ISSN 1061-9348, Journal of Analytical Chemistry, 2024, Vol. 79, No. 9, pp. 1214–1228. © Pleiades Publishing, Ltd., 2024.

ARTICLES

Azolotriazine-Based Fluorescent Test Systems for the Field Diagnosis of Endometritis in Cows

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Received December 7, 2023; revised April 11, 2024; accepted April 15, 2024

Abstract—The results of an experiment assessing the applicability of solutions of 6-oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazine-7(6*H*)-yl)acetic acid on plates and cellulose substrates for detecting excessive levels of volatile organic compounds associated with endometritis inflammation relative to biologically normal levels are considered. The fluorescent properties of the dye are studied using gynecological mucus from cows collected in various periods (pre- and post-partum) and nasal mucus from newborn calves. The test system responses were compared with clinically established diagnoses and the results of a microbiological study. An evaluation of the test systems revealed a false positive rate of no more than 11% and a false negative rate of 2%. Other characteristics, such as specificity, accuracy, and precision of the test systems based on 6-oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazine-7(6*H*)-yl)acetic acid were also assessed. The potential application of this fluorophore to the rapid on-site diagnosis of endometritis inflammation in cows is considered promising based on these findings.

Keywords: on-site analysis, test systems, organic fluorophores, inflammation, mucus analysis, endometritis, cows

DOI: 10.1134/S1061934824700588

The productive implementation of the genetic potential of high-yield cows is hindered by several factors. Among them, the most pressing global issue is a decrease in the fertility and longevity of animals due to uterine inflammatory diseases (metritis) developing in the postpartum period. In high-yield herds, these diseases are recorded in 30–70% or more of the animals, leading to a significant economic loss related to reproductive dysfunction, lactation disorders, and high treatment costs [1–5].

Currently, postpartum metritis is considered a typical multifactorial infectious pathology [6–8]. The development of an inflammatory process in the uterus of animals after childbirth is associated with the infection of the reproductive tract by various pathogenic and potentially pathogenic microorganisms. These often (in 75% of cases) occur in combination with fungi of the genera Candida, Mucor, and Aspergillus [6, 7, 9, 10]. The severity of the disease is associated not only with the pathogenic and conditionally pathogenic microflora, but also with the toxic products of their metabolism, which cause disruptions in the immune and metabolic processes in uterine tissues. This infectious pathology is not limited to cows. Microbiological studies of the upper respiratory tract of newborn calves have revealed identical microflora to that found in cows with postpartum metritis, contributing to the development of respiratory diseases in the young cattle [11]. This, in turn, leads to reduced calf survival rates and necessitates expenditures for treatment and health monitoring.

Numerous studies have established a theoretical foundation and developed preventive and control measures for this disease. Diagnostic methods and their capabilities play a crucial role in preventing livestock losses. The primary methods for diagnosing acute postpartum metritis include clinical-obstetric [12, 13], ultrasonographic [1, 12, 13], histological, and cytological ones [13]. These methods are accurate but are typically used in the late postpartum period. Additionally, they are ex0pensive, require highly skilled personnel, and are time-consuming, making them less practical for farm conditions. The sampling required for biopsy negatively impacts cow fertility [13], which is why histological and cytological methods are not widely used in practice for the on-site and real-time

diagnosis of uterine inflammatory diseases in cows. In most cases, clinical examination is used, which can be noninformative in the early days postpartum, and is labor-intensive and time-consuming.

Improving the existing algorithms and developing new methods for the prediction and early diagnosis of acute postpartum metritis in cows is an important research direction that helps to address this problem. There is a need in developing diagnostic equipment and other tools that can diagnose the condition noninvasively, quickly, and accurately, assess therapeutic efficacy, and evaluate the risks of an infectious pathology.

The most promising and under-researched approach to addressing such challenges is the use of simple and sensitive test systems for field analysis based on fluorescent probes. This method is based on measuring the fluorescence intensity of probe molecules in small volumes of biological media. A thorough literature review and a patent search for the early diagnosis of postpartum endometritis using such systems yielded no results, indicating a lack of systematic studies.

In developing field test systems, the use of organic agents, such as fluorophores, particularly azolotriazines, is relevant due to their pronounced and stable luminescence [14–16]. Regarding luminescent properties, imidazo[1,2-*b*][1,2,4]triazines are of particular interest among possible combinations of triazine and azole cycles in a biofluorophore molecule. The luminescence of imidazotriazines, depending on the nature of the medium and its bioactivity, is well-studied, making them suitable as fluorescent probes for various biomedical applications [17–20]. A drawback of imidazotriazine dyes, compared to linearly linked analogs [21, 22], is the low intensity of the analytical signal [23–25]. To address this issue, various electron–acceptor functional groups and/or an additional pyridine ring are introduced into the structure of these fluorophores [26, 27]. Preliminary tests on model biological samples [28] have shown that these modified dyes exhibit a maximum analytical response compared to their predecessors.

The goal of this study was to develop a method for detecting endometrial inflammations in cows on-site based on the results of slime test analysis with the visual fluorescence detection of organic fluorophores.

The tasks of the pilot experiment were as follows:

— Testing fluorescent dyes containing azolotriazine skeletons on model solutions and vapors of volatile organic compounds (VOCs)—inflammation markers;

— Selection of the optimal fluorophore for subsequent testing on cervical mucus of cows and nasal discharges of calves;

— Search for optimal reagent substrates for application in the form of a solution (plate) and without a solvent (paper strips, swabs);

— Evaluating the selectivity and sensitivity of test systems based on organic fluorophores to products of altered metabolism in the uterine environment of cows, associated with disease development, represented by volatile organic compounds (amines, acids, ketones, alcohols);

— Finding a correlation between the type (quenching, enhancement, wavelength shift) and the intensity of the analytical signal of the fluorophore in the cervical mucus of cows and the presence and severity of endometrial inflammation;

— Assessing the accuracy of predicting the state of endometrium in cows based on the results of using test systems with the proposed fluorophore in field conditions, including different periods postpartum, on a limited group of animals;

— Evaluating a possibility of using test systems for monitoring the biological colonization of the nasopharynx of newborn calves from the studied group of cows.

EXPERIMENTAL

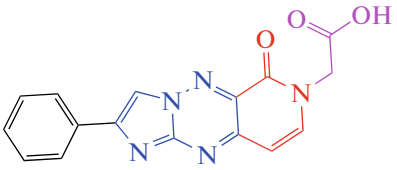
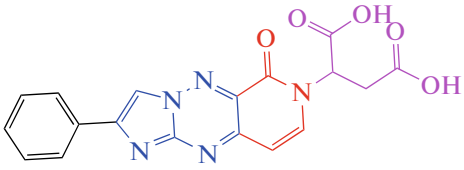
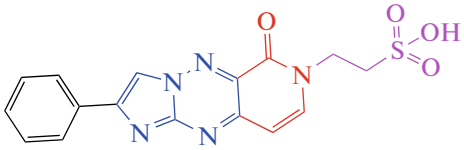
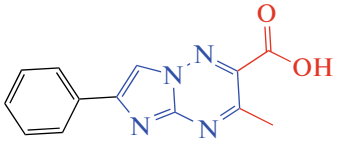
Fluorophores. Compounds Z1–Z4, containing the imidazo[1,2-*b*][1,2,4]triazine fragment, were investigated as potential analytical reagents, with their key characteristics presented in Table 1. The synthesis of these reagents was conducted following a previously described procedure [26]. For subsequent dye testing, their solutions were prepared in 2-propanol and chloroform at a reagent concentration of 1 mg/cm^3.

Preliminary experiments showed that the selected reagents possess diverse fluorescence and other physical properties, despite their structural similarity. For analytical applications, it was necessary to select a reagent with enhanced properties, one that maintains its characteristics across various environments and matrices, while also enabling the observation of significant changes upon interaction with analytes. These changes must be visually detectable.

Test substances. The potential interaction of fluorophores with aqueous solutions of VOCs and the resulting changes in their spectral properties were assessed. Solutions for each VOC were prepared at two concentration levels: the normal range boundary (low concentrations, X) and disease levels (high concentrations, X*), individually selected [29–40]. Test substances from the classes of ketones, carboxylic acids, and amines were selected as widely recognized inflammation biomarkers (KhromLab, EKROS, Russia; substance labeling in the study is presented in Table 2).

Research methods. Dye solutions were examined at the Voronezh State University and at the Vernadsky Institute of Geochemistry and Analytical Chemistry of the Russian Academy of Sciences using standard techniques of absorption, infrared (IR), and fluorescence spectroscopy employing a USB2000+ spectrophotometer (OceanOptics, United States). Photoluminescence spectra were acquired using an automatic spectral complex equipped with an MDR-4 mono-

Table 1. Investigated organic fluorophores based on imidazotriazines

Structure and name according to IUPAC	Lebel	Molar weight, g/mol	Absorption maxima, nm*
 2-(6-Oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl)acetic acid	Z1	321	372 496
 2-(6-Oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl)succinic acid	Z2	379	374 494
 2-(6-Oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl)ethanesulfonic acid	Z3	371	379 504
 3-Methyl-6-phenylimidazo[1,2-*b*][1,2,4]triazine-2-carboxylic acid	Z4	254	361 523

* Data obtained by analyzing solutions of dyes in dimethylformamide ($c = 1 \times 10^{-5}$ mg/cm^3).

chromator (LOMO, Russia), coupled with a R928P photomultiplier tube (Hamamatsu, Japan) and a PDF10C/M photodiode (Thorlabs, United States).

Visual fluorimetry on plates with digitalization of the interaction results between fluorescent probes Z1–Z4 and test substances was conducted under daylight and using the Denscan densitometer (NTs Lenkhrom, Russia) at two excitation wavelengths (365 and 254 nm). Plates were prepared following a unified pattern of the addition of substances with duplication (Fig. 1). For substances with clear or dubious analytical responses, wells were additionally replicated.

Fluorescence on cellulose substrates was examined visually and with a laser, and spectra were recorded using a Maya 2000 Pro spectrometer (OceanOptics, United States). This approach for evaluating the interaction effect between the fluorescent probes and test substances is most informative as it allows for the study of the reagent's reaction on substrates of any nature and the vapor of test substances, while excluding the effect of solvents.

Characterization of biosamples. Samples of cervical-vaginal secretions and uterine exudate were collected using the method described in [41], employing sterile swabs and a Pankov obstetrical spoon. After collection, biosamples were placed in sterile tubes. A total of 27 recently parturient animals participated in both the training and validation sets. Each animal was examined at least three times during the periods before and 3, 6, and 18 days after parturition.

Biosamples from the animals were concurrently analyzed for the presence of microorganisms (*Escherichia coli*, *Actinomyces pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Fusobacterium necrophorum*, *Bacillus subtilis*, *Staphylococcus saprophyticus*, *Bacillus licheniformis*, as well as microscopic fungi).

The selected animals exhibited varying microbiocenoses. These characteristics, along with the clinical

Table 2. Volatile organic test substances (biomarkers of pathology)

Test substance		Label
Ketones	Acetone	J
	Butane-2,3-dione	E
	Cyclohexanone	K
Carboxylic acids	Propionic acid	C
	Butyric acid	F
	Lactic acid	G
	Acetic acid	N
	3-Phenyllactic acid	M
Amines	Ethanolamine	H
	Dipropylamine	P
	Butylamine	A
	Isobutylamine	L
	Cyclopentylamine	O
	Triethylamine	D
	Morpholine	B
	Ammonia	Q

signs of the animals' conditions in the sample, formed a basis for dividing them into "normal" and "endometritis" groups. The endometritis group included animals and biosamples from them with clear or doubtful signs of illness, as well as samples from cows after treatment (chronic form).

Nasal mucus samples from calves ($n = 4$) were collected using sterile cotton swabs inserted into the calves' nasal passages. The swabs were gently rotated five times in circular motions and then placed in sterile tubes. Samples from calves were collected once, 1–3 days after birth.

Procedure for applying test systems for bioassay. The diagnosis of inflammation in cows and the assessment of the microflora status in calf nasal mucus on-site using optimal fluorophores involved the following steps: collection of 5–10 mL of cervical mucus from the cow, placing it in a sterile tube, adding 3–5 mL of water, shaking, and immersion in a solution of test systems of various natures impregnated with a chloroform solution of the reagent. The system was allowed to stand for 2–5 s, and changes in the intensity and color of the reagent zone compared to the initial (zero) state of the reagent were visually observed under daylight and using a portable UV lamp with a wavelength of 365 nm (Raritet International, Russia). If the intensity of the reagent color decreased or disappeared under daylight, and green fluorescence appeared in the biosample illuminated with UV light at a wavelength of 356 nm, the response of the test system was considered positive, indicating the presence or the development of inflammation in the uterus and endometritis. The intensity of the change was correlated with the severity of inflammation [42]. Samples from the training and validation groups were tested in wells (after dilution with twice-stilled water) and using swabs. Additionally, to verify the correctness of decision-making, the reagent solution was added to the smears of these same biosamples, previously collected after water addition. For a separate group of samples (collected in the same day), a possibility of assessing the condition was tested not only by adding the reagent solution to the well smears but also concurrently with the use of swabs (sample nos. 100, 4918, 4648, 5068, 2238, and 4768).

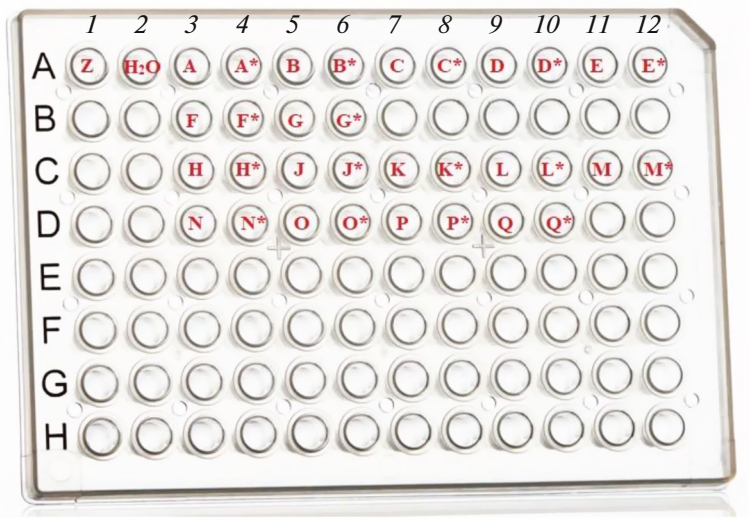


Fig. 1. Pattern of adding solutions of test substances with different concentrations to the solution of the investigated fluorophore. Systems where solutions with high concentrations of substances were applied are marked with an asterisk (X*).

Table 3. Results of microbiological studies of cows with physiological and pathological course of the postpartum period

Sample code	Sampling after parturition	Microbiology	Degree of microbial contamination, CFU/mL secretion	Preliminary diagnosis/group
100	2 weeks after parturition	Lactobacilli and bifidobacteria **were not isolated**. Isolated: *E. coli*, *A. pyogenes*, and *S. aureus*	2470.0	Endometritis/endometritis
4918	2 weeks after parturition	Lactobacilli and bifidobacteria **were isolated**. Isolated: *S. epidermidis*, *S. saprophyticus*, and *B. licheniformis*	738.0	Healthy/norm
4648	1.5 weeks after parturition	Lactobacilli and bifidobacteria **were isolated**. Isolated: *S. epidermidis* and *B. licheniformis*	625.0	Healthy/norm
5068	1.5 weeks after parturition	Lactobacilli and bifidobacteria **were isolated in small quantities**. Isolated: *E. coli*, *Pr. vulgaris*, *S. aureus*, and microscopic and yeast-like fungi	1470.0	Healthy/norm
2238	1.5 weeks after parturition	Lactobacilli and bifidobacteria were not isolated. Isolated: *E. coli*, *F. necroforum*, *S. aureus*, *Mycoplasma bovis*, and microscopic fungi	2520.0	Endometritis/endometritis
4768	1.5 weeks after parturition	Lactobacilli and bifidobacteria **were isolated in small quantities**. Isolated: *E. coli*, *A. pyogenes*, and microscopic fungi	2720.0	Endometritis/endometritis

RESULTS AND DISCUSSION

Recent advancements in analytical technologies aimed at recognizing and determining volatile organic compounds (VOCs) in human and animal matrices, including exhaled breath, urine, saliva, blood, feces, sweat, semen, and skin breath [30–40, 43–45], have garnered increasing interest due to the informativeness of VOCs for the early diagnosis, screening, and monitoring of various diseases and conditions [45–47]. As products of cellular metabolism, VOCs can give information about the state of intracellular metabolism. Additionally, microorganisms involved in the pathogenesis of most inflammatory diseases can synthesize a large number of volatile and semivolatile compounds, primarily organic compounds.

Gas chromatography–mass spectrometry is employed to investigate the VOC profile, allowing for the efficient separation of components within complex mixtures, their identification, and assessment of the relative abundance. Through this method, databases of identified volatile (VOCs) and medium-volatile (MVOCs) organic compounds have been composed (for example, the mVOC 3.0 database, https://bioinformatics.charite.de/mvoc/). This database encompasses over 2000 compounds emitted by various types of microorganisms (bacteria and fungi) [48, 49], as well as those formed during different diseases [47, 48, 50–57]. Alkynes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, complex ethers, amines, and sulfur-containing derivatives predominate among the marker VOCs/MVOCs [48, 49, 58–60]. In selecting target analytes or their mixtures for experimental design, it was considered that many VOCs/MVOCs are native and present in biosamples in the normal, healthy state of animals and humans. For such substances, an essential criterion for assessing pathogenicity is their concentration.

At the initial stage of the study, the microbiota of cervical-vaginal mucus and exudate from animals, categorized based on clinical signs into normal (absence of endometritis) and endometritis groups (Table 3), were investigated. In cows with a physiological postpartum period, the degree of microbial contamination was significantly lower, by more than 3.3 times ($P < 0.001$), compared to the animals suffering from postpartum metritis. In healthy animals, lactobacilli were isolated from cervical-vaginal secretions in 100% of cases, and bifidobacteria in 80% of cases. This exceeds similar indicators for biosamples from diseased cows by 4.0–5.0 times ($P < 0.001$), indicating a high level of biological protection of the reproductive tract in animals from the normal group. In addition, *B. subtilis*, *S. saprophyticus*, and *B. licheniformis* were isolated from healthy animals. In animals with obstetric pathology, *E. coli*, *A. pyogenes*, *S. aureus*, *S. epidermidis*, *Pr. vulgaris*, *F. necroforum*, *B. subtilis*, *S. saprophyticus*, *B. licheniformis*, as well as microscopic fungi, were isolated from uterine exudate. Based on combinations of microorganisms and biochemical processes involved in endometrial inflammation formation, as well as data from [47–57], the main pathogenic VOCs were selected for further testing. Among them, ammonia and low-molecular-weight amines dominate.

Selection of optimal fluorophore. The selection of a group of potential fluorophores to address the speci-

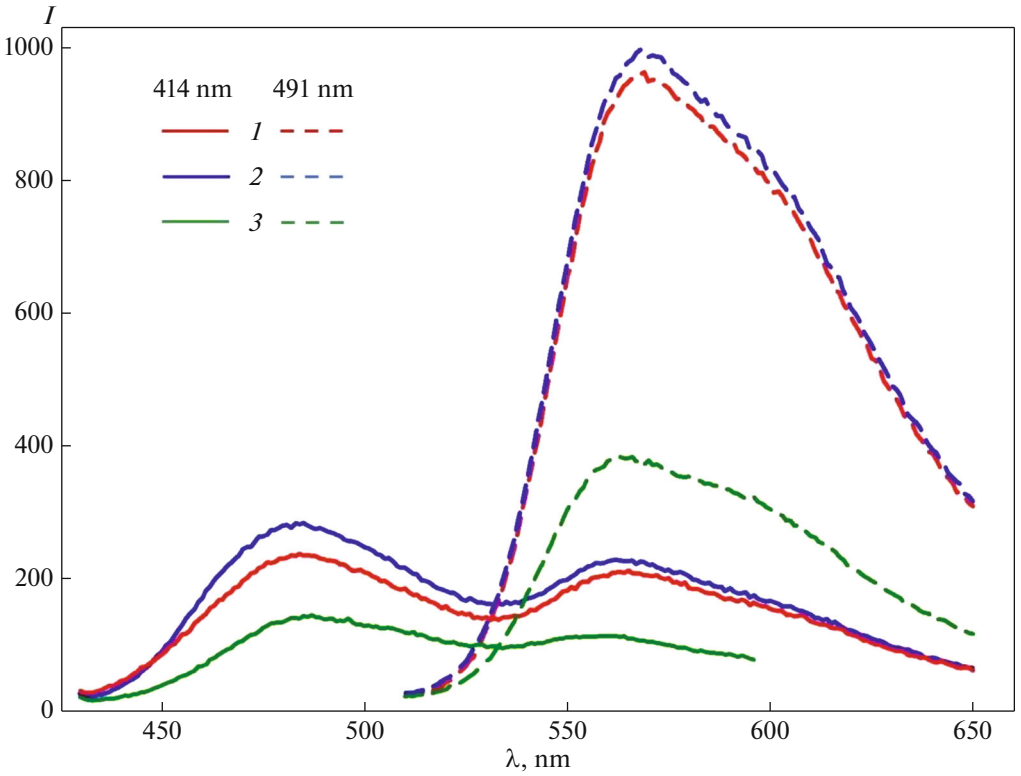


Fig. 2. Photoluminescence (using the example of the Z1 reagent) in water (*1*) without additives and with the addition of (*2*) a 14 mM solution of ammonia and (*3*) and 60 mM HCl.

fied tasks is based on the results of previous studies, during which various derivatives of azolotriazines were analyzed [26, 28]. The key factors in the selection process included the simplicity and reliability of the visual detection of the analytical signal, depending on the fluorescent properties of the reagents. Systems containing pyrazolo- and triazolotriazine skeletons are characterized by absorption maxima in the short-wavelength region of the spectrum (190–200 nm), limiting their application to creating visual test systems for preclinical diagnostics in field conditions. In addition, regardless of the solvents, solutions of such dyes undergo fluorescence degradation, depending on the method and the duration of storage. Meanwhile, solutions of imidazolotriazine derivatives are characterized by multiple absorption maxima in the wavelength region 361–504 nm, exhibiting high photostability during storage, fluorescence intensity, and pronounced analytical response. Further mathematical simulation of their interaction with biomatrices and VOCs allowed for the identification of four leading structures for noninvasive preclinical diagnostics, Z1–Z4 [26, 28].

To test the feasibility of using the selected reagents for the noninvasive preclinical diagnostics of endometritis, a series of experiments were conducted to study the intensity of their fluorescence on various substrates and in 96-well immunochromatographic plates.

The investigated reagents (Table 1) exhibited properties of fluorescent acid-base indicators. However, the results from intermediate tests showed that their fluorescence was significantly suppressed by 60 mM HCl, with a shift in the absorption maximum from 568 to 562 nm (Fig. 2). Meanwhile, solutions of ammonia of a similar concentration slightly increased the intensity of the reagent fluorescence, indicating an antagonistic nature of the reagent responses to acids and bases. These findings allowed for the assessment of a possibility of separately classifying the analytical response of the selected fluorescent reagents in the presence of organic acids or bases in biosamples. A hypothesis was put forward that, during inflammation (endometritis), biosamples contain a large amount of amines, leading, following preliminary studies, to an increase in fluorescence intensity in using these reagents in biosample smears. Other processes and microorganisms may increase the acidity of biosamples (fungi), resulting in fluorescence quenching up to the complete bleaching of the test system in aqueous extracts. To confirm this hypothesis, further experiments were conducted with standard solutions of test substances.

As biosamples represent a complex system with a high water content, we evaluated visually the analytical response of organic fluorophore solutions Z1–Z4 in the presence of test substances on a plate. Aqueous

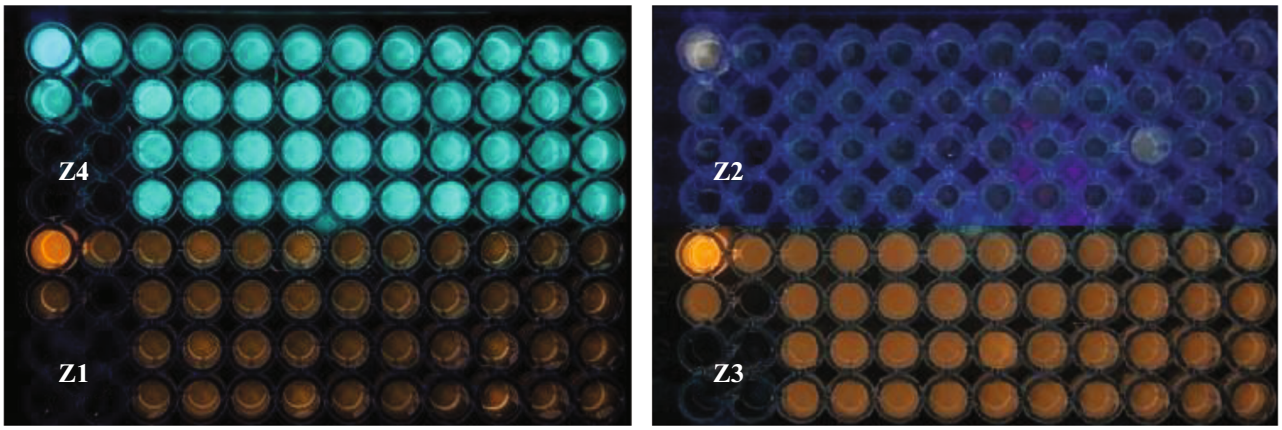


Fig. 3. Examples of plate photos when loaded with solutions of organic reagents by test substances under UV illumination with a wavelength of 365 nm.

solutions of test substances of minimal (physiological norm) and maximal (non-norm) concentrations were added to the reagent solutions. Examples of plates are shown in Fig. 3. Any visible changes under daylight and at 365 and 254 nm in the wells were considered analytical signals: suppression, enhancement of fluorescence, or color change.

The results of plate testing of the interaction between the fluorophores and test substances are presented in Table 4. For all investigated dyes, the partial or complete quenching of fluorescence (emission) in pure water was observed, which could be explained by the collision of molecules of hydrophobic probes.

Based on the results obtained, 6-oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl acetic acid (Z1) was selected as the most effective fluorophore for plate test systems. The carboxyl group in the molecule of the reagent ensures selective and reversible binding with nitrogen-containing analytes. This leads to a change in the intensity of fluorescence not only in the UV region but also under daylight, indicating a universal method for assessing changes in test systems based on it: under both daylight and UV illumination. This feature is important for enabling the rapid application of test systems in field conditions.

The increase in the luminescence intensity of compound Z1 in aqueous solutions of analytes may be associated with its belonging to the group of molecular rotors (Fig. 4). In this case, an increase in pH promotes the formation of hydrogen bonds between the carbonyl group and methylene protons, slowing the rotation of the aminoacetic acid fragment. Additionally, a slightly alkaline medium promotes the ionization of the dye molecules and changes in intermolecular interactions with water molecules and VOCs. Meanwhile, in an acidic medium, a change in conformation occurs due to the reduced probability of hydrogen bond formation.

Despite their simplicity and clarity, the use of plates in field conditions is not optimal. Therefore, we evaluated a possibility of transferring the fluorophore onto cellulose planar substrates (paper test strips) and swabs. For this purpose, equal volumes of a chloroform solution of Z1 were applied to the cellulose substrate; the swabs were dipped into the solution for 2 s and then dried at room temperature. The change in luminescence intensity was visually assessed upon the activation of the system with a UV lamp with a wavelength of 365 nm and a solid-state laser. On cellulose substrates, in the presence of vapors of VOCs that are a priority for inflammation, an inversion of the analytical response (Fig. 5) was observed compared to solution-based systems (plate tests). A gradual decrease in luminescence intensity, leading to complete quenching, occurs upon the interaction of ammonia and amines with the selected reagent (Fig. 5a). Under daylight, a similar inversion of the analytical response was observed (Fig. 5b). The results are consistent with the results of the photoluminescent analysis of paper test strips (Fig. 5c). This behavior is attributed to the possible absorption of some light by the cellulose substrate, a decrease in the "mobility" of the dye outside the solvent, and, consequently, a decrease in the efficiency of an electron transition to the excited state.

The results of model tests suggest a high selectivity of Z1 for amines and ammonia, which are priority biomarkers of inflammation. Therefore, the potential application of Z1 in preclinical, noninvasive diagnostics of inflammation and endometritis, based on mucus testing, is positively evaluated.

Comparison of veterinary examination results and the use of test systems on a training group of animals. In the training sample of animals, we determined a correlation between changes in luminescence and the effectiveness of the fluorophore as an indicator of animal health. The results of veterinary examinations were used as a basis for accurately assessing the response of the Z1 dye, although a portion of false

Table 4. Results of plate testing of organic reagents

Test substance	Analytical response							
	Z1		Z2		Z3		Z4	
	VL	365 nm	VL	365 nm	VL	365 nm	VL	365 nm
H_2O	0	↓	0	↓	0	↓	●	↓
Acetone	0	0	0	↓	0	↓	●	↑
Butane-2,3-dione	0	↓	0	↓	0	↓	●	↓
Cyclohexanone	0	0	0	↓	0	↑	●	↑
Propionic acid	0	0	0	↓	0	↓	●	↓
Butyric acid	0	0	0	↓	0	↓	●	0
Lactic acid	0	0	0	↓	0	↑	●	↑
Acetic acid	0	↑	0	↓	0	↑	●	↑
3-Phenyllactic acid	0	↓	0	↓	0	↓	●	0
Ethanolamine	●	↑	0	↓	0	↓	●	0
Dipropylamine	●	↓	0	↓	0	↓	●	0
Butylamine	●	↑	●	↓	0	↓	●	↓
Isobutylamine	●	↑	0	↑	0	↓	●	0
Cyclopentylamine	●	↑	0	↓	0	↓	●	0
Triethylamine	●	0	0	↓	0	↓	●	↓
Morpholine	●	↑	0	↓	0	↑	●	↓
Ammonia	●	↑	0	↓	0	↓	●	↑

VL, visible light. Symbols: ↑, increased luminescence; ↓, quenched fluorescence; 0, no change; ●, change in color intensity.

assessments by an expert is permissible. Different methods of applying the reagent solution were tested in the experiments.

Samples of cervical mucus, collected from five animals at various intervals after parturition, were diluted with water and placed in wells of a plate, to which the

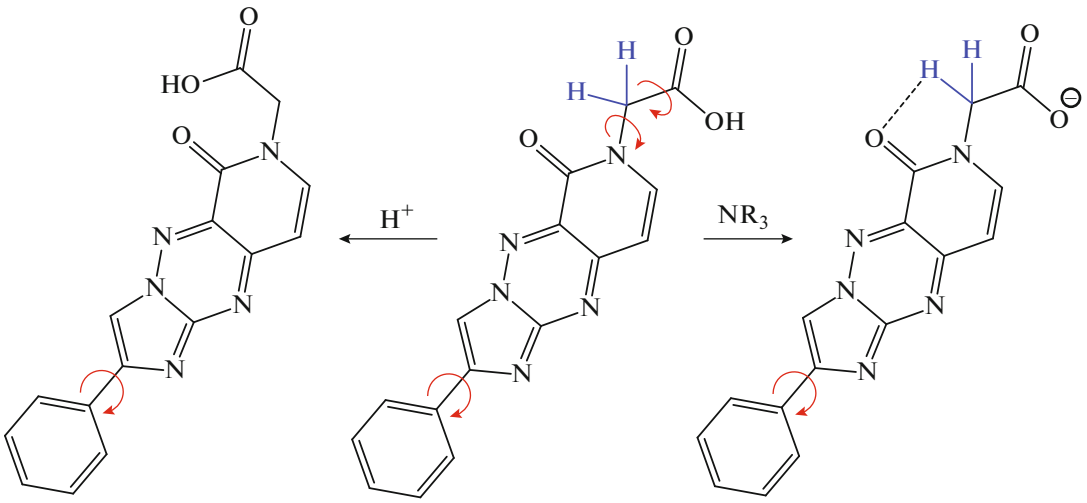


Fig. 4. Conformational dynamics of 6-oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl)acetic acid.

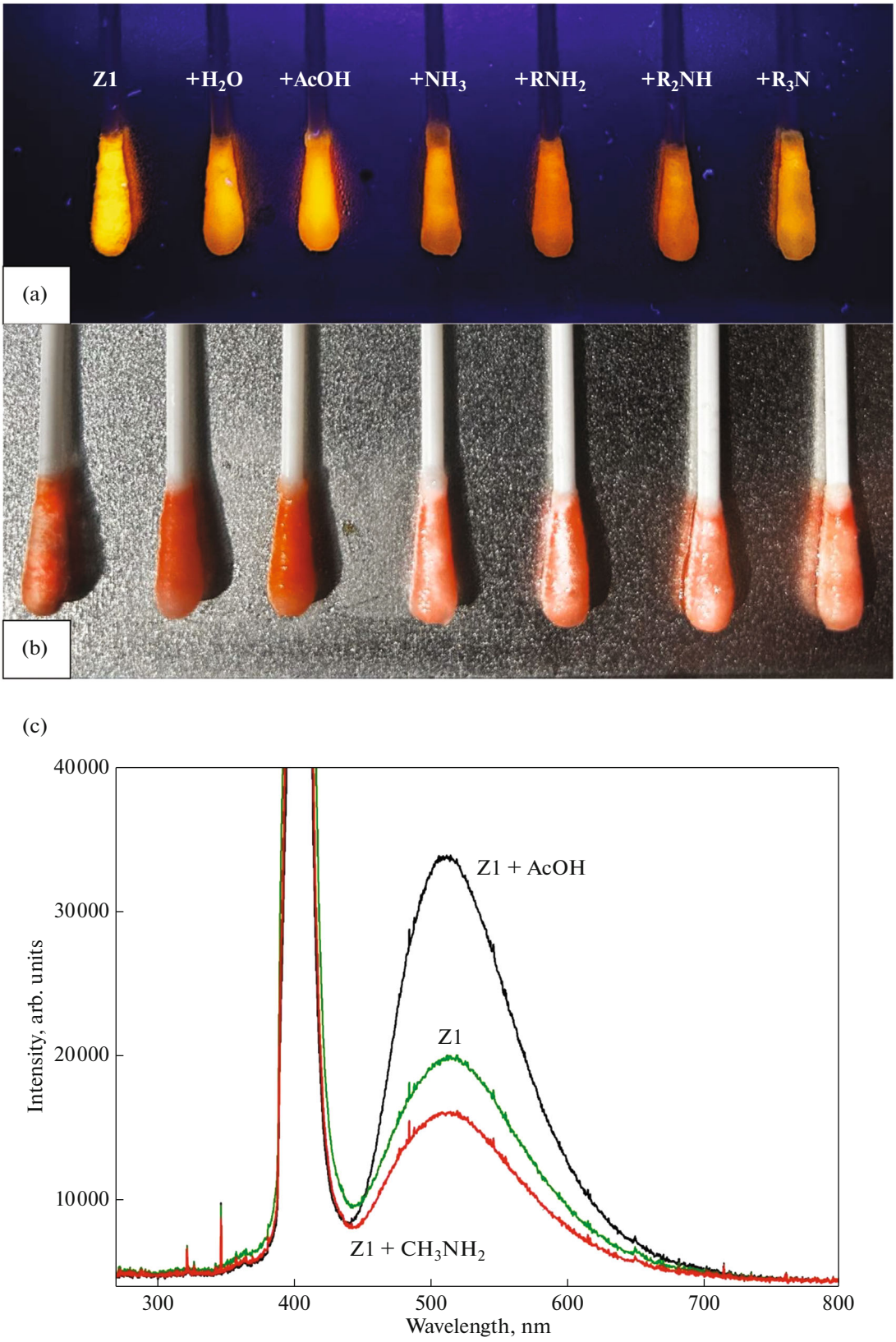


Fig. 5. Behavior of Z1 on swabs under (a) UV illumination at 365 nm and (b) natural illumination, and (c) its photoluminescence on paper test strips under the action of a solid-state laser with a wavelength of 405 nm when interacting with various volatile organic compounds.

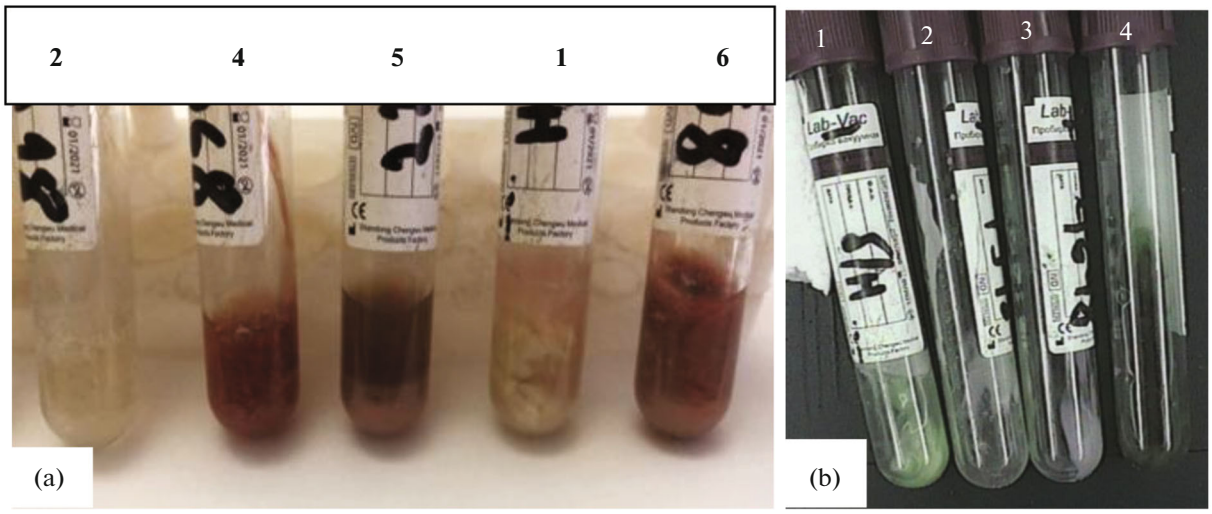


Fig. 6. Samples of cervical mucus with the addition of Z1 solution under (a) natural and (b) UV illumination.

Z1 solution was added. Depending on the degree of uterine inflammation in an animal, the fluorescence intensity of the wells under the irradiation with a UV lamp at a wavelength of 365 nm varied. This can serve as a visual indicator of the animal's health status in the presence of a baseline standard. It was also determined that the testing results were independent of the time of mucus sample collection after parturition but were solely determined by the level of inflammation. This indicates the potentially effective application of the Z1 dye for determining the animal's health status regardless of the timing of sample collection in the postpartum or antepartum period.

Due to the challenges encountered in using plates in field conditions, strips or swabs proved to be more convenient. During the preventive examination of a new group of cows, the veterinarian collected cervical mucus samples of at least 5–10 mL each (Fig. 5a). These samples were placed in sterile test tubes and mixed with a small amount of the tested fluorescent reagent solution (Fig. 6a). The results obtained under UV light were recorded (Table 5). In the mucus samples from animals no. 1 (100) and no. 4 (5068) (Fig. 6b), under a light source with a wavelength of 356 nm, green fluorescence was visually observed, indicating inflammation and endometritis. In samples no. 2 (4918) and no. 3 (4648), no fluorescence was observed. Similarly, changes were recorded in the tubes containing samples from animals with inventory numbers 2238 (no. 5) and 4768 (no. 6) (Table 5), in which fluorescence of varying intensity was observed under these conditions. Consistent with preliminary experiments, an inversion of the analytical response occurred in using swabs, noted under both daylight and at 365 nm. The testing results of samples using different methods—wells and swabs—demonstrated the accuracy of the diagnosis prediction, coinciding with the expert's diagnosis, at 91 and 67%, respectively (Table 5). False predictions in these samples, not corresponding to the clinical diagnosis, should be considered more accurate, as the reagent exhibits high sensitivity to inflammation markers, while clinical examinations and thorough examinations by experts are conducted infrequently. During the time between the examination and sample collection, the animal's condition could deteriorate. In this case, a false positive response from the test system is also favorable as it increases the likelihood of further examination and prevents the development of the disease in a more severe form or its early intervention. Neither false negative results nor false positives were observed in the wells or with the swabs. The direct introduction of the dye into the sample did not yield any false positive or false negative results. This method is the most accurate in prediction but is more costly in terms of reagent consumption.

For samples collected in different days from six animals, the prediction results coincided with the expert's assessment of the health status of five cows from different groups (norm and endometritis). Misclassification of cow no. 5068's sample into the endometritis group based on an analysis with the test reagent can be explained by the fact that, at the time of the initial examination, the animal was classified into the normal group, but signs of developing endometritis were observed by the veterinarian two weeks later. This indicates that, even at the time of the initial examination and sample collection (on the third day post-partum), the animal's condition was already different from normal and inflammation had begun. The use of the fluorescent reagent allows the detection of the onset of inflammation at an earlier stage than through expert examination alone.

To monitor the development of inflammatory processes, additional tests were conducted for mucus

Table 5. Results of an analysis of gynecological mucus of cows from the training set when samples interact with Z1

Sample code	Well number in Fig. 6	Sample number in Figs. 6a, 6b	Veterinarian diagnosis/group	Presence of reagent fluorescence at 356 nm	Compliance with diagnosis	Accuracy of prediction, %
Reagent solution in wells						91
10011	3		Healthy/norm	Extinguishing	Compliant	
10012	5		Healthy/norm	Extinguishing	Compliant	
10013	4		Beginning of purulent-catarrhal endometritis/endometritis	Fluorescence	Compliant	
10014	6		Beginning of purulent-catarrhal endometritis/endometritis	Fluorescence	Compliant	
10015	7		Beginning of purulent-catarrhal endometritis/endometritis	Fluorescence	Compliant	
100		1	Endometritis/endometritis	Strong fluorescence	Compliant	
4918		2	Healthy/norm	Absent	Compliant	
4648		3	Healthy/norm	Absent	Compliant	
5068		4	Healthy/norm	Weak fluorescence	The onset of inflammation was recorded	
2238			Endometritis/endometritis	Fluorescence	Compliant	
4768			Pronounced inflammation/endometritis	Strong fluorescence	Compliant	
Reagent solution on swab						
100			Healthy/norm	Without changes	Compliant	67
4918			Healthy/norm	Bleaching	Noncompliant	
4648			Healthy/norm	Bleaching	The onset of inflammation was recorded	
5068			Endometritis/ endometritis	Partial bleaching	Compliant	
2238			Pronounced inflammation/endometritis	Bleaching	Compliant	
4768			Endometritis/endometritis	Bleaching	Compliant	

samples collected from eight animals at different time intervals (Table 6). Visual assessment of the analytical response was performed under daylight using swabs after they were exposed to the mucus samples. The test systems on swabs effectively detected the inflammation and exacerbation of endometritis, even in pregnant cows (no. 14136). One false negative and one false positive assessment were obtained in the day of examination in this group, out of eight samples.

Upon retesting these animals 2 months later, the situation deteriorated sharply. Endometritis developed in only three out of the eight cows. In all calved animals (13303, 14136, and 14181), endometritis had significantly progressed by the time of retesting. The accuracy of the diagnosis based on signals from the swab during the initial testing was 75%, while in the retest of this group, it reached 100%.

The data obtained from the training sample of animals allow for several preliminary conclusions:

— The low cytochemical staining ability and high stability of Z1 solutions [28] explain the change in luminescence intensity due to binding with nitrogen-containing VOCs, which can be used to detect inflammatory processes.

— False-positive results are caused by the presence of blood in the sample;

— False-negative results in the dual assessment of the analytical effect (under natural light and UV irra-

Table 6. Results of testing samples using a swab with reagent Z1

Sample code	Changes on swab/group		Veterinary diagnosis	Accuracy of prediction, %
	06.02.22	04.04.22		
11354	No change/norm	No change/norm	Healthy (06.02), healthy (04.04)	75% on the testing date June 2, 2022 100% as of the retest date April 4, 2022
183300	Discoloration/endometritis	Severe discoloration/endometritis	Endometritis (25.01), deterioration of condition (04.04)	
3420	No change/norm	No change/norm	Endometritis (25.01), undergoing treatment; healthy (06.02 and 04.04)	
2172	No change/norm	No change/norm	Healthy (06.02), healthy (04.04)	
13303	No change/norm	Discoloration/endometritis	8.5–9 months of pregnancy; healthy (06.02), endometritis (04.04)	
18222	No change/norm	Discoloration/endometritis	Healthy (06.02), endometritis (04.04)	
14136	Slight discoloration/group not determined	Discoloration/endometritis	8.5–9 months of pregnancy; healthy (06.02), endometritis (04.04)	
14181	No change/norm	Discoloration/endometritis	8.5–9 months of pregnancy; healthy (06.02), endometritis (04.04)	

diation) are only associated with the degree of inflammation.

The accuracy of predicting the condition using a control group of animals. Eight samples from animals not included in the training sets were selected for validation. According to clinical examinations, the condition of the animals was assessed as suspicious of inflammation (15119), pronounced inflammation (1409 and 1417), and conditionally healthy with delayed placenta expulsion pathology (7012).

The analytical signal for sample 1409 is not clearly defined (Table 7), yet the condition of the sample indicates evident strong tissue decomposition processes, rendering the test diagnostically meaningless in this case. For sample 7012, the absence of changes in the analytical response is attributed to other reasons for postpartum condition disruption (placental retention), which is not inflammation. The evaluation of samples with inflammation and endometritis of varying severity coincided with the expert's assessment (Table 7).

For the cases of pronounced inflammation and healthy animal groups, no false positive or false negative test results were detected, which allows for a high assessment of its potential for field testing. Further research enables a more precise ranking of transitional states (treated, onset of illness). The accuracy of diagnosis was 87% (Table 7).

Sensitivity and versatility of the test systems. To assess the sensitivity and universality of the test systems based on Z1, we selected nasal mucus samples from calves born to cows from the validation sample. One of the main causes of perinatal pathology in newborn calves is microecological disturbances in the maternal herd's organism before insemination and during pregnancy. In some cases, various types of dysbiosis, the presence of potentially pathogenic and pathogenic microflora in the birth canals of the dam cows, become the primary sources of infection for the newborn [61, 62]. The composition of both VOCs and microorganisms significantly differs between nasal and cervical mucus in calves and their mothers, respectively. Under equal conditions, the levels of microbiota in a calf's nasal mucus and its mother's gynecological mucus vary significantly and are lower in the nasal passages. In other words, if the test tool allows for the identification of calves born to sick cows, its sensitivity meets the needs of multifaceted straightforward test analysis in farm conditions.

Samples from calves were collected once (1–3 days after birth) and placed in a sterile test tube to which 3–5 mL of water was added. After shaking, a swab with Z1 was inserted into the obtained sample. The research results are presented in Table 8. It was found that false negative results were observed with a small volume of nasal mucus samples. A potentially high risk of postnatal complications in calves was identified

Table 7. Results of an analysis of gynecological mucus of cows from the test sample when adding a swab with a Z1 solution to the samples

Sample code	Initial diagnosis	Changing the color of the swab in natural light	Compliance with diagnosis	Accuracy of prediction, %
Swab with Z1		Without changes		87
105	Start inflammation	Slight discoloration	Compliant	
107	Healthy	Without changes	Compliant	
108	Healthy	Without changes	Compliant	
106	Endometritis	Severe discoloration	Compliant	
15119	Suspicion on inflammation	Bleaching	Compliant	
1409, sample with flakes	Endometritis	Undefined	Pronounced inflammation according to the condition of the sample	
1417, blood test	Purulent inflammation, lochia	Complete discoloration	Compliant	
7012, color-changed proof	Delay postpartum placenta	Undefined	Compliant	

Table 8. Results of testing swabs with Z1 placed in samples of nasal mucus of calves

Cow code	Calf sample code	Clinical diagnosis (1–3 days after birth)	Analytical response of test systems	Clinical diagnosis after 3 weeks
1409	1t	Bronchopneumonia	Norm (not enough sample)	Bronchopneumonia
1417	2t	Healthy	Bleaching	Respiratory syndrome
7012	3t	Healthy	Bleaching	Respiratory syndrome
15119	4t	Respiratory syndrome, laryngotracheitis	Bleaching	Respiratory syndrome, laryngotracheitis

using the proposed test tool analysis a week before the signs of illness. The proportion of false positive results for mucus samples is similar to the same errors for cow's mucus. These results increase the informativeness and effectiveness of the proposed test tools for field observations of animals in agricultural complexes.

CONCLUSIONS

The results of the pilot study suggest that 6-oxo-2-phenylimidazo[1,2-*b*]pyrido[4,3-*e*][1,2,4]triazin-7(6*H*)-yl)acetic acid (Z1) can be used as a fluorophore reagent in test systems of various types (plates, paper test strips, swabs) for the rapid on-site diagnosis of endometritis inflammation in cows.

The fluorescent reagent ensures high sensitivity, ease of use, and reliability in detecting analytical responses and decision-making. It significantly simplifies procedures and enhances monitoring productivity, decreasing the economic and time costs for clinical and laboratory investigations. Analyzing their results allows for the early detection of the onset of endometrial inflammation in cows. However, a decrease in mucus sample volume, presence of blood, or mucus can lead to false or inconclusive results, increasing the frequency of false negative assessments of the animal's condition.

The aggregate of the obtained results enabled the evaluation of the characteristics of the proposed testing method (Table 9). Reagent Z1 enhances decision-making reliability by enabling the registration of analytical signals both under daylight and the artificial (UV) illumination of test tools or samples after its introduction. The reagent was selected as a priority for the further research and application as an additional modifier to cadmium sulfide quantum dots. It has been previously found that quantum dots significantly stabilize organic reagents and contribute to extending their active operation time in analytical systems. The effectiveness of using the Z1 reagent as a modifier for other sensors (nonoptical) is explored in our future work.

FUNDING

The work was supported by the Russian Science Foundation, grant no. 23-23-00609.

Table 9. Characteristics of the proposed test method

Mode of application	Specificity	Accuracy	Precision	Number of false positive results	Number of false negative results
Wells	0.857	0.910	0.857	1 of 11	0
Swab	0.813	0.867	0.813	3 out of 30	1 out of 30
Solution	0.667	0.833	0.750	1 of 6	0

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All studies were conducted in accordance with the principles of biomedical ethics as outlined in the 1964 Declaration of Helsinki and its later amendments. They were also approved by the Ethics Committee of Voronezh State University of Engineering Technologies (Voronezh), protocol no. 7 dated November 30, 2023.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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Translated by O. Zhukova