Tonkie Khimicheskie Tekhnologii = Fine Chemical Technologies.
2025;20(6):565–581

ISSN 2686-7575 (Online)

Biochemistry and biotechnology

Биохимия и биотехнология

UDC 577.11
https://doi.org/10.32362/2410-6593-2025-20-6-565-581
EDN SCSQPA

RESEARCH ARTICLE

Development of immunochromatographic assay for simultaneous detection of tetracyclines and streptomycin in milk

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Abstract

Objectives. To optimize indirect antibody immobilization on gold nanoparticles (GNPs) using anti-species antibodies for enhanced conjugate stability and to develop an immunochromatographic assay (ICA) for antibiotic detection in milk.

Methods. The GNPs were synthesized by reduction of tetrachloroauric acid in the presence of seed particles. The size of GNPs was determined spectrophotometrically according to literature data using a *Thermo Fisher Scientific* Varioskan LUX instrument. Monoclonal mouse antibodies to tetracycline and streptomycin were immobilized on the surface of the GNPs via anti-mouse antibodies. Conjugates of bovine serum albumin with tetracycline and streptomycin were obtained through Mannich reaction and click-reaction, respectively. The coupling ratio was determined by MALDI-TOF mass spectrometry on a *Bruker* RapifleX instrument. Immunoreagents were dispensed onto a nitrocellulose membrane using a *BioDot* ZX1010 dispenser. The assembled multi-membrane composite was cut into test strips using a *KinBio* ZQ4500 guillotine cutter. The test results were interpreted visually and using an *Allsheng* TSR-100 test strip reader.

Results. Following conjugate formation via indirect immobilization using anti-species antibodies, it was necessary to block residual binding sites on the anti-species antibodies in order to enhance solution-phase conjugate stability. As a result of optimizing the concentrations of immunoreagents, an ICA was developed for the simultaneous detection of streptomycin and tetracyclines in milk. The detection limit of the optimized ICA for tetracyclines and streptomycin was 2–7.5 and 25 ng/mL, respectively, for visual result interpretation, and 0.29–2.15 and 1.34 ng/mL, respectively, when using a test strip reader.

Conclusions. It is shown that the stability of the resulting conjugates in solution can be enhanced by blocking the free binding sites of the anti-species antibodies to prevent cross-linking of the nanoparticles caused by anti-species antibody binding.

Keywords

lateral flow immunoassay, antibiotics, rapid test, gold nanoparticles, tetracycline, streptomycin, immobilization

Submitted: 30.04.2025
Revised: 07.08.2025
Accepted: 11.11.2025

For citation

Maksin I.V., Polyakova D.I., Kesareva V.A., Sysuev A.A., Ivanov V.S., Simonova E.I., Khunteev G.A., Kirillova Yu.G. Development of immunochromatographic assay for simultaneous detection of tetracyclines and streptomycin in milk. *Tonk. Khim. Tekhnol.* = *Fine Chem. Technol.* 2025;20(6):565–581. https://doi.org/10.32362/2410-6593-2025-20-6-565-581

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НАУЧНАЯ СТАТЬЯ

Разработка иммунохроматографического анализа для одновременного обнаружения тетрациклинов и стрептомицина в молоке

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Аннотация

Цели. Оптимизация стабильности конъюгатов, полученных методом непрямой иммобилизации специфических антител на наночастицах золота (НЧЗ) при помощи антивидовых антител, и разработка на их основе иммунохроматографического анализа (ИХА) для определения антибиотиков в молоке.

Методы. НЧЗ синтезировали восстановлением золотохлористоводородной кислоты в присутствии зародышей. Размер определяли спектрофотометрически по литературным данным на приборе *Thermo Fisher Scientific* Varioskan LUX. Моноклональные мышиные антитела к тетрациклину и стрептомицину иммобилизовали на поверхности НЧЗ посредством анти-мышиных антител. Конъюгаты бычьего сывороточного альбумина с тетрациклином и стрептомицином получали реакцией Манниха и клик-реакцией соответственно. Степень конъюгации определяли при помощи MALDI-TOF масс-спектрометрии на приборе *Bruker* RapifleX. Иммунореагенты наносили на нитроцеллюлозную мембрану на диспенсере *BioDot* ZX1010. Собранный мультимембранный композит нарезали на тест-полоски на гильотинном резчике *KinBio* ZQ4500. Результаты ИХА интерпретировали визуально, а также при помощи считывателя тест-полосок *Allsheng* TSR-100.

Результаты. После получения конъюгатов посредством непрямой иммобилизации при помощи антивидовых антител необходимо проводить блокировку оставшихся незанятыми сайтов связывания антивидовых антител, чтобы повысить стабильность конъюгатов в растворе. В результате оптимизации концентраций иммунореагентов был разработан ИХА для одновременного обнаружения стрептомицина и тетрациклинов в молоке. Предел обнаружения оптимизированного анализа тетрациклинов и стрептомицина составил 2–7.5 и 25 нг/мл при визуальной интерпретации результатов, 0.29–2.15 и 1.34 нг/мл при использовании считывателя тест-полосок соответственно.

Выводы. Установлено, что блокировка свободных сайтов связывания вторичных антител повышает стабильность полученных конъюгатов антител в растворе, предотвращая кросс-сшивки наночастиц, вызываемые связыванием антивидовых антител.

Ключевые слова

антибиотики, экспресс-тест, наночастицы золота, тетрациклин, стрептомицин, иммобилизация

Поступила: 30.04.2025
Доработана: 07.08.2025
Принята в печать: 11.11.2025

Для цитирования

Максин И.В., Полякова Д.И., Кесарева В.А., Сысуев А.А., Иванов В.С., Симонова Е.И., Хунтеев Г.А., Кириллова Ю.Г. Разработка иммунохроматографического анализа для одновременного обнаружения тетрациклинов и стрептомицина в молоке. *Тонкие химические технологии*. 2025;20(6):565–581. https://doi.org/10.32362/2410-6593-2025-20-6-565-581

INTRODUCTION

Detecting antibiotics in food products is a critical task for ensuring food safety and protecting public health. The uncontrolled use of antibiotics in veterinary medicine leads to their accumulation in animal products, potentially posing a threat to human health due to increased antibiotic resistance in microorganisms and occurrence of allergic reactions.

Highly sensitive and specific physicochemical analysis methods, including high-performance liquid chromatography and mass spectrometry, are effectively used for antibiotic detection. However, due to their reliance on expensive equipment and the expertise of highly qualified specialists [1], these methods are used only in large laboratory centers.

Efficient testing in non-laboratory settings is based on the use of simple and accessible methods such as immunochromatographic assay (ICA). The principle of ICA is based on a highly specific antigen-antibody interaction followed by separation of the resulting immune complex as the fluid moves along a porous membrane. ICA is characterized by its simplicity and rapidity, allowing for a qualitative assessment of the presence or absence of a target compound in a sample without using additional equipment [2].

However, one of the main factors hindering the widespread use of ICA is its relatively low sensitivity [2, 3]. Traditional approaches to increasing ICA sensitivity include the use of high-signal-intensity labels, such as quantum dots [4], signal enhancement by coloring with silver salts [5], analyte concentration using functionalized magnetic nanoparticles [6], and preincubation of the sample with the antibody-label conjugate before adding it to the test strip [7]. In applying these approaches, it is important that the key advantages of the method, i.e., its speed and ease of use, are not sacrificed in the pursuit of a low detection limit.

Orienting specific antibodies on sensor surfaces in order to maximize the exposure of binding sites is a relatively new and promising approach in immunoassay, allowing for a significant reduction in the detection limit of immunosensors. Numerous approaches aimed at targeted immobilization of antibodies have been recently reviewed [3, 8]. For example, the research group led by B.B. Dzantiev has developed a number of highly sensitive test systems [9–11], including those with the simultaneous detection of multiple analytes on a single test strip [12, 13]. Nevertheless, the development of new systems remains relevant and requires an expanded scope of application.

The present work sets out to develop an immunoassay for the simultaneous determination of streptomycin and tetracyclines in raw milk with instrument-free interpretation of the results. To achieve a low detection limit of the ICA, we used secondary antibodies specific to the Fc fragment of mouse immunoglobulin G to immobilize mouse antibodies to tetracycline and streptomycin on the surface of gold nanoparticles (GNPs). We also applied the approach of preincubating the sample with the antibody-label conjugate before adding it to the test strip.

MATERIALS AND METHODS

Materials, reagents and solutions

The following reagents were used: bovine serum albumin (BSA), fraction V5 (*Proliant*, New Zealand), streptomycin sulfate (*PanReac Applichem*, Spain), propynyloxypropionic acid hydrazide, γ-azidobutyric acid oxysuccinimide ester (*Primetech*, Republic of Belarus), aurichlorohydric acid (*Aurat*, Russia), tetracycline hydrochloride (*Biotopped*, China), HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sucrose, tris(hydroxymethyl)aminomethane (*Dia-M*, Russia), tris-hydroxypropyltriazolylmethylamine (THPTA) (*Lumiprobe*, Russia), guanidine hydrochloride, sodium citrate (*Helicon*, Russia), L-ascorbic acid (*Sigma-Aldrich*, USA), hydroxylamine hydrochloride (*Panreac*, Spain), formaldehyde (*neoFroxx*, Germany). The following state standard samples were used as analytes to determine the detection limit and cross-reactivity: streptomycin sulfate, tetracycline hydrochloride, doxycycline hyclate, chloramphenicol (*VGNKI*, Russia), as well as oxytetracycline hydrochloride (*Thermo Fisher Scientific*, USA), penicillin G sodium salt (*Biotopped*, China), chlortetracycline hydrochloride, cefoperazone sodium salt (*Molekula*, United Kingdom), gentamicin sulfate, kanamycin sulfate (*neoFroxx*, Germany). The remaining reagents used were of Russian manufacture and had a qualification of at least chemically pure. Milk samples with a fat content of 3.2% were purchased from a retail network in Moscow. When preparing standard solutions of antibiotics in milk, the concentrations were calculated based on the pure active substance, taking into account the molecular weight of the corresponding salts. In addition, polyclonal goat antibodies to chicken egg antibodies, polyclonal goat antibodies to mouse antibodies, chicken egg IgY antibodies (*Arista Biologicals*, USA), mouse monoclonal antibodies to tetracycline, and mouse monoclonal antibodies to streptomycin (*Eastmab*, China) were used. Deionized water (Milli-Q®, *Millipore*, USA) was used to prepare solutions, GNP preparations, and conjugates based on them.

Thin-layer chromatography (TLC) was performed on RP-18 F254s reverse-phase aluminum plates (*Merck*, Germany).

SM31-40 adhesive backing (*KinBio*, China), 70CNPH analytical nitrocellulose membrane, FR-1 sample membrane, and AP45 absorbent membrane (*Advanced Microdevices*, India) were used to manufacture ICA strips.

Preparation of BSA–streptomycin (STR) conjugate

Preparation of STR–alkyne conjugate. Streptomycin sulfate (408 mg, 0.56 mmol) and propynyloxypropionic acid hydrazide (30 mg, 0.21 mmol) were added to 0.85 mL of 0.45 M Na_2CO_3 solution. The reaction mixture was incubated for 24 h at 4°C. Reaction completion was monitored by TLC in a methylene chloride/methanol = 40 : 1 system based on the disappearance of the starting material (retention coefficient $R_f = 0.3$). After elution, the plates were treated with ninhydrin solution and heated for visualization. The STR–alkyne concentration in the solution was assumed to be 0.247 M.

Preparation of BSA–azide conjugate. To a solution of 3.2 mL of BSA with a concentration of 20 mg/mL (0.962 µmol) in 0.1 M sodium bicarbonate, pH 8.5, 0.6 mL of a solution of γ-azidobutyric acid oxysuccinimide ester with a concentration of 6 mg/mL (15.91 µmol) in dimethyl sulfoxide was added. The reaction mixture was then incubated for 24 h at 4°C. The conjugate was purified by dialysis against deionized water. After dialysis, the protein concentration was determined by ultraviolet–visible (UV–Vis) spectrophotometry on a UV5Nano instrument (*Mettler Toledo*, Switzerland). The resulting conjugate was stored at 4°C.

Preparation of BSA–STR conjugate (copper reducing agent—L-ascorbic acid). To a solution of 0.5 mL BSA–azide with a concentration of 8.46 mg/mL (0.063 µmol, 1 equiv.), 0.1 mL of a 1 M HEPES solution with pH 7.2 and 13 µL of STR–alkyne (3.211 µmol, 50 equiv.) were added. In a separate test tube, solutions of 6.25 µL of 20 mM $CuSO_4$ (0.125 µmol, 2 equiv.) and 12.5 µL of 50 mM THPTA (0.625 µmol, 10 equiv.) were mixed. Next, the entire volume of the resulting solution was transferred into a mixture of BSA–azide and STR–alkyne. Then 25 µL of 100 mM guanidine chloride (2.5 µmol, 40 equiv.), 25 µL of 100 mM L-ascorbic acid (2.5 µmol, 40 equiv.), and 25 µL of 100 mM NaOH (2.5 µmol, 40 equiv.) were added. The test tube was filled with argon and left under stirring for 24 h. The conjugate was purified by dialysis against 10 mM phosphate-buffered saline (PBS), pH 7.4. This conjugate is referred to hereinafter as BSA–STR.

Preparation of BSA–STR conjugate (copper reducing agent—hydroxylamine). To a solution of 0.5 mL BSA–azide with a concentration of 8.46 mg/mL (0.063 µmol, 1 equiv.), 0.1 mL of a 1 M HEPES solution with pH 7.2 and 13 µL of STR–alkyne (3.211 µmol, 50 equiv.) were added. In a separate test tube, solutions of 6.25 µL of 20 mM $CuSO_4$ (0.125 µmol, 2 equiv.) and 12.5 µL of 50 mM THPTA (0.625 µmol, 10 equiv.) were mixed. The entire volume of the resulting solution was transferred into the mixture of BSA–azide and STR–alkyne. Then 50 µL of 100 mM hydroxylamine hydrochloride (5 µmol, 80 equiv.) were added. The test tube was filled with argon and left under stirring for 24 h. The conjugate was purified by dialysis against 10 mM PBS, pH 7.4. After dialysis, the protein concentration was determined by UV–Vis spectrophotometry using a UV5Nano instrument (*Mettler Toledo*, Switzerland). The prepared conjugate was stored at 4°C.

Synthesis of BSA–tetracycline (TET) conjugate

BSA–TET conjugate was prepared using a published method with modifications [14]. 8 mL of a solution of 18.75 mg/mL of BSA, 2 mL of a 3 M sodium acetate solution, and 12 mL of a water solution of 8.3 mg/mL of tetracycline hydrochloride were mixed. Then, 3.75 mL of a 37% formaldehyde solution were added dropwise with stirring. The container was protected from light with foil. The reaction mixture was stirred for 6 h at room temperature (20–22°C). Dialysis was then performed against 10 mM of PBS, pH 7.4.

MALDI-TOF[1] spectrometry

Mass spectrometric analysis was performed in positive ion registration mode on a RapifleX instrument (*Bruker*, Germany) at the Skolkovo Institute of Science and Technology, Advanced Mass Spectrometry Shared Use Center. A solution of HCCA (α-cyano-4-hydroxycinnamic acid) matrix was used for the analysis: a weighed portion of dry HCCA matrix (*Sigma-Aldrich*, USA) was dissolved in 50% aqueous acetonitrile with 0.5% of trifluoroacetic acid with a concentration of 20 mg/mL. Samples (1 µL) were applied on a Ground

[1] Matrix-activated laser desorption/ionization, MALDI is a process in which the analyte is ionized by a laser in the presence of a special matrix. After ionization, the ions are separated by time-of-flight (TOF), allowing the molecule to be identified according to the thus-determined mass-to-charge ratio.

Steel target (*Bruker*, Germany). Following desiccation of the droplet, 1 μL of the matrix was applied over the sample. Crystallization of the sample and matrix occurred at room temperature in a laminar flow hood. Each sample was analyzed in a single technical replicate in positive ion measurement mode. The *m*/*z* measurement error was no more than 500 ppm (up to 35 Da at a mass of 70000 Da). Spectra were visualized using Origin 9.8 (*OriginLab*, USA).

GNP synthesis

GNPs were synthesized according to the procedure [15]. First, a seed solution was prepared. 100 μL of a 5% tetrachloroauric acid solution was added to 50 mL of deionized water while stirring at 700 rpm. The mixture was brought to a boil. Then 1.5 mL of a 1% sodium citrate solution was added, and boiling was continued for 15 min. The prepared seed solution was brought to room temperature prior to use. Then, 1 mL of the resulting GNP seed solution, 0.5 mL of 1% sodium citrate solution, 0.1 mL of 5% tetrachloroauric acid, and 0.25 mL of 0.5% hydroquinone solution were added to 49 mL of deionized water with stirring at 700 rpm. The resulting sol was stored at 4°C for two months.

Test strip preparation

A ZX1010 dispenser (*BioDot*, USA) was used. After applying BSA–STR and BSA–TET conjugates to the test zone of a nitrocellulose membrane, chicken egg immunoglobulins IgY were applied to the control zone. The membrane with the applied reagents was dried in an oven at 45°C for 1 h. The multimembrane composite was cut into 4 mm wide test strips using a ZQ4500 guillotine cutter (*KinBio*, China) and stored at room temperature in a sealed bag with silica gel.

Selection of the optimal pH and protein concentration for sorption on the GNP surface

The selection was performed according to the method described in [16]. The optimal antibody concentration and pH values for the conjugation were determined on the basis of the nanoparticle conjugate stability to the strong electrolyte (10% NaCl solution). The selection was performed using spectrophotometry on a Varioskan LUX instrument (*Thermo Fisher Scientific*, USA). As the nanoparticle sol aggregates, the peak in the solution's absorption spectrum broadens along with a decreased optical density (OD) of the solution at the wavelength of maximum light absorption and increases in the long-wavelength region. Therefore, the degree of aggregation can be measured using the conventional spectral coefficient $K = OD_{536}/OD_{750}$, which reflects the degree of aggregation (aggregation coefficient). As the sol aggregates, the K value will decrease.

Conjugation of antibodies with GNPs

20 μg of polyclonal goat antibodies to mouse immunoglobulins were added to 10 mL of GNP solution adjusted to pH 8. The mixture was incubated for 1 h on an orbital shaker. Then, following the addition of 0.4 mL of 10% BSA solution, the mixture was further incubated for an additional 30 min and centrifuged for 30 min at 5000g. The supernatant was collected and the pellet resuspended in 0.5 mL of 4 mM tris buffer, pH 8, containing 5% of sucrose and 0.1% of BSA. The resulting conjugate was designated as GNP–anti-mouse. A conjugate of GNPs with polyclonal goat antibodies to chicken IgY antibodies was prepared similarly and designated as GNP–anti-IgY. The resulting conjugates were stored at 4°C.

Antibodies to streptomycin or tetracycline were added to the GNP–anti-mouse conjugate, and the mixture was incubated on an orbital shaker. After 10 min, mouse immunoglobulins were added to block the remaining binding sites. The resulting conjugates were designated as GNP–anti-STR and GNP–anti-TET, respectively. The prepared conjugates were stored at 4°C.

Conducting ICA

A solution of the GNP–anti-TET, GNP–anti-STR, and GNP–anti-IgY conjugates was mixed with 200 μL of milk sample in a well of a microtiter plate. The milk was used without prior treatment or dilution. The range of immunoreagent concentrations tested is shown in Table 1. The well was then placed in a preheated 40°C incubator for 5 min. A test strip was then immersed in the well for 5 min. The test strip was removed from the solution along with the sample membrane to stop the reaction. The color intensity of the test strip line was read using a TSR-100 reader (*Allsheng*, China).

Processing ICA results

Origin 9.8 software (*Origin Lab*, USA) was used to analyze the relationship between signal intensity (y) and analyte concentration in the sample (x). A sigmoid function was used to approximate the concentration dependencies:

$$y = (a - b)/[1 + (x/c)^d] + b,$$

where *a* is the maximum signal; *b* is the minimum signal; *c* (or IC_{50}) is the antigen concentration at which the signal decreases by 50% of its range of changes; *d* is the slope of the approximating dependence at point *c*.

The instrumental detection limit was defined as the analyte concentration causing a 10% decrease in the recorded analytical signal [17]. The visual detection limit was defined as the minimum concentration of the analyte at which the intensity of the test line coloring will be weaker than the intensity of the control line coloring or will be comparable.

RESULTS AND DISCUSSION

To achieve the aim of this study, several tasks had to be completed: (1) synthesize streptomycin and tetracycline conjugates with a carrier protein for immobilization on the analytical membrane; (2) optimize the immobilization of specific antibodies to antibiotics on the GNP surface mediated by secondary antibodies; (3) determine the detection limit of the ICA using the obtained immunoreagents.

Synthesis of BSA conjugates with antibiotics

BSA–STR conjugate **1** was obtained using a click reaction (Fig. 1) [18]. BSA was pre-conjugated with the oxysuccinimide ester of γ-azidobutyric acid to obtain BSA–azide conjugate **2**. According to MALDI-TOF mass spectrometry, the conjugation efficiency was 14 azide groups per one BSA molecule. Alkyne adduct **3** was prepared by reacting streptomycin with propynyloxypropionic acid hydrazide to form the corresponding hydrazone **3**. The reactivity of streptomycin with adipic acid dihydrazide has been described previously [19]. This formed the basis for the procedure used in this study.

Sodium L-ascorbate is typically used as a copper reducing agent in the click reaction. It has been reported [18] that the dehydroascorbate oxidation byproduct can interact with protein side chains (primarily arginine). For this reason, it is recommended to add a dehydroascorbate trap (“scavenger”) into the reaction mixture, for example, aminoguanidine. To protect the guanidine groups of streptomycin from the undesired reaction, we performed the click reaction using two different copper reducing agents: sodium L-ascorbate with the addition of guanidine as a “scavenger” and hydroxylamine. Conjugation results, according to MALDI-TOF mass spectrometry, were 6.0 and 5.6 hapten molecules per 1 BSA molecule when using sodium L-ascorbate and hydroxylamine, respectively (Fig. 2). In a comparative experiment in the ICA, the conjugates were no different from each other in performance (data not shown). We subsequently used a conjugate obtained by reduction in the presence of hydroxylamine alone.

The BSA–TET conjugate **5** was obtained under standard conditions using the Mannich reaction [14]. The conjugation efficiency was 1 tetracycline molecule per 1 BSA molecule (Fig. 2).

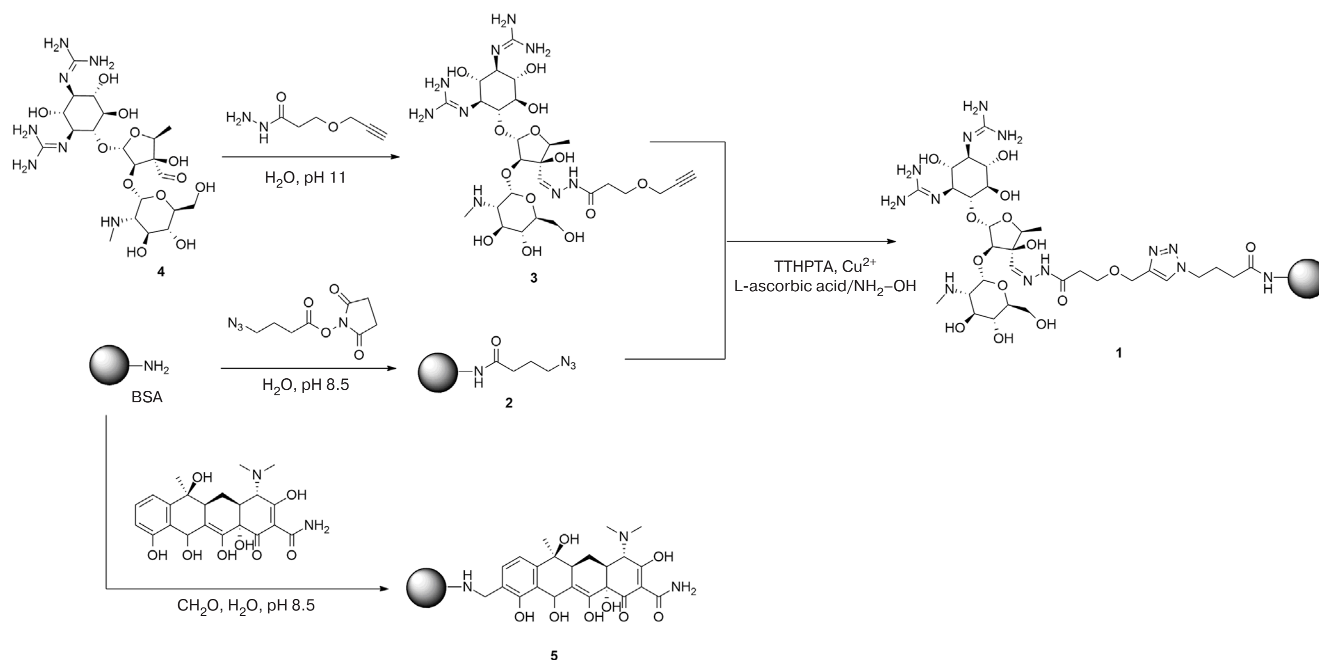

Fig. 1. Scheme of the synthesis of BSA–STR **1** and BSA–TET **5** conjugates

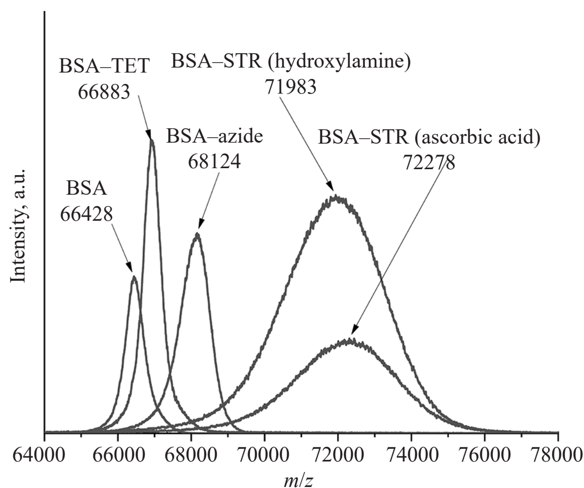

Fig. 2. MALDI-TOF mass spectra of the BSA conjugates

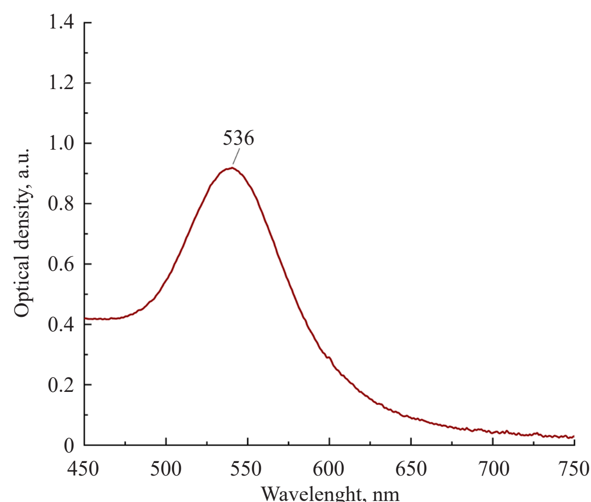

Fig. 3. Light absorption spectrum of the colloidal GNPs

Synthesis of GNPs

According to UV–Vis spectrophotometry data, the wavelength of maximum light absorption of the obtained sol is 536 nm, which corresponds to an average particle size of approximately 55–60 nm (Fig. 3) [20].

Scheme of competitive ICA for the determination of tetracyclines and streptomycin

The ICA strip for tetracycline and streptomycin detection was designed as follows (Fig. 4). BSA–TET, BSA–STR, and chicken egg immunoglobulin IgY conjugates applied to a nitrocellulose membrane formed two test lines (TL) and a control line (CL), respectively, on the assay membrane. After mixing the analytical GNP–anti-TET, GNP–anti-STR conjugates and the control GNP–anti-IgY conjugate with the sample in a microwell immediately before analysis, the mixtures were incubated for 5 min. The time of antibodies interaction with the analyte increases due to the stage of their incubation with the sample. This results in a decrease in the detection limit [7] since providing better kinetic conditions for the formation of the antibody-antigen complex as compared to the traditional ICA format: the reaction in this format is limited by the diffusion of immunoreagents in the porous structures of the test strip membrane [21].

Following immersion of the test strip in the sample mixed with the GNP conjugate, the mixture moves along the test strip coated with the reagents due to capillary action. If there are no analytes in the sample (Fig. 4a), antibodies will bind to the analytes attached to BSA. This will lead to the accumulation of gold particles on the TL and the appearance of a characteristic color. If one or both analytes are present in the sample (Fig. 4b), it (or they) will prevent the binding of the GNP conjugate to the BSA-conjugated analyte on the TL. As a result, the color of the TL will be dim or absent.

Since the assay conditions and milk sample composition can affect the visual interpretation of the result, we considered it necessary to use a control line as a standard for comparing coloring intensity. Therefore, the CL is colored due to the specific binding of the GNP–anti-IgY to chicken egg immunoglobulins IgY. Thus, the coloring intensity will be constant and independent of the fraction of the analyte conjugate retained on the CL.

The assay result is determined by comparing the brightness of the TL and CL. If the TL is brighter than the CL, the result is negative. If the TL is less bright than the CL, or their brightness is comparable, the result is positive. We considered the chosen method of result evaluation to be more acceptable taking into account the subjectivity of visual interpretation. This makes it possible to lower the detection limit of the assay when visually interpreting results, since a result will be considered to be positive not only in case of complete disappearance of the test line (typical for high analyte concentrations), but even in the case of a visible decrease in its intensity for relatively low analyte concentrations.

Conjugation of antibodies to GNPs

Secondary antibodies were conjugated to GNPs by physical adsorption at pH 8 and a concentration of 2 μg/mL. The selected parameters were obtained on the basis of an experiment to select optimal conditions (Fig. 5).

Preliminary experiments with the immobilization of antibodies to streptomycin on GNPs using secondary antibodies showed that the resulting conjugate was unstable. Within 24 h of conjugate preparation,

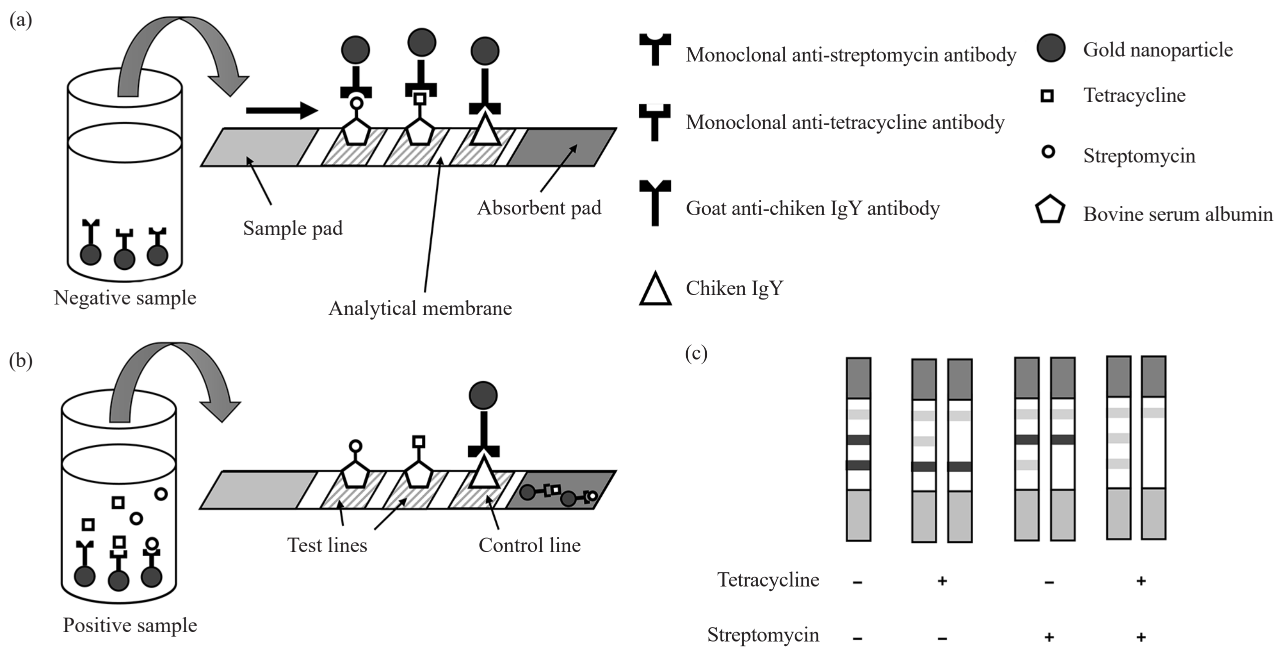

Fig. 4. Schematic diagram of the developed competitive ICA of tetracyclines and streptomycin.
(a) Negative sample; (b) positive sample; (c) result interpretation scheme

a statistically significant loss of activity was observed (Fig. 6, striped column, $p < 0.05$) along with significant aggregation, as evidenced by a decrease in the aggregation coefficient of the conjugate solution. When using two conjugates in the assay (GNP–anti-TET and GNP–anti-STR), an increase in the intensity of tetracycline coloring was observed upon the addition of streptomycin to the sample. We assume that both of these phenomena are due to the formation of nanoparticle aggregates caused by cross-linking via the binding of secondary antibodies carrying both streptomycin and tetracycline antibodies at the same time. As a result, when the sample contains streptomycin, the portion of the resulting polyvalent conjugate that was retained on the streptomycin line may migrate to the tetracycline line.

To prevent aggregation and the formation of polyvalent aggregates, we considered it rational to block unoccupied secondary antibody binding sites by using nonspecific mouse immunoglobulin G. A model experiment without specific antibodies to streptomycin and tetracycline showed that the dependence of aggregation on the amount of mouse

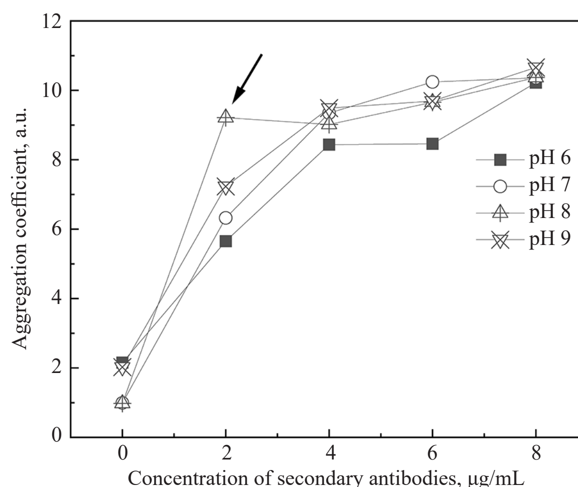

Fig. 5. Selection of optimal conditions for the sorption of antibodies to mouse immunoglobulins. The arrow indicates the selected concentration and pH value

antibodies administered was nonlinear and had a distinct minimum (Fig. 7). An increase in the degree of aggregation was observed in the concentration range of 1–7 µg/mL. This can be explained by the fact that, at low concentrations, the number of secondary antibody binding sites significantly exceeds the number of mouse antibodies, while the mouse antibodies themselves bind adjacent nanoparticles. Starting at a concentration of 9 µg/mL, a stabilizing effect occurred, which can be explained by the fact that the amount of mouse antibodies is now comparable to the number of secondary antibody binding sites to prevent the formation of large aggregates. When a concentration of 20 µg/mL was achieved, some aggregation was still observed ($p < 0.05$), but we considered it acceptable for further use (Fig. 7). The selected concentration was used to synthesize the GNP–anti-TET and GNP–anti-STR conjugates. The GNP–anti-STR conjugate prepared using blocking antibodies at a concentration of 20 µg/mL retained its functionality 24 h after preparation (Fig. 6, grey column). The next stage of work was to select the optimal concentrations of the test system immunoreagents to achieve the lowest detection limit.

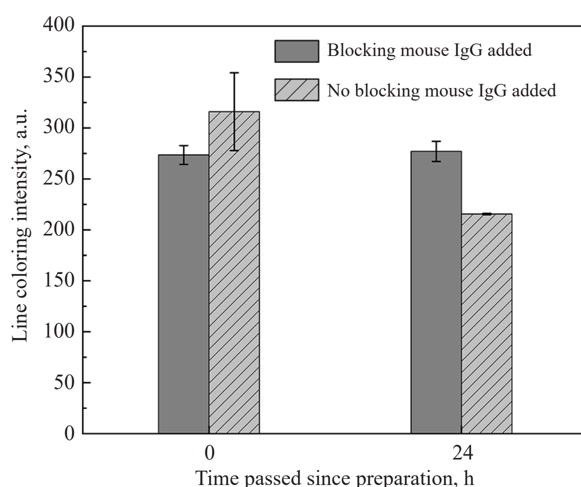

Fig. 6. Activity of the GNP–anti-STR conjugate
in the presence and in the absence of blocking immunoglobulins (20 µg/mL)

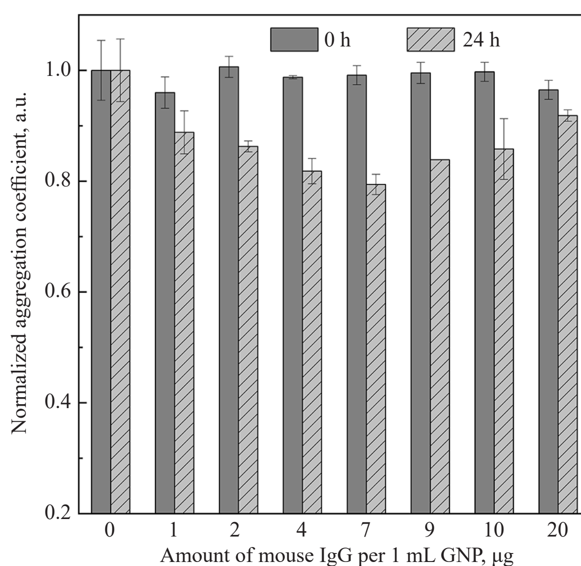

Fig. 7. Selection of the optimal concentration of mouse immunoglobulins for blocking free binding sites of secondary antibodies of the conjugate

Optimization of the detection limit

To achieve a low detection limit, we used well-known methodological approaches [22], which involved selecting the antibody-to-label ratio and immunoreagent concentrations (Table 1). Visual interpretation of the result involves comparing the brightness of the test lines with the control line. Therefore, when selecting the immunoreagent concentration, we aimed to ensure that the brightness of the control line was significantly lower.

The detection limit of the developed assay and a comparison with some previously published studies are presented in Table 2 and Fig. 8. According to the Technical Regulations of the Customs Union "On the Safety of Milk and Dairy Products" (TR CU 033/2013[2]), the maximum permissible concentrations of streptomycin and tetracycline antibiotics are 200 and 10 ng/mL, respectively. The achieved detection limits, which are deemed sufficient for use of the test system in accordance with TR CU 033/2013, are at the same level as previously published studies (Table 2).

Table 1. Selected immunoreagent amounts

Immunoreagent	Studied concentration range	Selected amount
BSA–TET	0.5–2.0 mg/mL	1 mg/mL
BSA–STR	0.1–1.0 mg/mL	0.2 mg/mL
GNP–anti-TET	10–20 µL	20 µL
GNP–anti-STR	10–20 µL	20 µL
GNP–anti-IgY	1–5 µL	3.5 µL
Anti-TET antibody	2–9 µg/mL	4 µg/mL
Anti-STR antibody	2–9 µg/mL	5 µg/mL
Mouse immunoglobulin	0–20 µg/mL	20 µg/mL

Table 2. Comparison of the developed ICA with published methods. Instrumental limit of detection (iLOD), visual limit of detection (vLOD)*

<table>
<tr><th>Antibiotic</th><th>Detection method</th><th>Sample type</th><th>iLOD, ng/mL</th><th>vLOD, ng/mL</th><th>References</th></tr>
<tr><td rowspan="11">Tetracycline</td><td rowspan="2">Fluorescence analysis</td><td>Water</td><td>750</td><td>–</td><td>[23]</td></tr>
<tr><td>Buffer</td><td>2.86</td><td>–</td><td>[24]</td></tr>
<tr><td>High performance liquid chromatography</td><td>Milk</td><td>21</td><td>–</td><td>[25]</td></tr>
<tr><td>Enzyme-linked immunosorbent assay</td><td>Milk</td><td>$IC_{50} = 0.72$</td><td>–</td><td>[26]</td></tr>
<tr><td>Colorimetric paper-based sensor with signal detection via smartphone imaging</td><td>Milk</td><td>0.5</td><td>–</td><td>[27]</td></tr>
<tr><td rowspan="6">ICA</td><td>Human serum</td><td>0.4</td><td>11</td><td>[28]</td></tr>
<tr><td>Milk</td><td>–</td><td>0.8</td><td>[29]</td></tr>
<tr><td rowspan="4">Milk</td><td colspan="2">Chlortetracycline</td><td rowspan="4">This work</td></tr>
<tr><td>2.15</td><td>7.5</td></tr>
<tr><td colspan="2">Oxytetracycline</td></tr>
<tr><td>0.29</td><td>2</td></tr>
</table>

[2] Technical Regulations of the Customs Union "On the safety of milk and dairy products" (TR CU 033/2013) (as amended on June 23, 2023) (in Russ.). URL: https://docs.cntd.ru/document/499050562. Accessed June 14, 2025.

Table 2. Continued

Antibiotic	Detection method	Sample type	iLOD, ng/mL	vLOD, ng/mL	References
Tetracycline	ICA	Milk	Doxycycline		This work
			0.78	6	
			Tetracycline		
			1.27	4	
Streptomycin	Surface-enhanced Raman scattering	Milk	$2.13 \cdot 10^{-3}$	–	[30]
	Aggregation-based L-histidine functionalized GNP assay	Milk	0.66	–	[31]
	Electrochemical sensor	Milk	$0.33 \cdot 10^{-3}$	–	[32]
	Resolved fluorescence of nanoparticles in ICA	Milk	1.10 ng/g	–	[33]
	ICA	Milk	0.4	50	[34]
		Milk	1.34	25	This work

* The table does not aim to provide a comprehensive overview and includes only selected examples from the literature.

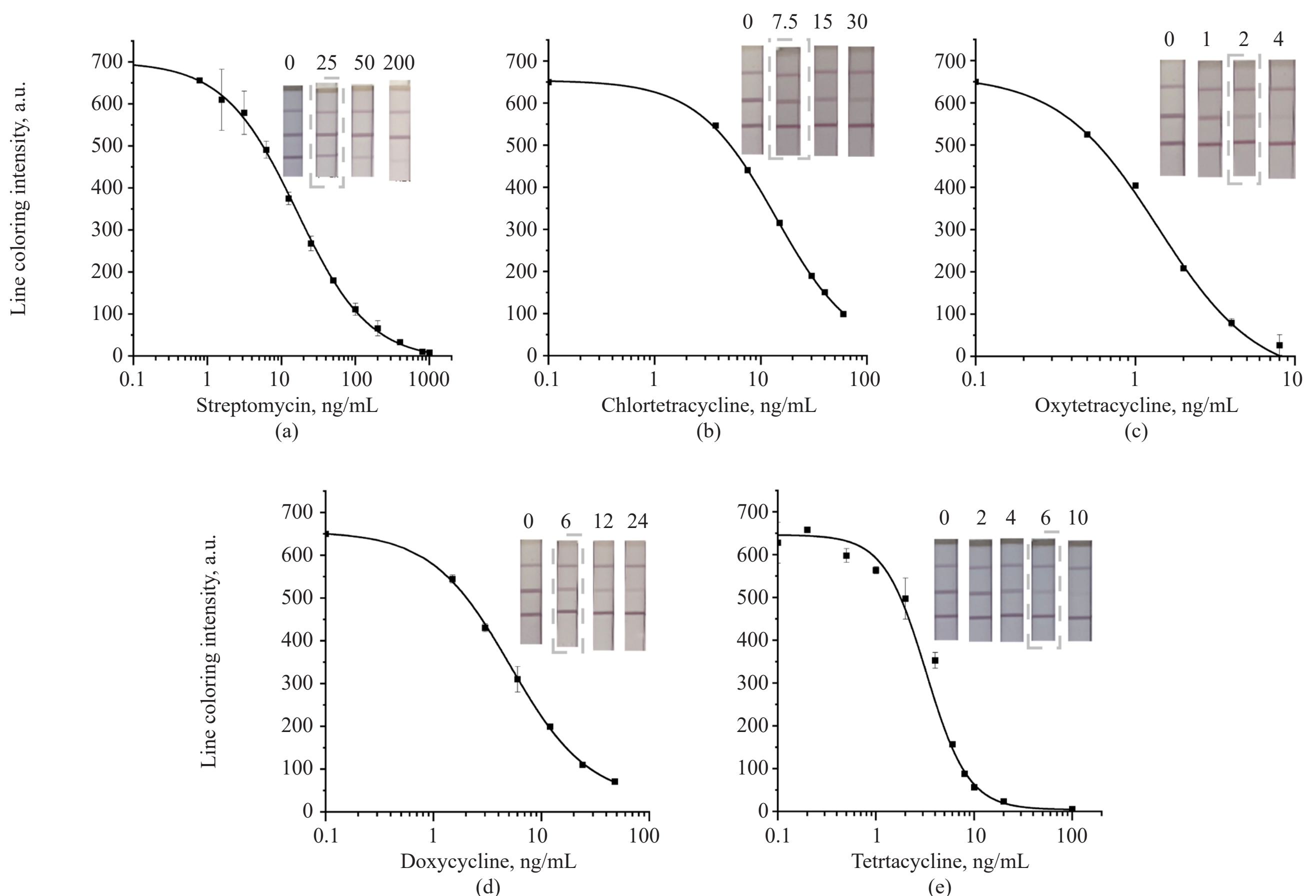

Fig. 8. Calibration curves showing the dependence of the test line color intensity on the concentration of streptomycin and tetracyclines in milk, and photographs of ICA results for the detection of (a) streptomycin, (b) chlortetracycline, (c) oxytetracycline, (d) doxycycline, and (e) tetracycline. The numbers above the photographs indicate the tested concentration of the corresponding analyte

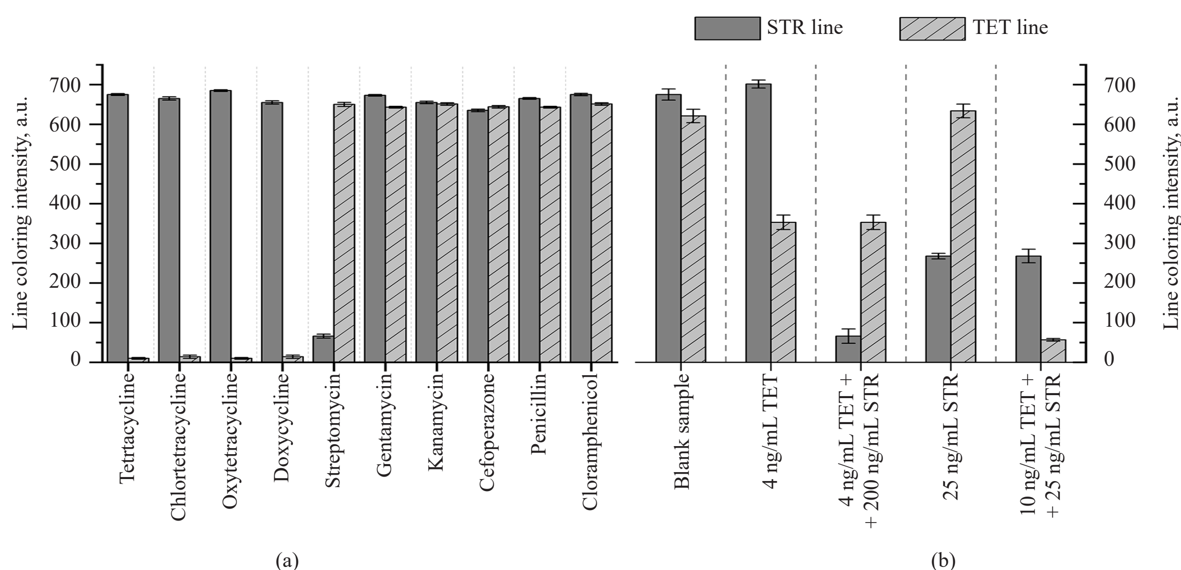

Fig. 9. (a) Assessment of the test system's cross-reactivity; (b) evaluation of the effect on the signal caused by the simultaneous presence of tetracycline and streptomycin in the sample

Additionally, the specificity of the developed assay to other aminoglycosides—gentamicin and kanamycin, as well as penicillin and chloramphenicol—was tested. No statistically significant effect of cross-reagents at a concentration of 1000 ng/mL in milk on the signal was demonstrated (Fig. 9a). The lack of cross-reactivity with aminoglycosides can be explained by the high specificity of antibodies to streptomycin and the more significant differences in the structures of the antibiotics under study. There was also no mutual interference between streptomycin and tetracycline (Fig. 9b).

CONCLUSIONS

A test system for the simultaneous detection of tetracyclines and streptomycin in undiluted milk has been developed. The visual detection limits for streptomycin, chlortetracycline, oxytetracycline, doxycycline, and tetracycline are 25, 7.5, 2, 6, and 4 ng/mL, respectively. By blocking the free centers of secondary antibodies, it becomes possible to improve the stability of the conjugates. The developed test system meets regulatory requirements and can be recommended for practical use in monitoring milk contamination with antibiotics. The methodological approaches used in this study for the simultaneous detection of tetracyclines and streptomycin are suitable for use in the development of a test system for the simultaneous detection of three or four groups of antibiotics.

Acknowledgments

The authors thank Maria Gennadievna Zavialova, a member of the Skoltech Advanced Mass Spectrometry Core Facility at the Skolkovo Institute of Science and Technology. We thank the *Rapid Bio* for providing the materials and equipment for the study.

Authors' contributions

I.V. Maksin—conceptual development of the study, interpretation and graphical presentation of the experimental data, processing results, and writing the text of the article.

D.I. Polyakova—selecting the ICA parameters and processing results.

V.A. Kesareva—selecting the ICA parameters and processing results.

A.A. Sysuev—synthesis of BSA conjugates with streptomycin and tetracycline.

V.S. Ivanov—methodological support for the study and discussion of the results.

E.I. Simonova—conceptual development of the study.

G.A. Khunteev—discussion of the results and conceptual development of the study.

Yu.G. Kirillova—conceptual development of the study, writing the text of the article, and discussion of the results.

Conflict interest declaration

Authors—I.V. Maksin, D.I. Polyakova, V.A. Kesareva, V.S. Ivanov, E.I. Simonova, and G.A. Khunteev—are employees of *Rapid Bio* (Moscow, Russia), a company engaged in the development and production of immunological test systems. This company provided financial support for this study. All authors confirm the absence of other potential conflicts of interest.

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   [Original Russian Text: Panferov V.G., Safenkova I.V., Zherdev A.V., Dzantiev B.B. Methods for Increasing Sensitivity of Immunochromatographic Test Systems with Colorimetric Detection (Review). *Prikladnaya biokhimiya i mikrobiologiya*. 2021;57(2):107–116 (in Russ.). https://doi.org/10.31857/S0555109921020112]
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Translated from Russian into English by M. Povorin
Edited for English language and spelling by Thomas A. Beavitt