od badanych z fałszywie dodatnim wynikiem testu immunofluorescencyjnego (IFA) w kierunku babeszjozy (10 od chorych z seropozytywnym reumatoidalnym zapaleniem stawów, 15 od chorych na toksoplazmozę).*
Wyniki. *W toku przeprowadzonych badań wykryto przeciwciała przeciwko B. divergens (6,9%) i B. microti (3,4%), z istotnie statystycznie większą częstotliwością (p <0,05) u chorych HIV-pozytywnych (26,7%) oraz u badanych z chorobą z Lyme (16,7%), w porównaniu z honorowymi dawcami krwi (1,7%). Istotne diagnostycznie miana IgG stwierdzono u 23,3% chorych HIV-pozytywnych i u 13,3% badanych z chorobą z Lyme, a także po 1,7% u honorowych dawców krwi oraz u chorych z seropozytywną latentną postacią toksoplazmozy. Swoiste IgM wykryto u 20,0% chorych HIV-pozytywnych oraz u 13,3% badanych z chorobą z Lyme. U chorych HIV-pozytywnych oraz u badanych z chorobą z Lyme w 57,1% przypadków istotne diagnostycznie miana stanowiły zarówno IgG jak i IgM.*
Wnioski. *Badania metodą immunofluorescencji mają ograniczone zastosowanie w diagnostyce babeszjozy: w ostrej postaci choroby w przypadku ujemnego wyniku badania mikrobiologicznego lub PCR, w bezobjawowym i subklinicznym przebiegu postaci przewlekłej przy niskiej parazytemii, a także w retrospektywnych i epidemiologicznych badaniach, służących ocenie stanu immunologicznego populacji. Konieczne są zwiększanie świadomości klinicystów odnośnie babeszjozy oraz prowadzenie dalszych badań celem ustalenia optymalnych metod leczenia tej infekcji u osób z grup ryzyka (włącznie z chorymi HIV-pozytywnymi i honorowymi dawcami krwi).*

Key words: *Babesia divergens, Babesia microti, immunofluorescence assay, seroprevalence, HIV-infection, blood donors*

Słowa kluczowe: *Babesia divergens, Babesia microti, immunofluorescencja, seroprewalencja, zakażenie HIV, honorowi dawcy krwi*

Human babesiosis is a zoonotic disease caused by the haemoprotozoan piroplasm parasite classified within the genus *Babesia* [31]. Interest in *Babesia* species is gaining an increasing attention as an emerging tick-borne pathogen. Humans act as accidental host; infection is primarily transmitted through *Ixodes* ticks, and alternatively by blood transfusions from asympto-

matic donors [13,36]. The spread of *Babesia* through transfusions is increasingly a problem and is one of the most commonly reported transfusion-transmitted infections [10]. However, the actual number of cases of a transfusion-associated babesiosis is much higher, as many of them remain unrecognizable [3,23]. Donors of blood components and organs may be unknowingly infected with *Babesia* outside traditional areas of transmission, due to donor travel or shipment of blood products [22]. Furthermore, considering wide distribution of the vector in temperate latitudes, the prevalence of human babesiosis can be underestimated [3].

The disease has been reported both in immunocompetent and immunocompromised individuals. Depending on the immune status and risk factors, babesiosis presents as a broad spectrum of illness ranging from asymptomatic to life-threatening illness. The risk factors associated with severe, prolonged and fatal disease are asplenia, malignancy, HIV-infection, usage of immunosuppressive agents and the presence of associated borreliosis [6,11,20]. Simultaneously we have to conclude about consequences of immunosuppression in patients with HIV and other infections with somatic pathology [7,25], combined infections [19,32], and injured immune organs [1,35] in a European cohort also [24,30,34].

Babesia divergens is considered the main agent of human babesiosis in Europe and have been documented primarily in splenectomised or immunocompromised individuals [38], although some cases have recently been reported in immunocompetent patients [26]. *B. microti* cases have been recorded in both spleen intact and asplenic patients, usually with a relatively mild clinical infection except in immunocompromised or elderly individuals. *Babesia microti* is a natural parasite of microtine rodents and occurs mainly in the USA. However, a case of autochthonous *B. microti* infection has been confirmed in Germany [12], and serological evidence of human *B. microti* infections in a number of different European countries has been reported [13,16,29].

As for most emerging infectious diseases the proportion of undetected babesiosis cases, the prevalence of babesiosis in blood donors or in the risk groups is not well established. Investigations of the epidemiology of babesiosis involving serological studies have concentrated mostly on inhabitants whose immunological function was normal. Serological tests have been used widely in endemic areas to support or confirm the diagnosis [17]. These techniques are particularly useful in screening blood donors for *Babesia* infections since asymptomatic individuals can be easily missed in the blood smear examination [14].

By contrast, the aim of this study was resurch of *Babesia* seroprevalence in different groups of population with the usage of experimental *B. divergens* whole-cell slide antigen and commercial *B. microti* immunofluorescence assay (IFA) substrate slide.

MATERIALS AND METHODS

A retrospective experimental research was performed for patients examined in Regional Infectious Hospital, Kharkiv, Ukraine (clinical base of the Department of Infectious Diseases, National Medical University, Kharkiv, Ukraine) from January 2016 to December 2016. The inclusion criteria for cases were blood donors (clinically healthy people) and patients with potential presence of *Babesia* antibodies (Lyme disease and HIV-infected patients). Controls were individuals (with seropositive rheumatoid arthritis and toxoplasmosis) that may have false-positive IFA.

Experimental *B. divergens* whole-cell slide antigen in addition to commercial *B. 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 145 blood samples of different origins (30 – HIV-infected individuals, 30 – Lyme disease patients, 10 – seropositive rheumatoid arthritis patients, 15 – patients with seropositive latent toxoplasmosis, 60 – blood donors).

Experimental *B. divergens* whole-cell slide antigen has been created in Mechnikov Institute of Microbiology and Immunology, Ukraine. This diagnosticum was fixed on the surface of slide plates RBC's of a sheep (infected by merozoites of *B. divergens* with parasitaemia level of about 10%) that can enter into an immunological reaction with *Babesia* antibodies (specific total Ig, IgG and IgM) and adsorb them (fig. 1a). Commercial *B. microti* (item number: BM-12, item description: *B. microti* 12-well IFA Substrate Slides "Fuller Laboratories", USA) was fixed on the surface of slide plates RBC's of a mouse (infected by merozoites of *B. microti* with parasitaemia level of about 50%) (fig. 1b).

Completed diagnostic kit consists of whole-cell slide antigens of *B. divergens* and *B. 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 [2,33].

Data were described using standard descriptive statistics, i.e. counts, percentages, means, and standard deviations. Statistical significance was set at $p < 0.05$.

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

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
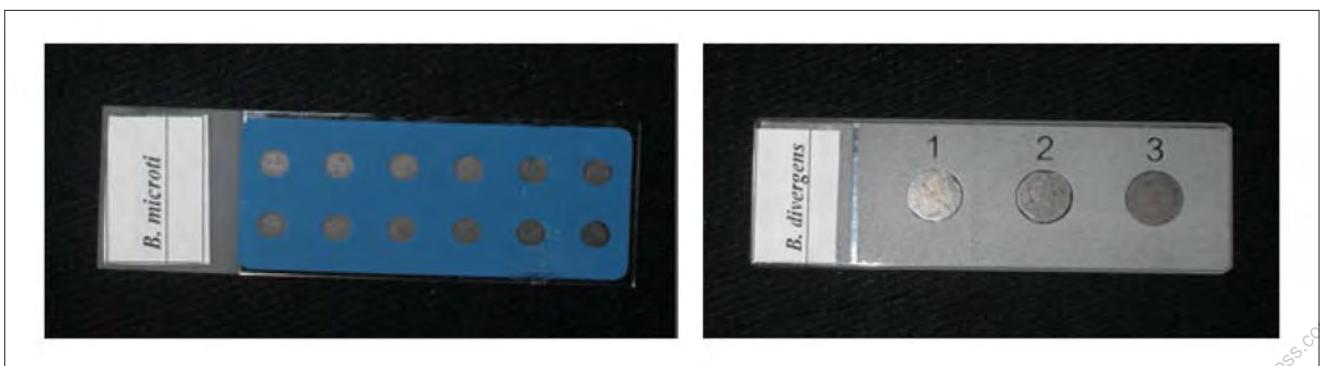

Figure 1. a) Commercial *B. microti* 12-well IFA Substrate Slides "Fuller Laboratories", USA (RBC's of a mouse infected by merozoites of *B. microti* with parasitemia level of about 50%); b) Experimental *B. divergens* whole-cell slide antigen (RBC's of a sheep infected by merozoites of *B. divergens* with parasitemia level of about 10%)

Rycina 1. a) Komercyjny 12-dołkowy substrat IFA w kierunku *B. microti* „Fuller Laboratories", USA (krwinki czerwone myszy zainfekowanej merozoitami *B. microti*, z poziomem parazytemii wynoszącym około 50%); b) Doświadczalny, pełnokomórkowy antygen *B. microti* (krwinki czerwone owcy zainfekowanej merozoitami *B. microti*, z poziomem parazytemii wynoszącym około 10%)

1:8 with detection of *Babesia* total Ig, by commercial fluorescent Anti-Human Ig was given in table I. 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 (fig. 2-3).

siosis, where the frequency reached 37.0% [4,8,16]. 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% [4,5,8,16,37].

Currently, universal criteria for evaluating the diagnostically significant titre of *Babesia* antibodies have not been developed,
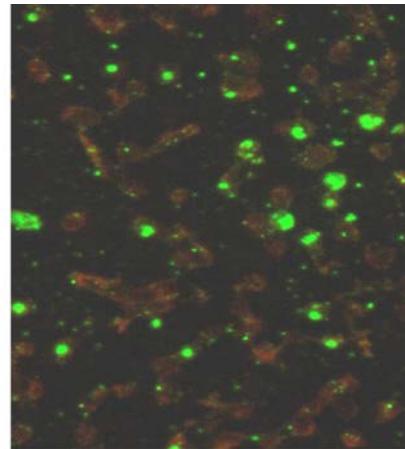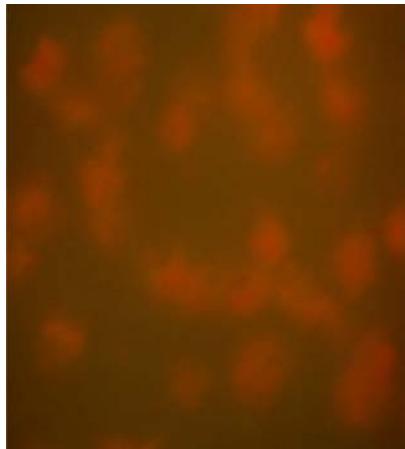
Figure 2. a) Negative IFA-reactivity of serum from human using *B. microti* parasites as antigen. Optical magnification (×1000). b) Positive IFA-reactivity of serum from human using *B. microti* parasites as antigen. Optical magnification (×1000).
**Rycina 2**. a) Ujemny wynik reakcji immunofluorescencji ludzkiej surowicy z wykorzystaniem antygenu *B. microti* (powiększenie 1000x). b) Dodatni wynik reakcji immunofluorescencji ludzkiej surowicy z wykorzystaniem antygenu *B. microti* (powiększenie 1000x).

Fifteen (10.3%) positive IFA samples were obtained. *Babesia* antibodies were found more frequently at patients with Lyme disease (16.7%) and in HIV-infected individuals (26.7%). At the same time, frequency of *Babesia* antibodies detection at patients with Lyme disease and HIV-infection exceeded ($p<0.05$) the corresponding figure (1.7%) in a clinically healthy group of blood donors. Antibodies were detected against both of the major human species – *B. microti* and *B. divergens* that confirms circulation of not only *B. divergens*, which is dominant in Europe, but also *B. microti* – a dominant pathogen of human babesiosis in America.

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 babe-

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

Blood samples	n	Positive IFA	
		B. microti	*B. divergens*
HIV-infection	30	3 (10.0%)	5 (16.7%)
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%)
Total	145	5 (3.4%)	10 (6.9%)
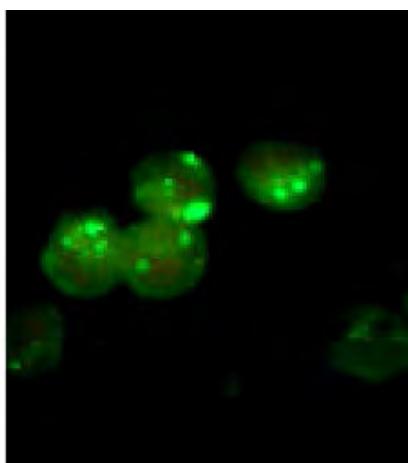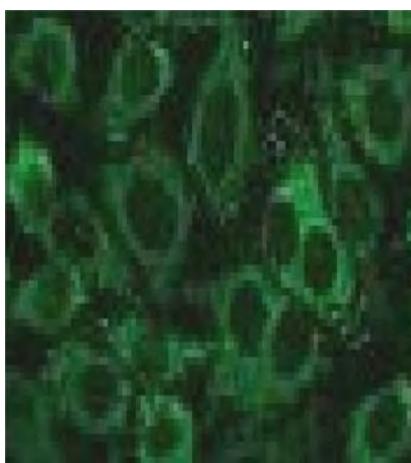
Figure 3. a) Negative IFA-reactivity of serum from human using *B. divergens* parasites as antigen. Optical magnification (×1000). b) Positive IFA-reactivity of serum from human using *B. divergens* parasites as antigen. Optical magnification (×1000).
Rycina 3. a) Ujemny wynik reakcji immunofluorescencji ludzkiej surowicy z wykorzystaniem antygenu *B. divergens* (powiększenie 1000x). b) Dodatni wynik reakcji immunofluorescencji ludzkiej surowicy z wykorzystaniem antygenu *B. divergens* (powiększenie 1000x).

due to differences in prototypes of diagnostic kits produced by different laboratories and the lack of unified IFA reproduction technologies [9,27,36]. We detected *Babesia* antibodies IgG and IgM titres in IFA (in three parallel reproductions) such diagnostic immunological criteria for positive tests: IgG in the titre ≥1: 128 and Ig M in the titre ≥1:32 [9].

According to the results of our study, diagnostic titres of anti-*Babesia* IgG were detected at seven (23.3%) blood samples of HIV-infected individuals, four (13.3%) blood samples of Lyme disease patients and by one (1.7%) sample of blood donors and patients with toxoplasmosis. IgG to *B. divergens* and *B. microti* were detected in 58.3% and 41.7% of these cases, respectively. Diagnostic titres of anti-*Babesia* IgM were detected at six (20.0%) blood samples of HIV-infected and four (13.3%) blood samples of Lyme disease patients. IgM to *B. divergens* and *B. microti* were detected in 70% and 30% of the cases, respectively. An important fact is that at 9 (30.0%) blood samples from HIV-infected individuals and 5 (16.7%) blood samples from Lyme disease patients, at which diagnostically significant anti-*Babesia* Ig were detected, 8 (57.1%) blood samples contained both fractions (IgG and IgM). 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 one blood sample from blood donors, and at one blood sample from a toxoplasmosis patient. In these cases, IgG indicates their anamnestic origin (previously asymptomatic, subclinical, or clinically expressed but not diagnosed babesiosis).

DISCUSSION

The use of IFA for immune diagnosis of babesiosis has both advantages and significant disadvantages. Advantages include 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 *B. microti*. The high level of sensitivity and specificity of IFA in *B. microti*-infection is based on the long practical experience of validated test systems usage, but in small proportion of cases false-positive results are possible [14,16,21].

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 parasitaemia <0.01%) were obtained, but with persistent suspicion of babesiosis probability (presence of epidemiological data). Limitation of the serological methods in babesiosis are becoming even more equitable in diagnosis of an acute phase of *B. divergens*-infection, due to the fact of a rapid course of illness and more prolonged period of seroconvergency, with relatively lower levels of IFA sensitivity (62-87%) and specificity (54-85%) [5,13,15,16,18,27,28].

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 [5,14,37].

In general, 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 non-specific cross-linked immunological reactions when used different types of whole-cell slide antigens (*B. microti* and *B. divergens*) with the same blood samples, as well as false-positive reactions between these antigens and blood sam-

ples from patients with seropositive rheumatoid arthritis and toxoplasmosis, which were indicated by other researchers [13,15].

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.

Nevertheless, despite the fact that IFA method remains the most accessible and popular in the practice of immunodiagnostics 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 *B. divergens* and close organisms) [5,15,16,18,27] substantiate the need to develop more sensitive, specific and more objective methods.

CONCLUSIONS

IFA has a limited use in babesiosis: in acute babesiosis with negative microscopy or/and PCR; in chronic, asymptomatic and subclinical disease with low level of parasitemia; in retrospective and epidemiological studies of the immune structure of population.

A preliminary laboratory-clinical trial of IFA performed by using a commercial (*B. microti*) and experimental whole-cell slide antigen (*B. divergens*) revealed *Babesia* antibodies to *B. divergens* (6.9%) and *B. microti* (3.4%) that were detected with higher (p <0.05) frequency in HIV-infected individuals (26.7%) and in Lyme disease patients (16.7%) than at blood donors (1.7%).

The increasing numbers of HIV-infected patients in the Ukraine combined with ecological changes that lead to expansion of areas where babesiosis is endemic are expected to gradually increase the incidence and prevalence of babesiosis in HIV-infected patients and other risk groups. Clinicians need to have increased awareness of babesiosis, and further studies are needed to clarify the optimal management of this infection in HIV-infected patients.

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