SCIENTIFIC REPORT

RealLine *Anaplasma phagocytophilum / Ehrlichia chaffeensis / E. muris*

Detection of Pathogens of Human Granulocytic Anaplasmosis and Monocytic Ehrlichiosis Using Real-Time PCR

Human granulocytic anaplasmosis (HGA), which was earlier referred to as human granulocytic ehrlichiosis, and human monocytic ehrlichiosis (HME) are acute transmissible tick-borne diseases. The pathogens of the diseases, obligate intracellular microorganisms (*Anaplasma phagocytophilum* for HGA, *Ehrlichia muris* and *Ehrlichia chaffeensis* for HME), are members of *Anaplasma* and *Ehrlichia* geni belonging to the *Anaplasmataceae* family [1]. The bacteria affect leucocytes (mostly granulocytes in HGA, and monocytes and mononuclear phagocytes in HME) and vessel endothelial cells.

HGA and HME pathogens have been detected in ticks in many European countries, in Russia, Korea, China, Japan, and the USA [2]. The life cycle of the microorganisms includes the stage of proliferation in Ixodes ticks, the carriers of the infection, and in vertebrates, their feeders (both serving as infection reservoirs).

Ticks get infection while feeding on infected hosts, *Anaplasma* and *Ehrlichia* actively penetrate the tick intestine epithelium, where their primary replication occurs. Then, the pathogens get into salivary glands, and the second reproduction cycle undergoes in their epithelial cells [3]. The main path for human infection with the HGA and HME pathogens is a bite by an infected tick. A few registered cases of diseases related to transfusion of infected donor blood or plasma, to transplantation of organs, as well as to ingress of the pathogens into human body via skin microtraumas or eye conjunctiva in the course of butchering infected animals, have been reported [4, 5].

Human granulocytic Anaplasmosis and Monocytic ehrlichiosis have similar clinical signs, with a wide spectrum including both symptomless (subclinical) and extremely severe forms threatening the life of infected individuals [1]. Mild forms of HGA and HME are manifested as an acute respiratory viral infection syndrome. Feverish conditions observed in more than 90% cases last from several days to several weeks. One third of HME patients have rash, which is three times more frequent than in HGA patients. Other manifestations include neurologic disorders, bleeding, myalgias, altered myocardial conduction, breathing difficulties, renal malfunction, GI disorders, increased activity of liver enzymes, anicteric hepatitis. Haemograms demonstrate thrombocytopenia and a pronounced leucocytopenia, which in turn promotes the development of opportunistic infections.

Severe forms of HGA and HME are often caused by delayed diagnostics and therapy initiation. Such disease progression is most common in patients who have earlier received immunosuppression therapy, AIDS patients, patients with diabetes and collagenoses, as well as elderly patients. According to the US statistics, lethal outcomes are observed in 0.5 – 3.5 % of all the patients with HGA and HME, and are mostly caused by the development of opportunistic infections (invasions): disseminated candidiasis, pulmonary aspergillosis, necrotising herpetic pharyngitis, cryptococcosis [4]. If timely diagnosed, HGA and HME are successfully treated with doxycycline and other tetracycline antibiotics [1, 4].

Both direct and indirect analytical methods are available for the laboratory diagnostics of HGA and HME. A microscopic examination of leucocytes from patients in acute period of the disease demonstrates the presence of specific aggregates of *Anaplasma* and *Ehrlichia* (morules), which must be differentiated from other cell inclusions. The morules can be found in only 0.1 % leucocyte cells, and thus the reliability of their detection significantly depends on the experience and skills of the examiner. Furthermore, the diagnostic sensitivity of the microscopic method in the first week of the disease varies from 2 to 75%, and then decreases [18]. On the other hand, the absence of morules in neutrophils does not exclude the presence of Anaplasmosis or Ehrlichiosis in the patient.

The culturing method of HGA and HME diagnostics is characterised by high sensitivity and specificity [19 - 22]. However, it is rather complex, requires special cell cultures, selective media, is expensive, and the analysis run takes two to three weeks.

The most common method for a serological diagnostics of the diseases is indirect immunofluorescence test, with enzyme-linked immunosorbent assay (ELISA) used less often. Specific antibodies to the HGA and HME pathogens can be detected in blood of some patients 7 – 10 days after the first clinical manifestations of the disease, and the initial levels of Class M immunoglobulins (IgM) only slightly exceeding the levels of immunoglobulin G (IgG) [23]. IgM is known to be less specific; therefore, if it is found in a patient, an additional examination is required to confirm seroconversion. The diagnostic sensitivity of the indirect immunofluorescence test for determination of IgG to *A. phagocytophilum* and *E. chaffeensis* is 82 – 100 % and 88 – 90 %, respectively [18]. However, it has been shown that, in certain regions, antibodies to Anaplasma can be detected in 5–10 % healthy individuals who possibly had had the infection earlier in a hidden form. Therefore, the diagnosis in seropositive patients is verified by studying paired blood sera collected in the 2nd and 4th weeks of the disease. A reliable confirmation for the presence of HGA or HME is a fourfold increase in the titre of specific IgG [23].

Of note that in the first week of the acute period of the diseases, a serological examination of patients yields mostly negative results; however, this does not exclude the possibility of *Ehrlichia* or *Anaplasma* infection. The most rapid and precise diagnostic information in this period can be obtained by detecting the HGA and HME pathogens DNA in the patients' blood using polymerase chain reaction (PCR). PCR method provides the same sensitivity and specificity as cultural approaches, but is less time-consuming [19]. The high sensitivity of PCR is due to the presence of several dozens to hundreds of *Anaplasma* or *Ehrlichia* in a single infected leucocyte. The unique specificity of this technique allows a reliable differentiation of HGA and HME from a series of diseases with similar clinical manifestations caused by respiratory, entero- and cytomegaloviruses, viruses of hepatitis and tick-borne encephalitis, as well as bacterial infections: meningoencephalites, leptospirosis, tularemia, tick-borne borreliosis, and some others.

The objective of this work is to develop and test a multiplex "RealLine *Anaplasma phagocytophilum* / *Ehrlichia muris*, *Ehrlichia chaffeensis*" assay kit for real-time PCR detection of the DNA of HGA and HME pathogens. The assay kit testing process included screening suspensions of Ixodes ticks (*Ixodes persulcatus*, *Ixodes pavlovskii*), as well as analysis of the blood of the *Anaplasma* and *Ehrlichia* carriers, small forest rodents.

Materials and Methods: The study used 1000 ticks (imago) of Ixodes genus collected by flagging in Novosibirsk region in 2009 and 2011. Extraction of total nucleic acids (NA) from tick suspensions after their individual homogenisations was performed using the "RealLine Extraction 100" assay kit (Bioron Diagnostics, Germany), as described earlier [24]. In addition, we also analysed 22 blood samples from red-backed and gray-sided voles (*Myodes rutilus*, *Myodes rufocanus*) caught in Omsk region (Russia) in 2011. The blood from each animal (100–500 µl) was collected into a separate sterile tube containing 30 µl 0.5 M EDTA solution, to which 2 volumes of lysing buffer (4 M guanidinium thiocyanate; 0.1 M Tris-HCl pH 6.4; 0.045 M EDTA pH 8.0; 1.3% Triton-X-100) was added. To extract total NA with the "RealLine Extraction 100" assay kit, we used 100 µl of the obtained blood lysates.

To perform PCR, 50 µl of total NA extracted from tick suspensions and 10 µl from animal blood lysates were used. The remaining part of total NA was frozen and stored at –70 °C. Detection of NA of the HGA and HME pathogens in the test samples was performed on a thermocycler with real-time fluorescence detection CFX (Bio-Rad, USA) using the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*" assay kit. The obtained results were evaluated using the RealLine Diagnostics service software [25].

To identify the detected bacteria, the HGA and HME pathogens, we determined the nucleotide sequences in the polymorphic regions of their genome. The sequencing was performed by Sanger method on both DNA strands using "*BigDye terminator sequencing kit"* on the automatic sequencer *ABI Prism 3100 DNA Analyser* (*Applied Biosystems*, the USA), followed by a phylogenetic analysis of the determined nucleotide sequences

using a software programme. The obtained nucleotide sequences were compared against data represented in GenBank with *BLAST* search system. Oligonucleotides were designed using software available online at www.idtdna.com.

Results and Discussion. Development of the assay kit for detecting the HGA and HME pathogens DNA: Following conservative gene regions were used as the amplification targets: for *Ehrlichia muris* and *Ehrlichia chaffeensis—gltA*, and for *Anaplasma phagocytophilum—p44*, wherein we could find such a region despite a wide polymorphism of this gene. The new assay kit was developed in compliance with the general principles for designing the RealLine line of kits for PCR with real-time detection of amplification products. The basis of the assay kit is a lyophilised ready master mix (RMM) containing all components required for PCR, which ensures extended storage time (up to two years) and the possibility of transportation at +4 °C [25]. An internal control sample (IC) of synthetic DNA is added to all test samples and together with them passes all the stages of analysis (including sample preparation), which allows tracking (i) any NA losses in the course of extraction and (ii) decrease in amplification efficiency if any inhibitors are present in the sample.

In the course of experiments, the entire set of conditions for PCR were optimised to ensure maximum sensitivity and specificity of analysis: the combination of primers and probes, their concentration, ready master mix composition and reaction conditions, ensuring optimal simultaneous and efficient amplification of all the targets (DNA of Anaplasma, Ehrlichia, or IC) in the test samples when following a standard protocol for analysis. The new assay kit simultaneously employs three independent channels for fluorescence detection in each test tube: for IC—FAM, for A. phagocytophilum DNA—HEX, for *E. muris* and/or *E. chaffeensis* DNA (without their differentiation)—ROX.

We changed the composition of RMM and the protocol of analysis to prevent false positive results that may arise due to contamination with products of an earlier amplification. Instead of deoxythymidine triphosphate (dTTP), RMM includes deoxyuridine triphosphate (dUTP) and contains an E.coli uracyl-DNA glycosidase (UDG) enzyme. When using RMM of this composition, PCR of natural targets produces amplicons whose nucleotide sequence contains deoxyuracyl residues instead of deoxythymidines. If the amplicons contaminate a newly tested sample, at the first stage of analysis (exposition for 2 minutes at 50 °C), UDG cleaves uracyl residues, leaving them unsuitable for further amplification. At the second stage (heating for 2 minutes at 95 °C), the enzyme becomes deactivated and loses its capability to cleave deoxyuracyl-containing amplicons at subsequent stages.

To assess the analytical sensitivity of the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis"* assay kit, standard reference samples (SRS) containing DNA fragments for the microorganisms were created. To obtain SRS for A. phagocytophilum and E. muris, we cloned selected DNA regions of the pathogens extracted from infected ticks. We used an artificial DNA fragment synthesised by polymerase elongation of overlapping oligonucleotides as SRS for *E. chaffeensis* [26]. Its nucleotide sequence was identical to a genome region of Ehrlichia of this species provided in GeneBank database.

The analytical sensitivity of the new assay kit for the detection of *Anaplasma* and *Ehrlichia* DNA was assessed using probit analysis based on the results of PCR of eightfold dilutions of three SRS in buffer solution; the dilutions contain 50 to 10^8 copies of the DNA fragments for each pathogen in 1 ml. The sensitivity of analysis at the confidence level 95% ($p < 0.05$) is 100 genome equivalents (GE) per sample with amplification efficiency (E) 97%.

To assess the specificity of the “RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*” assay kit, we assayed samples containing NA of the pathogens for other tick-borne infections (3 samples each for *B. afzelii*, *B. burgdorferi s.s.*, and *B. garinii*, causing tick-borne borreliosis; 5 samples each for *B. miyamotoi*, i.e. the tick-borne relapsing fever pathogen, and the tick-borne encephalitis virus). Each sample containing no less than 10^7 GE of the above pathogens was tested fivefold yielding only negative results of PCR analysis, which allows evaluating the specificity of the assay kit over the used set of samples as 100 %.

Testing 30 samples of DNA extracted from blood serum of healthy donors using the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*" assay kit also yielded negative results. However, the *A. phagocytophilum* and *E. muris* DNA were successfully detected in PCR if a tick suspension containing the genetic material of the pathogenic *Anaplasma* and *Ehrlichia* was added to the initial sera before sample preparation. The results of this experiment provide ground for application of the new assay kit for detection of the HGA and HME pathogens DNA in clinical samples.

Testing the Developed Assay Kit. Using the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*" assay kit, we examined 9 random samplings of Ixodes ticks (1000 imagos in total), collected in 2009 and 2011 in Novosibirsk region (Russia). PCR was carried out using total NA extracted from suspension of individual parasite. The studies demonstrated that depending on the sampling the *A. phagocytophilum* DNA was detected in 2–3 % ticks, while the *E. muris*/*E. chaffeensis* DNA was found in 4–10 %. The results indicate a high rate of infection with the HGA and HME pathogens of adult Ixodes ticks prevalent in Novosibirsk region, and do not contradict to earlier published data [27–29]. In particular, it was demonstrated that the detection rate of *A. phagocytophilum* and *E. muris* in ticks from Asian part of Russia is 0 to 5.6 % and 0 to 14.3 %, respectively [27].

Table 1

Results of analysis of 60 ticks using the "RealLine DNA *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*" and "AmpliSens TBEV, *B. burgdorferi s.l., A. phagocytophillum, E. chaffeensis*/*E. muris-FL*" assay kits

Assay Kit	Number of Samples		Number of Coincided Samples		
	Positive	Negative	Positive	Negative	total
Detection of Anaplasma phagocytophilum DNA					
"RealLine"	20	40	20	40	60 (100%)
"AmpliSens"	20	40			
Detection of Ehrlichia muris/Ehrlichia chaffeensis NA					
"RealLine"	20	40	20	39	59 (98.3%)
"AmpliSens"	21	39			

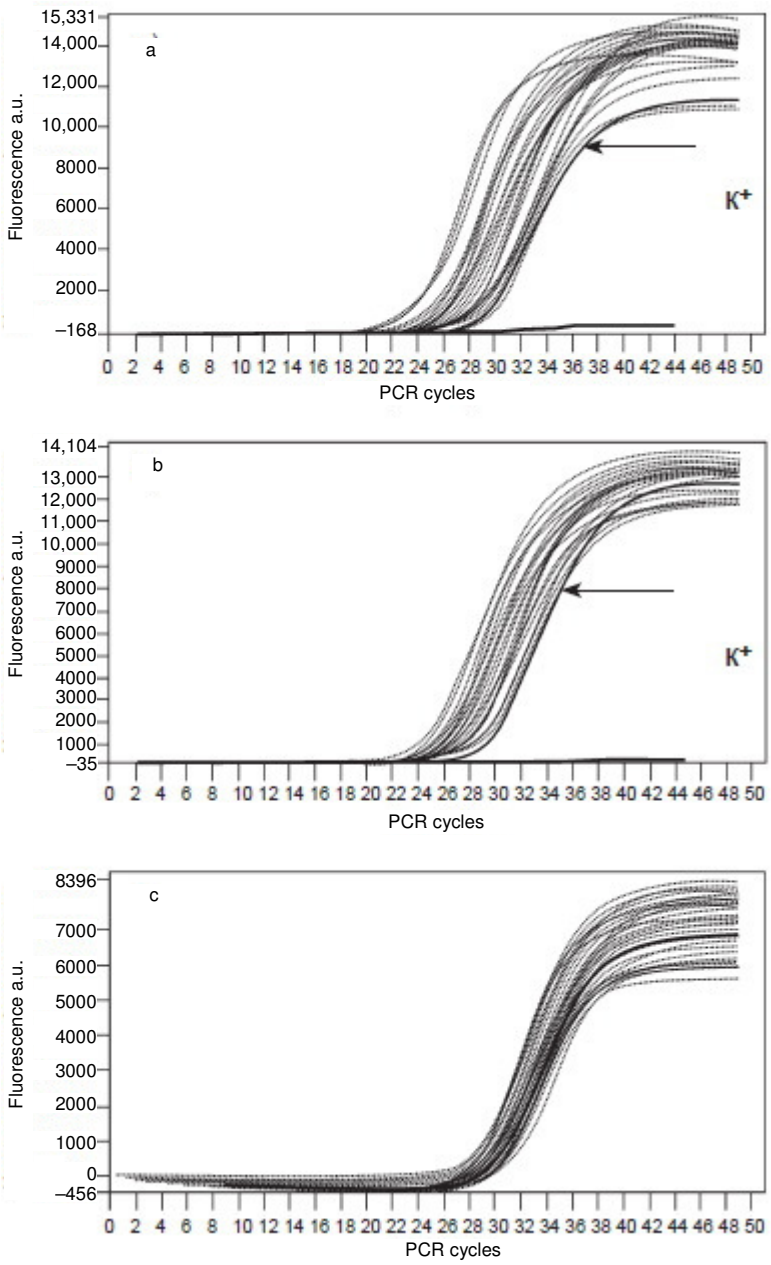

Figure. Curves of fluorescence build-up in three detection channels upon DNA amplification: a) *A. phagocytophillum* in 20 samples of tick suspensions—channel HEX; b*) E. muris* in 20 samples of tick suspensions—channel ROX; c) IC added to 60 samples of tick suspensions—channel FAM.

To assess the reproducibility of results obtained with the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris, Ehrlichia chaffeensis*" assay kit, we performed a repeated PCR analysis of 20 samples of total DNA extracted from the tick suspensions, where *A. phagocytophilum* DNA was found, and of 20 samples found positive for *E. muris* DNA. In all of 40 samples, the presence of the HGA and HME pathogens DNA was confirmed, and the fluorescence build-up profiles in HEX, ROX, FAM channels were demonstrated to be identical (see Figure).

To establish the species of Anaplasma whose DNA was detected in the tested ticks, a 540 bp gene region of the *groESL* operon of heat shock proteinswas sequenced in 10 randomly selected samples. Sequences

obtained were compared to GeneBank data; all 10 ticks of that sampling were infected with *Anaplasma phagocytophilum*.

In a similar study of Ehrlichia, *gltA* gene was sequenced for 10 randomly selected samples of total NA from those ticks, in which PCR had earlier detected fragments of E. muris/E. chaffeensis genome. The analysis of the obtained data demonstrated that all of this tick sampling was infected with *Ehrlichia muris*. Note that another HME pathogen, *Ehrlichia chaffeensis*, has not yet been found in the RF. Furthermore, it was also found that the fragment of gene *gltA*, which is used in the new assay kit as the target for detecting the *E. muris* DNA, is completely identical in all 10 sequenced nucleotide sequences and does not differ from the corresponding data in GeneBank (i.e., the correctness of choice of a conservative region of this gene has been confirmed).

To compare the diagnostic performance of the "RealLine *Anaplasma phagocytophilum / Ehrlichia muris*, *Ehrlichia chaffeensis*" assay kit and its sole commercial counterpart, a multiplex "AmpliSens TBEV, *B. burgdorferi s.l., A. phagocytophillum, E. chaffeensis/E. muris-FL*" assay kit (OOO Inter-LabService, Moscow), we performed a parallel analysis of 60 tick suspensions. In the reference assay kit, the genetic material of *A. phagocytophilum* is detected with a DNA target, while *E. muris* and *E. chaffeensis* are detected using 16S ribosomal RNA, from which prior to amplification cDNA must first be synthesised via reverse transcription. To perform this additional stage, we used the "REVERTA" assay kit (OOO InterLabService, Moscow).

The results of detecting the *A.phagocytophillum* DNA with two reference kits coincided in 100 % cases, and for detection of the NA of *E. muris*/*E. chaffeensis*—in 98.3 % (see Table 1). Only one sample that was found negative in the study using the newly developed assay kits, from the data of analysis using the "AmpliSens TBEV, *B.burgdorferi s.l., A. phagocytophillum, E. chaffeensis/E. muris-FL*" assay kit contained the RNA of Ehrlichia in a low concentration (the value of threshold cycle Ct = 37). However, in accordance with the recommended protocol for analysis, 5 additional hidden amplification cycles were not accounted for (i.e., the actual Ct value was equal to 42). On the other hand, when PCR is performed using the "RealLine *Anaplasma phagocytophilum/Ehrlichia muris, Ehrlichia chaffeensis*" assay kit, a sample is evaluated as positive if the value of Ct is less than or equal to 40.

The developed assay kit was also employed to detect the *A. phagocytophilum* and *E. chaffeensis/E. muris* DNA in the blood of red-backed and gray-sided voles, who are known to be the feeders for the ticks [2]. The positive results were found for 8 out of 22 blood samples for the rodents: in five samples, we found only the HGA pathogen DNA, while in three others, both HGA and HME pathogens DNA were found simultaneously. Note that the levels of *Anaplasma* and *Ehrlichia* DNA in the positive samples of this sampling differed by more than 2 orders of magnitude.

Conclusion. Hence, the studies performed have resulted in the development of a new "RealLine *Anaplasma phagocytophilum/Ehrlichia muris, Ehrlichia chaffeensis"* assay kit for simultaneous real-time PCR detection of human granulocytic anaplasmosis and monocytic ehrlichiosis DNA. The assay kit was shown to have a high specificity, sensitivity, and can reliably detect the pathogenic *Anaplasma* and *Ehrlichia* DNA in a concentration of 100 genome/equivalents in the samples of tick suspensions or lysates of mammal blood.

The results of analysis of the HGA and HME pathogens NA obtained with the new diagnostic kit and the reference assay kit "AmpliSens TBEV, *B. burgdorferi s.l., A. phagocytophillum, E. chaffeensis/E. muris-FL*" (OOO InterLab-Service, Moscow) coincide in 98.3 – 100 %.

The "RealLine *Anaplasma phagocytophilum / Ehrlichia muris, Ehrlichia chaffeensis*" assay kit was successfully used to test 1000 adult Ixodes ticks collected in Novosibirsk region in 2009 and 2011. The results of this study showed that 2–3 % ticks are infected with Anaplasma, and 4–10 % are infected with *Ehrlichia*, which is represented exclusively with the *Ehrlichia muris* species (data on sequencing a genome fragment of the microorganism in a sampling of 10 ticks).

References

1. Roudakov N.V., Shpynov S.N., Obert A.S. Human Anaplasmoses and Ehrlichioses—a New Challenge for Infection Pathology in Russia: Posobie dlya Vrachey. Omsk, 2006. 46 p. (In Russian).
2. Rar V., Golovljova I. // Infect. Genet. Evol. 2011. V. 11. N. 8. P. 1842–1861.
3. Ueti M.W., Knowles D.P., Davitt C.M. et al. // Infect. Immun. 2009. V. 77. P. 70–75.
4. http://www.cdc.gov/ticks/diseases/index.html.
5. Bakken J.S., Krueth J., Lund T. et al. // Clin. Infect. Dis. 1996. V. 23. P. 198.
6. Afanasieva M.V., Korenberg E.I., Vorobieva N.N. et al. // Materialy 9-go Syezda Epidemiologov. Moscow, 2007. V. 3. P. 152 (In Russian).
7. Roudakov N.V., Shpynov S.N. Samoilneko I.E. et al. // Bul. Sibirskoy Meditsiny. 2006. App. No. 1. P. 111–115 (In Russian).
8. Fomenko N.V., Rar V.A., Epikhina T.I. et al. // Bul. VSNT SO RAMN. 2007. No. 3 (App.). P. 177–180 (In Russian).
9. Tkachev S.E., Fomenko N.V., Rar V.A. et al. // Internat. J. Med. Microbiol. 2008. V. 298. Suppl. 1. P. 365–367.
10. Afanasieva M.V., Vorobieva N.N., Korenberg E.I. et al. // Infektsionnye Bolezni. 2006. V. 4. No. 2. P. 24–28 (In Russian).
11. Obert A.S., Roudakov N.V., Sedykh N.N. // Detskie Infektsii. 2009. No. 2. P. 30–32 (In Russian).
12. Samarov M.N. // Author's Abstract, Cand. Med. Sci. Thesis, Moscow, 2009. 20 p. (In Russian).
13. Shchuchinova L.D. // Author's Abstract, Cand. Med. Sci. Thesis, Omsk, 2009. 20 p. (In Russian).
14. Aleshkovskaya E.S., Blagov N.A., Druzhinina T.A. et al. // Epidemiologiya i Infektsionnye Bolezni. 2008. No. 2. P. 6–8 (In Russian).
15. Grigoryan E.V., Korenberg E.I., Vorobieva N.I. // Epidemiologiya i Infektsionnye Bolezni. 2000. No. 6. P. 20–23 (In Russian).
16. Borisov V.A., Kozlova I.V., Aitov K.A. et al. // Zhurn. Infektsionnoi Patologii. 2010. V. 17. No. 3. P. 35–37 (In Russian).
17. Rar V.A., Fomenko N.V., Melnikova O.V. et al. // Bul. Sibirskoi Meditsiny. 2008. V. 7. App. 1. P. 73–77 (In Russian).
18. Thomas R.J., Dumler J.S., Carlyon J.A. // Expert. Rev. Anti. Infect. Ther. 2009. V. 7. No. 6. P. 709–722.
19. Dumler J.S., Madigan J.E., Pusterla N. et al. // Clin. Infect. Dis. 2007. V. 45. P. 45–51.
20. Bakken J.S., Aguero-Rosenfeld M.E., Tilden R.L. et al. // Clin. Infect. Dis. 2001. V. 32. No. 6. P. 862–870.
21. Nefedova V.V., Korenberg E.I., Kovalevskii Yu. V. et al. // Vestn. Ros. Akad. Med. Nauk. 2008. No. 7. P. 47–50 (In Russian).
22. Sidelnikov Yu.N., Medyannikov O.Yu., Ivanov L.I. et al. // Klinicheskaya Meditsina. 2003. No. 81. P. 67–68 (In Russian).
23. http://www.cdc.gov/anaplasmosis/symptoms/index.html
24. Bondarenko E.I., Timofeev D.I., Ivanov M.K. // Novosti "Vector-Best". 2010. No. 1 (55). P. 2–7 (In Russian).
25. Ivanov M.K., Poryvaev V.D., Trukhina A.V. et al. // Novosti "Vector-Best". 2008. No. 4 (50). P. 16–19 (In Russian).
26. Jayaraj S., Reid R., Santi D.V. // Nuc. Acids Res. 2005. V. 33. P. 3011–3016.
27. Rar V.A., Epikhina T.I., Livanova N.N. et al. // Vector-Borne and Zoonotic Diseases. 2011. V. 11. P. 1013–1021.
28. Shikhin A.V., Bazhenova I.V., Romanenko V.N. et al. // Natsionalnye Prioritety Rossii. Sovremennye Aspekty Prirodnoi Ochagovosti Bolezney. 2011. No. 2 (5). P. 69–70 (In Russian).
29. Travina N.S., Skrynnik S.M., Karan L.S. C. // Natsionalnye Prioritety Rossii. Sovremennye Aspekty Prirodnoi Ochagovosti Bolezney. 2011. No. 2 (5). P. 76–77 (In Russian).

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BIORON Diagnostics GmbH
info@bioron.de