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Experimental-Biological Model of Bartonellosis

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Abstract: Bartonella are known to be important causes of zooanthroponotic diseases. The range of human infection varies from mild lymphadenopathy and asymptomatic bacteremia to life-threatening systemic disease in immunocompromised patients. Microbiological improvements in isolation methods and PCR amplification of organism-specific DNA sequences have resulted in a dramatic increase in reports describing human patients with bartonellosis. Nevertheless, clearly and successful isolation of Bartonella spp. from bacteremic animals and human patients remains an ongoing challenge. Technology of experimental bartonellosis due to intraperitoneal introduction of biological material samples containing causative agents to laboratory animals is presented in the article. White nonlinear mice with the artificially cyclophosphamide formed immunodeficient state may be used as an experimental model for further investigation of the biological alterations responsible for angiomatosis. On the other hand, we believe that this new method will enhance the diagnostic sensitivity and specificity needed to achieve a diagnosis of bartonellosis.

Key words: Biological model, *Mus musculus L.*, cyclophosphamide, bartonellosis, *B. henselae, B. quintana*.

1. Introduction

Bartonellosis is characterized by acute and chronic clinical forms with polymorphic symptoms that affect different organs and systems. The *Bartonella* genus now includes about 40 species. 13 species of *Bartonella* are pathogenic for human–*B. alsatica*, *B. bacilliformis*, *B. clarridgeiae*, *B. elizabethae*, *B. grahamii*, *B. henselae*, *B. koehlerae*, *B. melophagi*, *B. quintana*, *B. rochalimae*, *B. tamiae*, *B. vinsonii*, *B. washoensis* [1].

Two species of Bartonella can cause wide spectrum of syndromes in immunocompromised patients: *B. henselae* being the causative agent of cat scratch disease and *B. quintana* being the causative agent of trench fever. Immune status of the patient with these diseases is considered as a key factor that defines character of the infectious process. The unique biological ability of the *B. henselae* and *B. quintana* is to stimulate endothelial cells proliferation and to form tiny vessels in their capillary part with subsequent development of pseudoneoplastic process. Angioproliferation develops usually at CD_4 cells level less than 100 per mm^3 [2].

Laboratory models for an artificial reproducing of bartonellosis in the experimental animals are actively developed in different countries of the world [3]. The ultimate goal of the research was to determine effectiveness *in vivo* specificity, sensitiveness and reproducibility of diagnostics methods.

The aim of our research was the development of a generalized biological model for reproduction of experimental bartonellosis that is characterized by simplicity of technological reconstruction, affordability and availability for wide practical usage.

2. Materials and Methods

Algorithm to reproduce experimental bartonellosis consisted of such consequential stages: (1) artificial formation of the immunodeficient state in nonlinear white mice (*Mus musculus* L.); (2) preparation of the tested biological material samples; (3) inoculation of the laboratory animals with the artificially formed immunodeficient state using prepared biological samples; (4) sampling from the dead or slaughtered

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(due to chloroformed anaesthesia) experimental animals of sectional material (organs and target tissues); 5) verification of aetiology with PCR detection of causative agents in the selected samples of sectional material.

The artificial immunodeficient state in white nonlinear laboratory animals was produced by a single intracutaneous injection of 250 μg/kg cyclophosphamide ("Cyclophosphanum®", "Kyivmedpreparat", Ukraine) 3-4 h before the introduction of investigated biological material samples to the animals. The intracutaneous method (unlike intraperitoneal) provided more even entering of preparation to the system of blood circulation and hemopoietic organs and reduced speed of its elimination from the animal organism, that provided formation of more protracted (to 7-10 days) immunodeficient state sufficient for reproduction and accumulation of *Bartonella*. Optimal empiric dose of the preparation, fixed by us, is 250 μg/kg. The injection of this dose provides forming of the well expressing and stable immunodeficient state, and it does not result in unforeseeable death of a part (about 12% and more) of experimental animals that were marked with the introduction of 500 μg/kg and more [4, 5].

The selected samples of blood were brought in sterile capacities with an anticoagulant (with absent antimicrobial activity) to prevent blood coagulation. During realization of our research we used vacuum systems of blood sampling Venosafe™, Terumo Europe N.V. (Belgium) and test tubes VF-052SDK (working volume of 2 ml) with the anticoagulant K2-EDTA. The samples of blood were frozen within a single-phase with the temperature of -20 °C and unfrozen with a room temperature that provided destruction of blood cells and release of pathogen microcolonies.

Due to the information that bartonellosis could be transmitted by ticks, we used *Ixodes ricinus* (n = 12) and *Dermatocentor reticulous* (n = 119) for sampling. The samples of biological material of solid consistency carefully were homogenized (by trituration or grinding) and diluted with sterile distilled water in 1:9 ratios (volume/volume, respectively) with further mixing for formation of even suspension.

Before homogenization, the surface of ticks was disinfected by immersion of them in 70% ethyl spirit for 10 min. We homogenized all the body of the tick, as causative agents might be presented in its different tissues and organs (salivary glands, lymph, intestine etc.). To introduce them into the laboratory animals, we selected the supernatant (after settling and unassisted precipitation of fair-sized particles) of the homogenized samples of suspension.

All the manipulations with selection and preparation of biological material samples for the research were carried out in aseptic conditions to prevent their additional contamination by extraneous microflora.

Samples of different biological material (in the volume—0.3 mL) were injected intraperitoneally to nonlinear white mice with the artificially formed immunodeficient state, that provided a large area for the application of causative agents with high authenticity of their contact with target-cells (macrophages, leucocytes, erythrocytes etc.). The term of observation of the infected experimental animals was 8-10 days as in this period; the concentration of causative agents arrives at a maximal value in tissues and organs of animals [6].

To exactly verify infectious process aetiology from the dead and slaughtered animals (using method of chloroform hyper anesthetization), the most accessible and technologically cheap standard variant of PCR was applied. Verification of aetiology is based on the PCR detection of basic clinically meaningful species of *Bartonella* in the samples of blood (selected by puncture of heart with the observation of asepsis rules) of experimental animals infected by the investigated biological material samples. Expediency of the exact selection of blood samples is grounded by the simplicity of the used for this purpose manipulations, and also by the complete coincidence of our PCR

results (positive and negative) in parallel research of blood and other tissues, as well as organs' (spleen, bone marrow, liver, lymph nodes) samples, selected from the same experimental animals. For reproduction, we applied standard PCR, accessible in the territory of Ukraine, that are relatively low-priced and already geared-up for the direct use of commercial sets of reagents "IsoGene Lab. Ltd" (Moscow, Russian federation); "Universal probe preparation reagents kit"—Diatom®DNA Prep 100 (includes reagents for a selection and cleansing of DNA from the samples of biological material with the purpose of further amplification of its certain fragment); "DNA amplification reagents kit"—Gene Pak®DNA PCR Core (includes universal reaction mixtures, which do not contain primer system and were used for amplification of specific fragments of genome of *B. henselae* and *B. quintana* after addition of specially synthesized primers that correspond to gene *gltA*, with nucleotide sequences—CAT TTC TGT TGG AAA TCC TAG и TTT TAA TGT AAT GCC AGA ATA A); "Marker of DNA molecular mass M50, M100—GenePak™ DNA Ladder M50, M100 (includes DNA fragments mixtures of different molecular mass which differ in 50 or 100 pair of nucleotides, respectively, and are used for size comparative identification of formed amplicons in the reproducing of PCR with synthesized primers); "Universal inner control UVK-90" (includes the reagents set for monitoring of possible DNA losses during its selection from the investigated samples of the biological material and determining possible inhibition of PCR in the process of its reproduction) [7].

3. Results and Discussion

To create experimental bartonellosis foreign researchers used biological models of those types of animals that are natural reservoir for causative agents, such as cats, dogs, coyotes, primates and pack rats [3]. The exploitation of the indicated animals in the experiment allows recreating the natural course of infectious process with large authenticity. However, there are some substantial shortcomings due to inability for wide application in practice: high cost of such animals, difficulties in their maintenance in laboratory conditions and technical complications in experiment realization on them, and, also, limited possibility of standardized laboratory model creation for all *Bartonella* species, that is associated with ability of every species of pathogen to multiply and accumulate only in a certain type of sensible animals.

Therefore, the last years noted tendency to the increase of researches, aimed at the development of technically more suitable models to experimentally reproduce bartonellosis that are based on the exploitation of the most widespread laboratory animals, —mostly mice, considerably rare—rats, guinea—pigs, rabbits [3, 5, 8]. It should be noted that for ordinary white nonlinear laboratory mice experimental bartonellosis is characterized with the absence or extraordinarily poorly expressed symptoms of the disease without substantial reproduction and accumulation of causative agents in their organism, and with rapid elimination of them even in the conditions of bacteraemia in experimental animals on the initial stage after their infection. The mentioned above circumstances make the researchers apply the genetically cloned syngeneic lines of mice with an innate immunodeficiency, and that are characterized with a high level of sensitiveness to the causative agents of different infectious diseases [9]. However, such linear animals are relatively expensive and scarce enough; the special conditions for their isolated maintenance and reproduction are required, which would prevent deaths of animals due to casual infecting. It substantially complicates the stable providing of laboratories and their practical deployment with linear immunosuppressive animals being a biological model. Therefore, potentially most perspective for wide practical application, there can be a biological method,

based on the usage of white nonlinear laboratory mice with the induced immunodeficient state, which we may successfully do by the way of introduction to the experimental animal of compounds, that have the necessary spectrum of immunosuppressive action. Today there is a large list of medicinal preparations, which are characterized with the polytypical mechanisms of immunosuppression without the display of antibacterial activity. It allows a reasonable choice that enables to apply exactly those preparations that provide the adequate reproduction of the necessary immunosuppressive condition for experimental animals with absence of undesirable bactericidal or bacteriostatic action on *Bartonella*.

The choice of preparation "Cyclophosphanum®" ("Kyivmedpreparation", Ukraine) for the artificial formation of the immunodeficient state of white nonlinear mice is reasonable due to the detailed study of its mechanisms of immunodepressive action, absence of antibacterial activity, cheapness and availability [4].

The biological model of experimental bartonellosis created by us is suitable for realization of diagnostic, epidemiological, epizootic, exobiological and other researches with different origin of biological material samples, including samples of solid and liquid consistency material, with the absence and presence of contamination with an extraneous microflora.

When supervising the animals during the experiment, their premature death and onset of clinical signs of the disease were noted: decline of mobility and appetite, inertness to a tactile and sound irritation, hunched seat, lameness, flatulence, and loss of about 30% of the body mass. We performed determination and comparison of morbidity level (premature death and disease incidence) in three different groups of laboratory animals: group 1 (n = 7) are control intact animals; group 2 (n = 7) are control animals with the artificially formed immunodeficient state; group 3 (n = 42) are experimental animals with the immunodeficient state infected with the samples of the investigated biological material (Table).

The results of these researches showed that the experimental animals of group 3 produced clinically expressed disease in 46.3% of cases unlike the control animals from group 1 and 2. It was set with the usage of PCR method that bartonellosis was the reason of premature death of experimental animals in 13.0% cases resulted in an onset of the disease with clinical signs without death, while being supervised in 33.3% cases. Asymptomatic forms of infection were observed in 41.6% cases.

To confirm the reproduction and accumulation of *Bartonella* in experimental animals of group 3, comparative (taking into account the positive result of PCR) determination of corpuscular antigen amounts (cells and microcolonies) *Bartonella spp.* bacteria was done using the method of indirect immunofluorescence assay [10]. This was performed with the samples of the investigated biological material, which was inoculated to the experimental animals, and in the samples of sectional material from them, such as tissues and organs that can contain most of the causative agent's cells (blood, spleen, bone marrow, liver, lymph nodes) potentially. The results of this research showed the accumulation of bacteria in tissues and target organs of the infected animals, which exceeded the initial concentrations of these microorganisms in the corresponding samples of the investigated biological material more than in 102-103 times. In addition, comparative analysis of the results of Bartonella detection using PCR method in the same samples of biological material without and with the usage of the pathogen reproduction, and accumulation stage with growing in vivo conditions (offered biological model), demonstrates the substantial ($p < 0.05$) increase of causative agents detection from 14.3% to 28.5% (Table 1).

Table 1 Results of *Bartonella spp.* PCR detection from biologic material of different origin.

Material	Number	*B. quintana* detected by PCR		*B. henselae* detected by PCR	
		Direct method	Biological method	Direct method	Biological method
Blood from cats	10	1 (10%)	1 (10%)	4 (4%)	6 (60%)
Blood from patients suspected on cat scratch disease or bacillary angiomatosis	8	1 (12.5%)	2 (25%)	0	2 (25%)
Homogenate of *Ixodes ricinus*	24	0	0	0	1 (4.2%)
Total	42	2 (4.8%)	3 (7.1%)	4 (9.5%)	9 (21.4%)

4. Conclusions

The white nonlinear mice with the artificially formed immunodeficient state are proven to be effective biological model of experimental bartonellosis with the increased sensitiveness to the infection.

Biological model, based on intracutaneous introduction of immunosuppressive medication "Cyclophosphanum®" to the laboratory animals in a dose 250 μg/kg, is characterized with commonality and simplicity of reproduction, considered cheaper than the analogues and are affordable for practical deployment.

Inoculation of the white nonlinear mice with the artificially formed immunodeficient state with biological material samples, potentially infected with *Bartonella*, allows to promote the level of causative agents' detection (with further verification by PCR method) 2 times as much, which can be used for bartonellosis diagnostics in people and animals due to the detection of causative agents in the samples of the biological material.

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