

Original article

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Development and validation of an instrument-free system for dna target detection based on the ultra-monodisperse gold nanospheres

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Abstract:

Objective: to develop a simple and user-friendly technology for the detection of DNA targets for the diagnosis of socially significant diseases based on a colorimetric aggregation assay using gold nanoparticles.

Materials and Methods: Gold nanospheres were synthesized by liquid-phase chemistry methods. The physicochemical parameters of the nanoparticles were controlled using absorption spectroscopy, dynamic light scattering, and electron microscopy. Rational design of specific genomic target sequences and oligonucleotides included in the test system was carried out considering thermodynamic constants and using genomic databases. Analytical validation of the test system was performed using a model for the detection of *Babesia* species DNA – the causative agent of piroplasmiasis in humans and animals – by cross-comparison of characteristics with polymerase chain reaction (PCR) with electrophoretic detection in real-time format.

Results: A pilot version of the test system for specific detection of up to 10 ng of DNA in blood samples was developed, based on visual discrimination of the color of the reaction mixture with an analysis duration of approximately 30 minutes. The size effect of nanoparticles in the range from 15 to 60 nm on the minimum detection limit of DNA targets was established.

Conclusion: This study presents the results of the development and analytical validation of a pilot express test system for DNA diagnostics based on gold nanoparticle labeling and visual color discrimination of the reaction mixture.

Keywords: rapid test system, molecular diagnostics, gold nanoparticle, colorimetric test

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Introduction

The need for rapid and accurate determination of genetic targets of socially significant diseases [1], as well as direct identification of pathogens of infectious diseases [2], creates the preconditions for the development of simple and convenient genetic tests ready for use in everyday medical practice. The development of approaches in modern laboratory diagnostics and bioengineering has formed a separate direction for the creation and adaptation of express test systems designed for self-testing, referred to in English-language literature as POCT (point-of-care test systems) [3], outside of specialized medical institutions, the so-called “testing at the patient’s bedside” [4]. A key role in the creation of DNA biosensors [5] and POCT test systems is the development of specific markers consisting of two main components - a probe and a label. A probe is a molecule that selectively and specifically binds to a target fragment based on the principle of complementary binding, immunochemical or other bio-specific affinity. Traditionally, molecular or corpuscular carriers of a specific signal, triggered during the molecular recognition reaction “probe-target”, serve as labels. Thus, unambiguous detection of targets in the analyzed environments is achieved through high-affinity molecular recognition reactions which are characterized by distinct indicators such as a color change [6], a chemiluminescent

signal [7], and other responses that can be detected by the eye or with a use of simple indication systems. In addition to markers development, a key challenge is the search of new approaches for detection systems [8] that have to simultaneously meet a range of technical and socio-economic requirements. In particular, the parameters of POCT systems must meet the following criteria:

- detection of analytes (in our case, specific fragments of nucleic acids within diagnostically significant ranges);
- competitiveness and cost-effectiveness in comparison with traditional laboratory methods [9];
- user-friendliness and ease of implementation;
- minimal time and labor costs.

Meeting the listed criteria will facilitate the successful implementation of newly developed POCT systems, ensuring adequately meeting modern requirements for the provision of medical care within preventive and diagnostic services.

Among the many actively developing areas, colorimetric DNA test systems based on gold nanoparticle (GNP) labels are of particular interest [10]. More than 20 years after the pioneering publication in 1996 by C. A. Mirkin and colleagues [11], numerous studies have been proposed on the use of gold nanoparticles (GNPs) as labels for genetic POCT systems, some of which have progressed to clinical application [12].

The operating principle of most known colorimetric test systems lies in the biospecific or salt-induced aggregation of nucleic acids (NA), triggered by nucleotide hybridization of the «probe – target» complex. In particular, one of the strategies is implemented through the use of gold nanoparticles (GNPs) covalently labelled with specific oligo-DNA probes that have sequences complementary to the target fragment being detected [11]. The second strategy is based on electrostatic forces and is implemented in a label-free format through the use of specially selected surface modifiers for gold nanoparticles (GNPs) and the composition of the hybridization buffer [13]. The keen interest in the second strategy is due to a number of objective reasons, discussed in detail in our previous works [14]. We previously proposed a colorimetric DNA test [15], which is protected by a Russian Federation patent [16]. The principle of the method is based on the change in the color of the colloid in the presence of target DNA in the test sample, caused by the aggregation of gold nanoparticles (GNPs). The registration of the specific signal can be detected using several methods with varying sensitivity:

- by visual inspection (with the naked eye);
- instrumentally, using standard absorption spectrophotometry;
- by dynamic light scattering (DLS), which records changes in the average hydrodynamic particle size;
- by spectrofluorometry;
- by microscopic analysis;
- and through other approaches.

Objective: is to develop a simple and user-friendly technology for detecting target DNA sequences for the diagnosis of socially significant diseases, based on a colorimetric aggregation assay using gold nanoparticles (GNPs). As part of the concept being developed, we propose an improved version of the colorimetric DNA test based on supermonodisperse GNPs as labels. The key advantage of the proposed modification is an increase in detection sensitivity combined with reduced assay time. This is achieved due to the unique aggregation properties and supersphericity of the gold nanoparticles (GNPs), compared to the previously used quasi-spherical GNPs.

Cross-validation of the analytical characteristics of a test system using a model for detecting *Babesia* species DNA – the causative agent of babesiosis (piroplasmosis), a dangerous transmissible infection affecting both humans and animals. The reference method is polymerase chain reaction (PCR) with two detection formats:

- electrophoretic detection of results (PCR-ED);
- real-time fluorescent detection (PCR-RT).

Materials and Methods

Design of Oligonucleotide Primers and Probes

The sequences of specific oligonucleotide primers for performing reference- tests by PCR-ED (polymerase chain reaction with electrophoretic detection), namely, for identification of extended sections of genes encoding 18S rRNA of *Babesia canis canis*—were adopted from study [17]. Primers for detecting precursor genes of the cryptic antigen of *Babesia canis canis* were taken from the work [18],

respectively. The design of target-specific primer sequences for the amplification of short fragments of the 18S rRNA gene of *Babesia canis canis* by real-time PCR (PCR-RT) was carried out using generally accepted algorithms. Oligonucleotides with sequences similar to direct PCR primers were selected as probes for the colorimetric test on GNP labels. The specificity of all developed sequences was confirmed by comparison with genomic fragments of targets annotated in the worldwide GenBank database. Melting temperature optimization, as well as verification of the sequences for the presence of non-specific sites of mutual complementary binding of oligonucleotides, was conducted by an oligocalculator.

The characteristics of all used oligonucleotides are presented in *Table 1*. The synthesis of oligonucleotides was carried out using the method of automated phosphoramidite chemistry on a solid-phase support CPG-500 (glass with a controlled pore size of 500 Å) and an Polygen 12 oligosynthesizer (PolyGen GmbH, Germany). To cleave the synthesized oligonucleotides from the carrier, incubation was conducted in a 37% aqueous ammonia solution at a temperature of 55°C for 24 hours, then rough purification of the oligonucleotides from organic impurities was carried out by desalting in n-butanol and reprecipitation into an aqueous solution. Preparative purification of the oligonucleotides was performed by high-resolution gel electrophoresis in a 20 % polyacrylamide gel under denaturing conditions, followed by elution of the product from the gel into a buffer (10 mM aqueous solution of Tris-ethylenediaminetetraacetic acid at pH 8).

Sample preparation of biological specimens

Genomic DNA was isolated from dogs' blood samples with a clinically confirmed diagnosis of piroplasmosis (the samples were kindly provided by Professor S. A. Staroverov, Federal State Budgetary Educational Institution of Higher Education «Saratov State University of Genetics, Biotechnology and Engineering named after N. I. Vavilov») using a commercial genomic DNA extraction kit (DNA Blood Mini-Kit, Qiagen, Germany). The procedure was carried out in a laminar flow cabinet BMV-II («Laminar-S», Russia) following the manufacturer's instructions. 37 clinical samples were used in the study in total. The purity and concentration of the preparations (OD₂₆₀ = 1 corresponds to 50 µg/mL of DNA) were verified by ultraviolet (UV) spectrophotometry at wavelengths of 230/260/280 nm using a Nanobron Onec optical analyzer (Thermo FS, USA). The obtained DNA samples were stored in 1.5 mL microtubes at -20 °C (POZIS HL-340, Russia) until the commencement of the colorimetric test and PCR amplification.

Procedure for Performing Polymerase Chain Reaction (PCR) with Electrophoretic Detection of Results

PCR amplification was carried out in 200 µL thin-walled tubes (Scientific Specialties, Inc., USA) using a SimpliAmp thermal cycler (Thermo FS, USA).

The amplification mixture (total volume 30 µL) contained:

- 3 µL of 10× PCR buffer;
- 10 mM dNTPs;
- 25 mM MgCl₂;
- 5 units/µL Taq DNA polymerase;
- a mixture of forward and reverse primers – 50 pmol of each;
- 2 µL of genomic DNA (30 ng/µL);
- Milli-Q deionized water (Merck Millipore, Germany) – adjusted to the final volume of 30 µL.

Table 1. Oligonucleotides Used in This Study

| Notation | Functional type | 5'-3' sequence | Amplicon size, bp | Reference |
|------------------|---------------------------|--------------------------|-------------------|---------------|
| <i>BAB-P</i> | Labeling probe for GNPs | GTCTTGTAATTGGAATGATGG | – | [17] |
| <i>BAB_Fw_Un</i> | Forward primer for PCR-EF | GTCTTGTAATTGGAATGATGG | 559 | |
| <i>BAB_Rv_L</i> | Reverse primer for PCR-EF | CCAAAGACTTTGATTTCTCTC | | |
| <i>BAB_Fw_Un</i> | Forward primer for RT-PCR | GTCTTGTAATTGGAATGATGG | 61 | Present study |
| <i>BAB_Rv_S</i> | Reverse primer for RT-PCR | CACCAGACTTGCCCTCCAAT | | |
| <i>CBA_P</i> | Labeling probe for GNPs | ACCATGATGCTGCTCTTCGCCTTG | – | [18] |
| <i>CBA_Fw</i> | Forward primer for PCR-EF | ACCATGATGCTGCTCTTCGCCTTG | 1065 | |
| <i>CBA_Rv</i> | Reverse primer for PCR-EF | GCGAAAAACATGAGTGGGACC | | |
| OKO | Negative control | TCCTGCAGATACACTCCCACCAA | – | [15] |

The amplification conditions (for both primer mixes BAV and CBA) were as follows: 5 min of initial denaturation at 94°C, 45 cycles, each comprising: 60s of denaturation at 92°C, 60s of primer annealing at 52°C, 90s of elongation at 72°C and 7-minute final elongation at 72°C. The amplification results were visualized by horizontal agarose gel electrophoresis using the intercalating dye ethidium bromide (Helicon, Russia). 10 µL of the mixture of PCR products with loading buffer was added into each well. DNA length markers (50–1500 bp Step50 bp Plus, Biolabmix, Russia) were used to determine fragment sizes. The fragments were separated in a 1.5 % agarose gel (Agarose E, low EEO, #D00391, Dia-M, Russia) prepared in 1×TAE buffer (Thermo, Lithuania). The electrophoresis was performed using a Blue Marine 200 Cx electrophoresis system (Serva, Germany) connected to a 310 Marine Power Supply constant current source (Serva, Germany). The running conditions were as follows: voltage: 130 V; power: 30 W; current: 55 mA; duration: 90 min. The electrophoretic images were captured using a BlueCube-300 gel documentation system (Serva, Germany).

Procedure for fluorescent detection of results in real-time mode

The real-time PCR (qPCR) reaction mixture contained: 2 µL of genomic DNA (30 ng/µL); 10 µL of premixed amplification master mix containing the intercalating dye SYBR Green (2× HS-qSYBR-blue, Biolabmix, Russia); a primer mix (*BAB_Fw_Un* and *BAB_Rv_S*) – 0.4 pmol of each primer; Milli-Q water – adjusted to a total reaction volume of 20 µL. The amplification was

triple performed using a LightCycler 96 real-time PCR instrument (Roche, Switzerland). Amplification modes: 1 cycle 94°C (5 min), then 45 cycles [94°C (10 s), 60° (20 s) + detection on the FAM channel]. The amplification curves were analysed using the certified LightCycler® 96 Software, version 1.1.0.1320.

Production and characterization of colloidal gold nanoparticle labels

Preparation of quasi-spherical colloidal gold nanoparticle preparations (AuNP-cit) was carried out using the Frens method [19], with additional surface modification by cetyltrimethylammonium bromide (CTAB), as described in [15]. The preparation of monodisperse spheres in a micellar solution of cetyltrimethylammonium chloride (AuNP-CTAC) was carried out by liquid-phase chemical synthesis using the seed-mediated method of Xia [20]. The typical set of spectral and geometric characteristics of freshly prepared colloids was obtained using conventional procedures and methods of UV-Vis spectrophotometry and DLS (dynamic light scattering). The morphology and sizes of the particles were also determined from images obtained using a Libra 120 transmission electron microscope (Carl Zeiss, Germany) at the Shared Research Equipment Center in the Field of Physicochemical Biology and Nanobiotechnology “Symbiosis” of the FRC SSC RAS.

Determination of operating parameters of gold nanoparticles for a colorimetric test

The essence of the optimization was to sequentially determine the operating parameters of reaction mixture components based on the GNP labels, in which particle aggregation doesn't occur in the hybridization-salt buffer until the target DNA is added to solution. In brief, the step-by-step procedure is as follows. Freshly prepared GNP preparations, washed from excess reaction mixture by centrifugation at 12,000 rpm for 30 minutes, were resuspended in a micellar aqueous solution of CTAB with various concentrations (1, 3, 5, and 10 mM CTAB solution). Next, the salt resistance of GNP was tested for each of the obtained colloidal solutions of GNP-CTAB complexes. For this purpose, a series of mixtures were prepared consisting of 900 μ l of GNP-CTAB solution and 100 μ l of sodium chloride (NaCl) solutions with concentrations from 0.1 to 1M. Next, to determine the optimal concentration of the probe, mixtures of GNPs and solutions of BAB-P oligonucleotides were prepared in a hybridization buffer (7 mM Tris-HCl, pH 7.4; 170 mM NaCl) in a concentration range from 0 to 50 nM. Ten minutes after the preparation of the described test mixtures, 100 μ l of each were transferred into the wells of optically transparent 96-well plates with a flat bottom. The absorption spectrum was recorded in the wavelength ranges on a Multiskan SKYHIGH TC plate multi-reader (Thermo Scientific, USA).

Procedure for Performing a Colorimetric test

The colorimetric test was conducted according to the protocol described in our previous work [15] with minor modifications. A typical set of experimental and control reaction mixtures includes:

- 1) Blank – GNPs in water;
- 2) Standard 1 – GNPs in hybridization-salt buffer;
- 3) Standard 2 – a mixture of GNPs in hybridization buffer and probe;
- 4) NCS – a mixture of GNPs in hybridization buffer, probe, and a negative control sample;
- 5) PCS – a mixture of GNPs in hybridization buffer, probe, and control sample (a synthetic sample with the target DNA sequence at a known concentration);
- 6) Sample – a mixture of GNPs in hybridization buffer, probe, and the test sample (a pre-amplified fragment of the target DNA after 5–10 cycles of standard amplification).

The components were mixed in 2 ml microtubes in the following ratio: 600 μ l of GNP-CTAB suspension with an optical density of 0.2 at a wavelength of 520 nm, 900 μ l of hybridization buffer (a mixture of 50 mM Tris-HCl, pH 7.4; 1.2 M NaCl and Milli-Q water in a volume ratio of 1:1:2), 150 μ l of probe solution, 150 μ l of target solution (added last) and Milli-Q water until the final mixture volume reached 1800 μ l. The results were recorded with the visual effect after 5 minutes and spectrophotometrically (in a cuvette or in the wells of optically transparent 96-well plates) after 30 minutes by the DLS method after the final preparation of the mixtures.

Statistical Data Processing

The analysis was carried out using standard Microsoft Office Excel 2010 package. The obtained data are presented as the mean and its standard deviation ($M \pm SD$). The normality of distribution of quantitative data was tested using the Shapiro–Wilk test. The equality of variances was tested using the Levene's test. One-way analysis of variance (ANOVA) was used to assess the significance of differences in quantitative characteristics. The p-value was calculated with a critical threshold set at 0.05.

Results

According to the presented data of electron microscopic images (Fig. 1), GNP-cyte have significant quasi-sphericity, the degree of which increases with increasing particle size (Fig. 1, d), which is also reflected in the broadening of the extinction spectrum (Fig. 1, b). In

contrast, the seed growth method enables the production of monodisperse samples of gold nanoparticles (GNPs) with perfect spherical morphology and provides control over particle size across a broad range from 10 to 150 nm (Fig. 1, a, c).

The use of high-quality nanoparticle samples with low polydispersity and polymorphism as labels for DNA aggregation testing is fundamentally important. The registration of characteristic phenomena occurring during particle aggregation can be carried out: visually (qualitatively by observing the color change of the initial colloid from red to gray-blue or colorless) or quantitatively by UV-visible spectrophotometry and Dynamic Light Scattering (DLS). The absence of false peaks in the histogram, associated with the rotational diffusion of quasi-spherical GNPs, during DLS registration of particle aggregation events induced by DNA-DNA hybridization allows for more accurate analysis and thereby increases its sensitivity. Indeed, an independent assessment of the lower detection limit of tests based on GNP-cyt+CTAB and GNP-CTAC (based on the presence of a signal that is significantly different from the control) carried out on model synthetic oligonucleotides in the 3 above-mentioned detection methods showed significant advantages of monodisperse GNP. Subsequent testing of the assay on real samples (see Fig. 1) using the detection of specific gene fragments of *Babesia canis canis* as an example confirmed the high detection limit values. Species identification of the piroplasmosis/pathogen was simultaneously performed in parallel using PCR with electrophoretic analysis of the results. The presence of *Babesia canis canis* was detected in 37 canine clinical blood samples with 100% specificity; general clinical data (anamnesis, blood smear, hemodynamic parameters, commercial PCR test) were used as reference data.

Comparison of the detection limits between the colorimetric test on GNP-CTAC and PCR-EF (Fig. 2, g) demonstrated the superiority of the developed test by almost 3 orders of magnitude (Table 2). It should be noted that the proposed colorimetric test is most similar to the variant of quantitative PCR using hybridizing fluorescent probes, the role of which is performed by GNPs. The test procedure consists of preamplification (from 1 to 5–15 cycles, depending on the number of nucleic acid copies of target agents in the original sample) and detection in a buffer solution containing GNPs and recognizing probe molecules. According to the data presented in Table 2, significant quantitative indicators of detection limits were achieved in three variants of biospecific aggregation registration: with unaided eye (comparable to RT-PCR-RV), spectrophotometrically (sensitivity is an order of magnitude higher than PCR-RV), or by DLS method (100–1000 times more sensitive than PCR-RV). Finally, the advantages of the colorimetric method are its simplicity and efficiency: the analysis time is reduced from several hours (typical for RT-PCR with real-time detection) to 30–60 minutes, and no expensive equipment is required beyond a conventional thermal cycler and spectrophotometer.

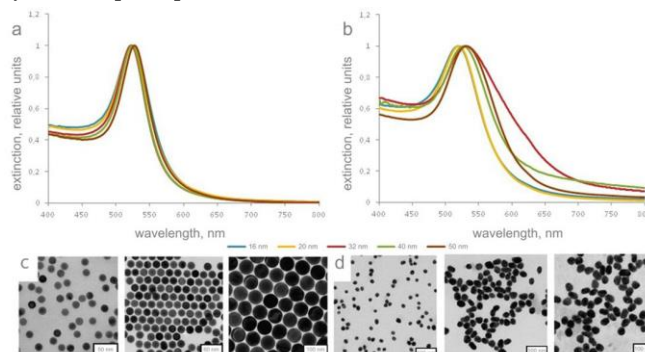


Fig. 1. Extinction spectra and transmission electron microscopy images of monodisperse spheres in a cetyltrimethylammonium chloride micellar solution (a, c) and quasi-spherical gold colloidal particles (b, d) with diameters of 16, 20, 32, 40, and 50 nm. Scale bars correspond to 100 nm

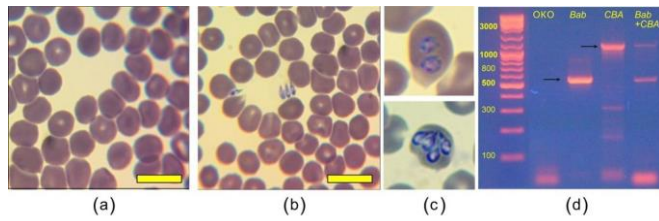


Fig. 2. Light microscopic detection in the culture fluid on the 14th day of cultivation (a–c) and detection of specific fragments of *B. canis* genes by the PCR-EP method (d). Staining was performed using the Romanowsky-Giemsa method. Lane labels of the electropherogram (d) from left to right: DNA length markers (100–10,000 bp); NTC (Negative Template Control) – negative control (water instead of DNA template); Bab – species-specific detection of 18S rRNA gene fragments; CBA – identification of cryptic antigen fragments; Bab+CBA – multiplex detection of both genes. The scale bars correspond to 20 μm

Table 2. Comparative Characteristics of Analytical Sensitivity of Colorimetric and PCR Tests Using the Example of 18S rRNA *Babesia canis canis* Gene Identification

| Test system | Amplicon detection method | Analysis duration (excluding sample preparation), min | Quantitative determination capability | Lower detection limit for target DNA |
|--|----------------------------------|---|---------------------------------------|--------------------------------------|
| PCR-EF | Visual (gel electrophoresis) | 300-400 | no | 10 ng |
| RT-PCR | Fluorescence | 120-180 | yes | 1 ng |
| Colorimetric test on GNP-cit-CTAB labels | Visual method (with unaided eye) | 2-20 | no | 100 ng |
| | UV-vis | 3-5 | yes | 10 ng |
| | DLS | 30-60 | yes | 1 ng |
| Colorimetric test on GNP-CTAC labels | Visual method (with unaided eye) | 2-20 | no | 10 pg |
| | UV-vis | 3-5 | yes | 1 pg |
| | DLS | 30-60 | yes | 0.1 pg |

Discussion

A comparison of our “label-free” version with known colorimetric tests based on conjugates of nanoparticles with chemically attached thio-derivatives of oligoDNA probes [11] allows us to make the following observations. Firstly, the simplicity and efficiency of the analysis. The comparatively labor-intensive and time-consuming stage of preparing conjugates is absent in our case. Detection occurs immediately after adding the target solution (containing NC isolated from the sample after 5–15 cycles of preamplification or without it) to the mixture of solutions of GNPs and recognition molecules. Thus, in its simplest version – unaided detection without a preamplification step from the moment of biomaterial collection – the test procedure comes down to nucleic acid extraction and colorimetric detection. The total time for the procedure takes 30–40 minutes. Secondly, the cost price plays a significant role, which is particularly important during practical implementation stages: commercial synthesis of unmodified oligonucleotides is much cheaper than their thio-derivatives. Thus, the developed approach is simple and accessible for subsequent commercialization as a prototype test system with the possibility of adaptation to various analytes and detection systems.

Simplicity, efficiency, and high sensitivity to the formation of aggregates in the system are competitive advantages of the non-disturbing DRS method.[15] On the other hand, the geometric parameters of nanoparticles (monomorphism and isodispersity) significantly affect the accuracy of signal measurement. Traditionally used in bioanalytics, GNP-cyt [19] have a significant degree of asphericity, which increases with particle size growth. In addition, while the particle size increases, their colloidal stability decreases, making further use of particles larger than 35 nm impossible. These fundamental drawbacks affect the effectiveness of using citrate nanospheres for colorimetric DNA tests. In this regard this work utilized nanoparticles with improved characteristics (isodispersity and monomorphism degree of at least 98%) and the ability to control particle size in a wide range from 10 to 150 nm [20]. It should be noted that the method of controlled growth of «seeds» in the micelles solution of GNP- CTAB used for synthesis results in particle production with positive surface charge, is ready for use in colorimetric testing after washing off the growth solution. On the contrary, an additional stage of surface modification of particles with a molecular layer of CTAB is required in the case of using GNP-cit [19]. Thus, we assume that replacing only one component – one type of GNP with another – will allow us to overcome the previously identified [15] diagnostic limit of sensitivity (approximately 10 ng of DNA, which is equivalent to the sensitivity of PCR-EF) while simultaneously simplifying the procedure.

Conclusion

This paper presents the results of pilot tests of the developed colorimetric assay based on ultramonodisperse spherical GNPs for rapid and sensitive detection of specific fragments DNA targets. Validation of quantitative indicators has shown significant advantages (up to 3 orders of magnitude in sensitivity) of the developed approach compared to the RT-PCR method. The developed prototype of the test system for specific detection of *Babesia* sp. can be adapted for further application in clinical laboratory diagnostics and veterinary practice to identify the causative agent of piroplasmiasis in humans and economically important animals. The obtained results may be in demand in laboratory and clinical practice as an alternative to the PCR method and as a basis for new developments in the fields of cell engineering, molecular diagnostics, pharmacology, etc.

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Contribution of authors:

S.S. Veretennikov – polymerase chain reaction research, writing the initial version of the article;

T.E. Pylaev – synthesis of gold nanoparticles, article design, data processing, writing and editing of the manuscript.

The authors approved the final version of the article before publication, expressed consent to be responsible for all aspects of the work, implying proper consideration and resolution of issues related to the accuracy or integrity of any part of the work.

Conflict of interest. The authors declare the absence of both explicit and potential conflicts of interest related to the publication of this article.

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