Application of luminescence spectral assay for assessment of the functional state of birds’ gastrointestinal tract by nucleic acids content in cells

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Abstract. The method of functional state evaluation of chickens’ glandular stomach mucous membrane epithelium, using luminescence spectral analysis with fluorescent dye “Ethidium bromide” was proposed. The dynamics of nucleic acids content in cells of chickens’ glandular stomach under experimental escherichiosis and in intact poultry was established using this method. A gradual increase in the nucleic acids content in epithelium of glandular stomach of intact chickens was revealed from 1th to 30th days. The nucleic acids content in cells of chickens under experimental escherichiosis was gradually increased from the fourth day of life (second day after infection). The minimal index of nucleic acids content was recorded at the 7th day of chickens’ life. From 8th to 30th days of life, periods of increase / decrease in nucleic acids quantity in the cells were noted. By the 30th day, nucleic acids content in the chickens glandular stomach epithelial cells of the experimental groups was less by 40.1% than in control group poultry.

1. Introduction
Poultry bacterial infections is one of the urgent problems in modern poultry farming. The largest share among them is occupied by E. coli caused diseases. This intestinal infection not only causes colossal economic damage, but also has a leading role in the occurrence of human diseases, primarily food poisoning [1, 2].

Reducing the use of antibiotics-growth stimulants on account of the state prohibitive measures or changes in demand on the poultry meat market promotes development of disease prevention, as well as the use of antibiotic substitutes. Currently, probiotics, prebiotics, synbiotics, enzymes and so forth have been developed and proposed for poultry farming [3, 4, 5]. However, antibiotic alternatives have not yet been widely used in practice.

A systematic approach to the development of new preventive and therapeutic agents requires the use of biomarkers capable of detecting changes in the state of the bird’s body, including at the cellular level. Currently, various indicators that could serve as biological markers (“villi height”, “crypt depth”, etc.) are proposed in the scientific literature for monitoring the gastrointestinal tract state, and some of which
have been tested on poultry [6]. At the same time, these biomarkers do not allow registering changes in the functional state of organs at the cellular level. Implementation of this approach requires interaction with the cell and visualization of the process at molecular level [7, 8].

Luminescent spectral assay can be used as a method capable of detecting biochemical changes at cellular and tissue levels. This method has demonstrated the effectiveness in assessing the functional state of different cells (lymphocytes, neurons, etc.) by determining the quantitative content of organic substance, such as proteins and nucleic acids. Fluorescence assay method is realized by means of two-wave microfluorimeter, which is a combination of a fluorescence microscope with a spectrum-analyzer complete with electronic recording and control modules. This equipment is used to study an intracellular metabolism status [9]. The data about using of a luminescence spectral assay to study the functional state of the gastrointestinal tract of farm animals and poultry at the cellular and molecular levels is poorly presented in scientific information sources.

The purpose of our research was to study the dynamics of nucleic acids content in chickens’ glandular stomach mucous membrane epithelium under experimental escherichiosis and in intact poultry using luminescent spectral analysis.

2. Materials and methods

Ethidium Bromide (EtBr) 95 % (Acros Organics, USA) was used as a luminescent dye because of the high sensitivity and selectivity of interaction with nucleic acids (RNA and DNA), as well as the correlation between intensity of fluorescence and concentration of nucleic acids in the specimen [10].

The staining method of histologic specimens of chickens’ glandular stomach for the determination of nucleic acids in mucous membrane epithelium included the following steps:

- double dewaxing with ortho-xylene;
- double processing with absolute alcohol exposure for 3 minutes;
- staining for 6 minutes with an alcohol solution of EtBr (10^(-4) M);
- rinsing with distilled water for 2 minutes;
- air drying at room temperature for 10 minutes;
- clarification in xylene for a few seconds;
- coating with a synthetic medium (distren dibutyl phthalate xylene).

Comparison of luminescence spectra of stained with EtBr and unstained histologic specimens was carried out to exclude tissue luminescence due to formalin fixation of proteins, containing amino-, imino-, and amido- groups. It was found that unstained histologic specimens had a blue-green fluorescence spectrum with the maximum luminescence intensity at 480 nm. Bright red fluorescence with the maximum luminescence intensity at 616 nm was observed against blue-green tissue luminescence background in stained with fluorochrome histologic specimens. It was due to ethidium bromide complexed with nucleic acids and most pronounced in mucous membrane epithelium, alveolar glands epithelium of the mucous membrane and in muscle layer cells.

Thus, the fluorescence maxima had a difference of more than 100 nm. This corresponds to the condition that, in order to perform separate recording of the luminescence intensities, the luminescence intensity maxima must be at least 60–100 nm apart from each other. Thus, the luminescence intensity at 616 nm was registered to calculate the nucleic acids (relative units) in the photometric area.

The absorption spectrum was recorded in a stained histologic specimen, the transmission spectrum was registered in an area next to the specimen, and then absorbency was calculated using the data.

The wave-length, when passing through the photometric area light was almost not absorbed by the fluorochrome EtBr and had the smallest value, been according to 540 nm. Therefore, the absorbency value at 540 nm was using when calculating the nucleic acids in relative units (RU).

Using the obtained data, the nucleic acids in the photometric area of the histologic specimen was calculated in relative units (RU) according to the following formula:
\[ I_{NK} = \frac{I_n}{D_n \cdot I_\lambda}, \]

where

- \( I_{NK} \) - nucleic acids content (relative units);
- \( I_n \) - luminescence intensity of the photometric area at 616 nm;
- \( D_n \) - optical density of the region at 540 nm, used as its thickness;
- \( I_\lambda \) - luminescence intensity of uranium glass at 540 nm, used as a reference.

To exclude the influence of autolysis on the object fluorescence intensity, after photometry of three regions with the highest luminescence intensity, the nucleic acids content was calculated, and the largest of the three obtained results was taken into account for task solution.

The object of the study was Hisex brown chickens (cockerels). The chickens were divided into 2 groups according to the analogy principle: experimental (165 chickens) and control (165 chickens). The division of chickens into groups was carried out randomly by the method of "Random numbers", body weight was used as a criterion (±10%).

Infection of chickens with 24-hour culture of Escherichia coli serotype 078 was carried out by oral inoculation of 2-day-old chickens with 0.2 ml of 2x10^8 colony forming units (CFU) / ml of E. coli. The control group chickens were inoculated with saline by oral route.

The chickens were decapitated on the 1–4, 6–8, 10, 15, 21, 30 days (n=15 each group) with subsequent dissection and registration of the results. Pieces of glandular stomach 0.5 x 1 cm were fixed in a 10% aqueous solution of neutral formalin for 7-10 days (after a day, the formalin solution was replaced with freshly prepared). Then the samples were removed from the fixing solution and washed with running water for a day. Dehydration of samples and paraffin embedding were performed according to standard methods [11]. Slices of 4-7 μm were made using a sledge microtome “Mikrom” HM450 (Germany) and placed on chemically pure glass slides. Then, after dewaxing, they were stained with EtBr, followed by fluorescence microscopy with registration of luminescence and absorption spectra, using a universal color analyzer the LOMO MSFU-K microscope-spectrophotometer (Russia). Measurements were made using a standard monochromator with a halogen lamp KGM 9V 70W and mercury vapor lamp HBO 100 W / 2 as light sources. Measurement step was 0.5 nm, and a scan point diameter was 10-4 mm at 480x magnification (12x40).

### 3. Results and discussions

During luminescence microscopic analysis of histologic specimens of the control and experimental groups of chickens, sectors with saturated bright red fluorescence of varying intensity in various parts of the mucous membrane, serous membranes and muscle layer of glandular stomach were observed against the background of weak blue-green tissue fluorescence. The most pronounced fluorescence intensity was noted in histologic specimens of the mucous membrane epithelium and the least pronounced - in histologic specimens of the muscle layer.

However, using visual microscopy, it was not possible to reveal any relationship between changes in the luminescence intensity, reflecting the dynamics of nucleic acids content.

Calculation of the nucleic acids content in the mucous membrane epithelium of glandular stomach was carried out using our assessment methodology of the functional state of birds’ gastrointestinal tract organs at the cellular level with the application of EtBr.

The obtained results were subjected to statistical processing. The research results are presented in table 1, and the identified trends are illustrated in figure 1.

| Table 1. The nucleic acids content in glandular stomach mucous membrane epithelium of the control and experimental groups of chickens. |
|-----------------|-----------------|-----------------|
| Age,days        | Nucleic acids content, RU |               |
|                 | Control group (n=165) | Experimental group (n=165) |
| 1               | 10.12±0.36         | 10.31±0.32       |
| Age, days | Control group | Experimental group |
|----------|---------------|---------------------|
| 2        | 10.29±0.42    | 10.11±0.32          |
| 3        | 10.20±0.38    | 10.40±0.36          |
| 4        | 10.41±0.37    | 7.38±0.25***        |
| 6        | 10.30±0.36    | 4.70±0.15***        |
| 7        | 10.60±0.41    | 3.12±0.12***        |
| 8        | 11.27±0.36    | 6.81±0.24***        |
| 10       | 11.89±0.38    | 7.97±0.25***        |
| 15       | 12.00±0.39    | 5.81±0.19***        |
| 21       | 12.81±0.43    | 5.89±0.21***        |
| 30       | 13.63±0.45    | 9.73±0.30***        |

*the difference in this indicator is statistically significant between the experimental and control groups (* - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001).

Figure 1. The trends of nucleic acids content in glandular stomach mucous membrane epithelium of the control and experimental groups of chickens.

As it follows from the presented in table 1 data and the reflected in figure 1 trends, the nucleic acids content in the studied cells of the control group chickens increases from 10.12±0.36 to 13.63±0.45 RU from 1 to 30 days. Thus, the cells functional activity increased by 34.7% during the experiment. Glandular stomach mucous membrane epithelium cells of the experimental group chickens were characterized by a decrease in the nucleic acids content from the fourth day of life. The minimum value of the indicator was recorded at the 7th day of life. An increase in the indicator value to 7.97 ± 0.25 RU was noted at 8th and 10th days of life, and at the 15th and 21th days the indicator value, on the contrary, decreased. By the 30th day, this indicator values were less than the same of the control group by 40.1%.

4. Conclusion
The results allow us to conclude that the developed luminescence spectral assay with EtBr is capable of revealing the nucleic acids distribution features in histologic specimens of glandular stomach mucous membrane epithelium in chickens and determining their quantitative content in normal and pathological
conditions. The nucleic acids content in the studied cells is characterized by a gradual increase in intact poultry, and a decrease in chickens with intestinal pathology (escherichiosis).

The indicator of quantitative nucleic acids content in glandular stomach mucous membrane epithelium, detected by the luminescence spectral assay with EtBr, can be considered as a biological marker of the poultry gastrointestinal tract status in addition to already known biomarkers.

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