Method for detection latent natural foci of wild animals babesioses in nature conservation reserves

V V Belimenko¹ and P I Khristianovsky²

¹ Federal State Budget Scientific Institution “Federal Scientific Centre VIEV” (109428, Russia, Moscow, Ryazanskiy prospekt, 24/1)
² Orenburg State Agrarian University (460014, Russia, Orenburg, Chelyuskintsev str., 18).

E-mail: vlad_belimenko@mail.ru

Abstract. Babesioses are large group of natural focus transmissive tick-borne diseases, which are widespread and damage many species of domestic and wild animals. Babesia spp. the causative agents of babesiosis in livestock also infect the phylogenetic species of wild animals. Vectors of Babesia spp are hard ticks. In agriculture for control of tick the insectoacaricides are used usually. But this way is impossible for nature conservation reserves because the employment of chemical and biological drugs for acaricidal treatment is prohibited by Federal Low N 33 from 14.03.95. The purpose of the manuscript is providing the methodology for detection of natural foci of animal babesiosis for evaluation of epizootological situation in nature conservation reserves. The Method is based on Babesia lifecycle and observation of Babesia in tissues and egg of ticks.

1. Introduction
Babesioses are large group of natural focus transmissive tick-borne diseases, which are spread everywhere in Russia and effect many species of domestic and wild animals. A development of high yield animal agriculture is allied with real difficulties at spread places of this group of diseases. High yield brood animals die in most cases while the Babesia infection, and convalescent ones drastically decrease a productivity. Vectors of Babesia spp are hard ticks (Ixodidae) — arachnids, typically 3 to 5 mm long, part of the order Parasitiformes. Along with mites, they constitute the subclass Acari. Ticks are ectoparasites (external parasites), living by feeding on the blood of mammals, birds, and sometimes reptiles and amphibians. Ticks had evolved by the Cretaceous period, the most common form of fossilisation being amber immersion. Ticks are widely distributed around the world, especially in warm, humid climates.

Almost all ticks belong to one of two major families, the Ixodidae or hard ticks. Adults have ovoid or pear-shaped bodies, which become engorged with blood when they feed, and eight legs. In addition to having a hard shield on their dorsal surfaces, hard ticks have a beak-like structure at the front containing the mouthparts, whereas soft ticks have their mouthparts on the underside of the body. Both families locate a potential host by odor or from changes in the environment [1].
Ticks have four stages to their lifecycle, namely egg, larva, nymph, and adult. Ixodid ticks have three hosts, taking at least a year to complete their lifecycle. Because of their habit of ingesting blood, ticks are vectors of at infectious and parasitic diseases that affect humans and other animals [2, 3, 4].

In agriculture for control of tick the insectoacaricides are used usually. But this way is impossible for nature conservation reserves because the employment of chemical and biological drugs for acaricidal treatment is prohibited by Federal Law N 33 from 14.03.95.

At the same time in the Russian Federation stable biotopes of hard ticks have been formed up and being occurred constantly. these ticks eat animal and human blood and are agent transmitters of many transmissive infectious and invasion diseases [5, 6, 7]. They are very stable towards adverse factors of nature environment. They can overwinter and bear overflowing of their inhabitation places for 12 days. Ixodid tick females can live in hungry state for 3 years. Besides, they are very fertile. Tick female can lay less than 5 thousand eggs. An extremely important capacity of ticks is to transfer disease agents transovarianally to next generations. So, the territorial areas inhabited with the invasion ticks stay dangerous for decades and represent natural focuses of illnesses. Besides, ticks are capable to move on to new territories thanks to master-feeder and as a result they gradually inhabit new territories, forming new for them natural foci of diseases [8, 9].

*Babesia spp.* the causative agents of babesiosis in livestock also infect the phylogenetic species of wild animals. For example, the causative agent of northern bovine babesiosis *Babesia divergens* MacFadyean, Stockman, 1911 infects wild ruminants *Capreolus capreolus* (European roe deer), *Cervus dama* (Fallow deer), *Rangifer tarandus* (Rein deer), *Babesia bigemina* Smith et Killborn, 1893 infects wild ruminants from genes *Gazella*, *Odocoileos*, *Bufoal*, *Mazamo*, *Cynacerus*, *Bison* ect., ovine pathogens *Babesia ovis* Babes, 1892 and *Babesia moelasi* Wenyon, 1926 infects *Cervus elaphus* (Red deer), *Cervus dama* (Fallow deer), *Capreolus capreolus* (European roe deer) ect., equine *Babesia caballi* Nuttell at Strickland, 1910 infects wild horses and donkey and canine *Babesia canis* Piana et Galli-Valerio, 1895 and *Babesia gibsoni* Patton, 1910 infects foxes, wolves, Raccoon dogs (*Nyctereutes procyonoides*), jackals (*Canis aureus*, *Canis mesomelas*, *Canis adustus*) and coyote (*Canis latrans*) [10].

Thus, diseases (not tick-born only) can cause death of wild animals, damage their population and obstruct the introduction of the Red Book animals in nature conservation reserves.

For a successful tick and tick-born diseases control, it is necessary to know the regularities of the formation of these diseases natural focuses and be able to discover these focuses on a territory. It will allow to work out and apply effective measures on their elimination taken to particular conditions. Besides, a study of these focuses can serve as rather effective approach of transmissive disease prophylactics, because in this case, abruption of epizootological chain “parasite-vector-host” takes place.

The purpose of the manuscript is providing the methodology for detection of natural foci of animal babesiosis for evaluation of epizootological situation in nature conservation reserves. The Method is based on Babesia lifecycle and observation of Babesia in tissues ang egg of ticks.

2. Methods of collection of ticks

Ticks can reside in natural and host biotopes. To first ones. the pastures, animals drinking and run places are referred, to second ones – bird nests, holes, lairs of ground vertebral animals, spaces for house animals, various practical units. Ixodid ticks can be collected just on territory or natural biotopes as well as from animals.

To pick-up of ticks in natural biotopes the following appliances are used:

1. Travois — represents piece of wool light tissue of 1x1.5 m size, better with animal sweat traces, or dressed lambskin. It is carried along the grass and is observed every 50-100 steps.
2. Hand flag — gauze or white sheeting cut of 80х60 cm size attached to 1 m handle. It is slowly carried along grass and bushes, is observed from time to time, and hung on ticks are taken off from time to time.

3. G.S. Pervomyaskiy towel — piece of light heavy but not waffle tissue of 3х1.5 size attached from both sides to transverse poles of 2 m long. Its tag is run across greens back and forward and then hung on ticks are taken off.

4. Cultivator of Pomerancev — plywood of 35х50 cm size bordered with gauze and attached to handle. The cultivator is carried with force through plants cover touching ground with narrow end hung on ticks are taken off then.

5. Screen of Pomerancev — right angled frame from poles of 200х75 cm size bordered with white sheeting ruled by eight transverse lanes of 25 cm each. The screen is applied for ticks which picked from tall bushes and trees up. The screen is carried in vertical position through plant thicket with ticks taken off and height marked where ticks were noticed.

3. Lifecycle of Babesia spp.

It is known that Babesia spp. are characterized by natural patchiness of special type when they are transferred transovarially from generation to generation of ticks. Therefore, Ixodid ticks and their eggs are the major reservoir of babesioses in nature. Hence, Babesia biological lifecycle, including their capability for transovarial transfer in the ticks, effects diseases epizootology essentially.

Babesia lifecycle consists of two parts: development in mammalian organism and development in vectors — the ixodid ticks. The most spectacular example, which all stages of babesia development are shown, is equine babesiosis causative agent Babesia caballi life cycle.

In obtained smears from animal blood, there are observed different agent single forms — trophozoites: round (11.4 %), oval (25.2 %), amoeboid (13.1 %), annulate (19.3%), pear-shaped (12.3%); as well as coupled pear-shaped forms — merozoites (18.7%). Infection intensity alternates from 18 to 55 affected erythrocytes in 100 ones in a view. Hence, reproduction by the way of simple division is observed on this stage of Babesia development cycle (table 1).

During film investigation from different ticks’ organs, schizonts of various maturation degree are observed in salivary glands, with schizont’s 4-18-micron diameter and 12-25 nucleoli. Single club-shaped forms (sporozoites) of 8-10 x 2-3-micron size with well-recognized nucleus and 1-2 large vacuoles are observed at females. In a whole, II of salivary glands by Babesia is rather pronouncing (from 1-2 to 10-12 specimens in 1 view).

Schizonts and single club-shaped forms (kineties) are observed in midbowel in essentially less quantity than in salivary glands.

10-12 days after gonotrophic cycle passage, all females start egg laying. The eggs are laid by portions during 12-14 days. According to many authors data, all eggs are appeared to be infected, while egg Babesia infection is higher at first three days of laying than at following days.

Larva development in the eggs proceeds for 18-25 days. During first 12-36 hours of incubation, it is revealed in films at x900 zoom the parasite very tiny round cells of 0.6-1-micron diameter. Further (up to 6-14 days), middle-size and large single babesia are being found, wherein, significant polymorphism is observed (table 1). 11 kinds of single forms are observed and described, which spindle-shaped and club-shaped ones dominate amongst (74.0% and 9.8%. correspondingly, from the overall parasite amount; their sizes are 10-12 x 2-4 microns).

Single forms number constitutes 3-97 specimens in 100 of view. 2 peaks of egg infection are noted that are of babesia single forms — big peak corresponding to 3-5th day of the incubation and peak of small intensity corresponding to 10th day of incubation. It is worth to note that small intensity peak was not observed in all egg laying. In the incubation process, schizonts are observed in the eggs that look like dense
granulated balls of 12-15 micron size with 200 nucleus number. Their number is liable to particular periodicity – single schizonts appear from incubation first days, observation maximum is at 6-14th days, then their number is being decreased again. Schizont decay process is also recorded.

Beginning from 10-12th day of incubation the babesia single forms become tiny (2-4 x 0.5-1 microns). During last 3-8 incubation days, neither single forms nor schizonts are observed in the researched material. Organs of future ticks’ larva are well recognized in egg mass at this time. Probably, babesia are already in larva organs at this period and are not stained by Romanovsky-Gimza method.

Table 1. Babesia caballi polymorphism in tick’s eggs depending on day of incubation.

| Incubation days | Spindle-shaped | Club-shaped | Pear-shaped | Round | Finger-like | Leech-like | Sar-like | Triangle | Half-moon | Infusorial | Flagellates | Schizonts |
|-----------------|----------------|-------------|-------------|-------|-------------|------------|---------|----------|-----------|------------|-------------|-----------|
| 2               | 73.7           | 4.6         | 3.5         | 5.6   | 1.5         | 4.5        | 0.5     | 0.5      | 5.1       | 0.5        |             |           |
| 3               | 72.5           | 4.1         | 5.7         | 1.6   | 4.8         | 0.8        | 0.8     | 6.5      | 3.2       | 0.5        |             |           |
| 5               | 70.0           | 16.3        | 1.3         | 2.1   | 1.8         | 2.1        |         | 9.0      | 0.6       | 0.8        |             |           |
| 6               | 70.5           | 11.3        | 1.3         |       |             |            |         |          |           |            |             |           |
| 7               | 74.9           | 18.1        |             |       |             |            |         | 5.0      |           |            |             |           |
| 8               | 65.4           | 19.2        | 13.0        | 3.2   | 6.9         | 4.8        |         | 1.0      |           |            |             |           |
| 10              | 73.0           | 7.9         | 13.0        |       |             |            |         |          |           |            |             |           |
| 12              | 86.0           | 13.2        |             |       |             |            |         |          |           |            |             |           |
| 13              | 72.2           | 21.8        | 5.2         |       |             |            |         |          |           |            |             |           |
| 14              | 99.5           |             |             |       |             |            |         |          |           |            |             | 0.5      |
| 16              | +              |             |             |       |             |            |         |          |           |            |             | +         |
| 17              | +              |             |             |       |             |            |         |          |           |            |             | +         |
| 18              | +              |             |             |       |             |            |         |          |           |            |             | +         |

+ single parasite

Within incubation 1-5 days frame, many researchers saw parasite flagellate forms. They are not observed further in the films. These forms presence allow to suppose the existence of sex process in babesia on this stage of the development.

Hence, Babesia transfer from large to tiny forms in schizogenesis process proceeds that allows them to penetrate into ticks larva cells. As a result, parasite circulation in ticks goes on and territory stable problems on babesioses is being maintained.

4. Babesia discovery in hard ticks’ organism

Revealing of babesia in organism of hungry ticks, collected from the territory, is connected with real difficulties. Babesia revealing in fed ticks organism is more efficient. Nevertheless, hungry ticks usage is
also possible but they are preliminary fed during 3-4 days on rabbits. Parasite largest number in ixodid tick’s organism is revealed in salivary glands. But Babesia also can be observed in blood plasma, subepidermal layer, bowel and female gonads. Due to research multiple data, babesia begin to occur in salivary glands on the 4th day of ticks feeding. So, to reveal the agent one should take off the fed-up ticks from animal, prospect organs, make a film from them on object plate and stain it by Romanovsky. For research results validity, it is recommended to prepare no less than 10-15 ticks.

To accomplish this job following instruments are necessary: eye sharp scissors, pinchers with maximally narrow shanks, preparative needle, pins, paraffin extinguished Petri dish with cavity in the middle, fat-free clean object plates, glass sticks, spreaders.

Preliminary, ticks is washed with distilled water or carried through 96 % alcohol, dried with filtering paper, then head end of its body (when back is upward) is locked between last phalanges of thumb and index finger, and the intersection starts. Microscopic preparations must be ready simultaneously.

First, films from tick’s blood plasma are made. For this, one or two limbs are cut at their basement with sharp small scissors, and shown on stump the blood plasma drop is put on fat-free object plate, the drop is spread across the plate with thin uniform layer, then it is dried and fixated with methanol or Nikiforov mixture and then stained. Club-shaped babesia are predominantly represented in blood plasma.

Then belly backside rim is cut. Into the appeared small whole, scissors branch is put and chitin cover is cut with it along right and left flanks making the cut till rostel basement.

After this, the ticks is transferred into physiological solution poured into Petri dish, which bottom is filled with melted paraffin, fastened with pins, and then ticks back chitin cover elimination and gonads and salivary glands preparation are performed. It is worth to perform manipulations under mesoscope control (for instance, MBC-4).

Backside rim of spinal chitin is caught by thin pinchers, pulled upward cautiously wherein releasing interior surface from fixated therein muscle batch, then it is cut at the rostel bottom and eliminated. Simultaneously, scrape of subepidermal layer of spinal chitin is made with pinchers branch and is put with thin layer onto object plate.

Furthermore, bowel sprays are caught by pinchers; bowl represents dark, tortuous tubes of various width. It is released with the help of preparation needles from tracheas and adipose body, is put aside and then salivary glands, located at flanks of body front part, and gonad, located at body spinal half, having a form of thick streak abundantly covered with egg follicles, are denuded.

Salivary glands and gonad are transferred with pinchers onto separate object plates, mashed with preparation needle or spreader to uniform mass and spread with thin layer, dried out during 1-2 hours at room temperature, fixated and stained with regular solution of Romanovsky-Gimza stain for 30-40 minutes. Then stain is cautiously washed out with water and film is being dried out then.

Films microscopy is pursued in immersion system at x630 zoom, no less than 200 views must be looked. It is happened to discover single round (2-2.5 microns), pear-shaped (2.5-3.5 microns), club-shaped (8-10 microns) shapes in the films. Cytoplasm has a gray-blue color, nucleus – red one (under fortunate staining). Schizonts are also discovered – multinuclear cells, one of babesia development stages. They have a look of balls consisting of red nuclei variety. Besides Romanowsky stain one can use Nocht or Pappenheim staining.

Babesia should be distinguished from nuclei differing by size and shape and from shells of distracted salivary gland cells stained into lilac-red or red color or from secreta drops of yellow-pink or dark-gray color.

5. Detection of babesia in eggs of ticks

Ixodid tick’s biology specificity is in that during blood feeding, males copulate with females. Females eat up to 4-6 days, whereafter leave host organism and lay eggs in different wrinkles and cracks in the soil. Egg
laying proceeds up to a month, after which the female dies. In environment, larvae are developed in the eggs. Larva development process in the egg (incubation) goes on for 18-25 days.

It is known that babesia got into tick female organism with blood, penetrate into female gonad and forming eggs, In the laid egg, babesia are transferred into larva, nymph from there and then to imago (transovarian transfer). This process is repeated during many generations even while ticks feeding at non-specific hosts.

It is not difficult to reproduce egg-laying process and egg incubation in vitro. For this, slopped female ticks must be taken away from animal (namely females increase multiple times while blood nutrition) and placed in Petri dish on filtering paper and closed with lid. The dish is kept at 20-22°C, the paper is dampened with water after 48 hours.

Usually, 10-12 days after gonotrophic cycle passage, females begin egg-laying. It goes on by portions and proceeds 12-14 days. Our researchers revealed that eggs of first 3-5 days of laying are the richest in babesia, namely they should be used for films preparation.

The specificity of babesia development in the eggs is very difficult to notice at first 12-36 incubation days because they have very small sizes (0.6-1.0 microns) just in this period. Beginning from incubation 3-4th day various babesia forms are revealed in contaminated eggs, they are mostly spindle- and club-shaped ones, their sizes are 10-12 microns. During staining by Romanovskiy-Gimza, agent cell cytoplasm obtains blue-like or gray color (sometimes colorless), nucleus – red color. Schizonts are also observed at that time, they look like balls of red nuclei of 12-15 micron sizes. Sometimes, one is capable to catch schizont decay process.

6. Technique of smear preparation from ticks’ eggs
Methodology constitutes the following. Small part of egg mass is transferred on scalpel tip from Petri dish onto object plate, physiological solution drop is added, all is rubbed with glass stick and dried on air. Then the film is fixated with ethanol till drying off and is stained with regular solution of Romanovsky-Gimza stain for 30-40 minutes. After stain washing out with water and drying out the film is microscoped in immersion system at x630 zoom.

Thus, in a whole, the methodology of babesia revealing in ticks’ eggs looks in a following way:
1. To take off several slopped ticks females from animal.
2. To place them into Petri dish for egg-laying at 20-25°C temperature and 70-80% humidity.
3. Eggs of first 3 days laying should be incubated for 3-4 days.
4. Films should be prepared from these eggs and stained by Romanovsky-Gimza. Observation of Babesia single forms and schizonts testifies on excitant circulation in ticks of given territory and is a feature of territory misfortune on the matter of Babesioses.

7. Conclusion
The methodology permits to reveal with validity sufficient degree the animal Babesia spp. foci on all stages of parasite lifecycle (as in ticks-carriers and their eggs as well as in hematothermal animals-babesia carriers). This methodology can serve as one of the objective criteria of fortune evaluation of some territory on babesia during epizootological inspections.

Basing on this assessment the current and perspective plans on prophylactics measures of animal babesioses are being arranged.

Also it is necessary to create special epizootological geoinformation systems for collect and analysis of information about ticks and tick-borne diseases in nature conservation reserves [11, 12, 13, 14].

References
[1] Olenev N O 1931 Parasitic Ixodoidea ticks of USSR fauna (Leningrad: Izdatelstvo AS USSR)
[2] Belimenko V V 2016 *Protozoan diseases in pets* (Moscow: Infra-M) DOI: 10.12737/17436

[3] Sirotkin M B and Korenberg E I 2018 Influence of abiotic factors on different developmental stages of the Taiga tick *Ixodes persulcatus* and the sheep tick *Ixodes ricinus* *Entomological Review* **98**(4) 496-513. DOI: 10.1134/S0013873818040115

[4] Fernandez P J and White W R 2010 *Atlas of transboundary animal diseases* (France: OIE)

[5] Belimenko V V, Christianovskiy P I, Novosad E V and Gulyukin A M 2019 Formation of hard ticks' biotopes on urban territories *IOP Conf. Ser.: Earth Environ. Sci.* **315** 042024 https://doi.org/10.1088/1755-1315/315/4/042024

[6] Uskov A N, Lobzin Yu V and Burgasova O A 2010 Tick-borne encephalitis, ehrlichiosis, babesiosis and other topical tick-borne infections in Russia *Infection Diseases* **2** 83-8

[7] Korenberg E I, Kovalevskiy Yu V, Gorelova N B and Nefedova V V 2015 Comparative analysis of the roles of *Ixodes persulcatus* and *I. trianguliceps* ticks in natural foci of ixodid tick-borne borrelioses in the Middle Urals, Russia *Ticks and Tick-Borne Diseases* **6**(3) 316-21 DOI: 10.1016/j.ttbdis.2015.02.004

[8] Korenberg E I, Sirotkin M B and Kovalevskiy Yu V 2016 A general scheme of the circulation of ixodid tick-borne borrelioses pathogens in natural foci of Eurasia *Zoological Journal* **95**(3) 283-299 DOI: 10.7868/S0044513416030090

[9] Belimenko V V and Gulyukin A M 2020 Tick-born diseases epidemiological monitoring system in the Russian Federation *IOP Conference Series: Earth and Environmental Science* **548** 42039

[10] Krylov M V 1996 *Key to Protozoan parasites* (St. Peterburg: Zoological Institute RAS)

[11] Belimenko V V, Rafienko V A, Droshnev A E, Laishevtsev A I and Kapustin A V 2019 Application of geoinformational systems for veterinary geology *IOP Conference Series: Earth and Environmental Science* **315** 032015

[12] Gulyukin A M 2014 Significance of modern methods for laboratory detection of rabies agents and identification of the zoonose immunological survey *Voprosy virusologii* **59**(3) 5-10

[13] Makarov V V, Svyatkovsky A V, Kuzmin V A and O I Sukharev 2009 *Epizootological research method* (Moscow: Infra-M)

[14] Gulyukin A M, Belimenko V V, Shabaykin A A, Droshnev A E and Laishevtsev A I 2020 Epizootological geo-information systems *IOP Conference Series: Earth and Environmental Science* **421** 042013