Application of luminescence spectral assay to evaluate the results of a scientific experiment

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Abstract. The method of characterization of functional state of epithelium of mucous membrane of alveolar glands of poultry proventriculus, using luminescence spectral assay with fluorescent dyes Ethidium bromide (EtBr) and 5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF) is offered. The dynamics of nucleic acids and proteins content in chickens’ cells under experimental klebsiellosis in antibiotic-treated poultry is established using this method. It is found that at the 2th – 7th days of treatment the markers of the cells functional status in the experimental group chickens are similar to those of poultry of the intact group.

1. Introduction

Modern education is closely related to scientific and experimental behavior. New highly effective means of treatment and prevention of infectious illnesses are in demand for modern poultry farming. Next-generation pharmaceuticals have to conform consumer needs, as well as government demands to reduce use of antibiotics for non-therapeutic purposes.

A systematic approach to development of new prophylactic and therapeutic agents requires using of biomarkers to detect changes in birds’ physiological status. A combination of biomarkers is required to track all aspects of the status, including gastrointestinal health. Currently, various biological markers, such as villi height or crypt depth, are used for monitoring of a gastrointestinal tract state, and some of them have been tested on poultry [1]. These biomarkers do not allow registering changes in the functional status at cellular level. But while solving such problems requires visualization of the process at molecular level [2, 3].

Luminescent spectral assay can be applied as a method capable of detecting biochemical variations at cellular and tissue levels. This method has demonstrated the efficiency in assessing the functional status of various kinds of cells by determining of the organic substance quantitative content, such as nucleic acids and proteins. Fluorescence assay is realized by means of two-wave microfluorimeter, which is the combination of the fluorescence microscope with the spectrum-analyzer complete with electronic recording and controlling modules. This equipment is applied to analyze the status of intracellular metabolism [4].

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However, there is no information in the scientific literature about application of luminescent spectral assay to assess a functional status of a gastrointestinal tract of farm animals, including chickens. The purpose of our research was to study an assessment of functional status of the alimentary tract of poultry under experimental klebsiellosis, in antibiotic-treated and intact poultry, using luminescence spectral assay.

2. Materials and methods

Ethidium bromide (EtBr, Acros Organics) was used as a luminescent dye for nucleic acids (NA) labeling, and 5-[(4.6-Dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF, Sigma-Aldrich) was used as a marker of proteins (Pr).

The developed staining method of simultaneous nucleic acids and proteins detection in poultry proventriculus cells 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 DTAF (10^{-4} M);
- rinsing with distilled water for 1 minute;
- 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).

Crimson-red fluorescence, most pronounced in mucous membrane epithelium of alveolar glands of poultry proventriculus, was observed against blue-green background in stained with fluorochromes histologic specimens. Crimson-red fluorescence indicates the localization of NA, associated with EtBr, and blue-green - the localization of Pr, associated with DTAF. The intensity of fluorescence of both dyes is determined by the Prs and NAs content, detected by this method at wave-lengths, corresponding to the maximum the dyes’ fluorescence intensity. DTAF quantitively binds to Prs and EtBre quantitively binds to NAs, as the result two emission bands are detected in the fluorescence spectrum, and they are correlating with the proteins and nucleic acids concentration in the specimen. With this staining method the maximum Prs fluorescence intensity is recorded at a wave-length of 528 nm, NAs - 624 nm. The amount of Prs and NAs is determined according to the fluorescence intensity index. Thickness of the tested area and equipment parameters affect the fluorescence intensity too. They are varying. Therefore, to get comparable data, the content of Prs and NAs should be evaluated in relative units (RU), taking into account the thickness of the standard and photometric area. The optical density, using as the thickness of the studied area, is directly proportional to the thickness of the absorbing layer at 648 nm, which is experimentally established. Luminescence spectrum of uranium glass, which has a thickness of ~ 1.5 mm, was taken into account as a reference at wave-length of 548 nm.

The Prs content in the studied area of the histologic specimen was calculated according to the following formula, in relative units (RU):

\[ B = \frac{I_b}{D \times \varnothing}, \]

\( B \) – proteins content (relative units);

\( I_b \) – luminescence intensity of the studied area at wave-length of 528 nm;

\( D \) - the region optical density at wave-length of 648 nm, used as its thickness;

\( \varnothing \) - uranium glass luminescence intensity at wave-length of 548 nm, used as a reference.

The NAs content in the studied area of the histologic specimen was calculated according to the formula, in relative units (RU):
\[ N = \frac{I_n}{D \times \Omega}, \text{ where} \]

\( N \) – nucleic acids content (relative units);  
\( I_n \) – luminescence intensity of the studied area at wave-length of 624 nm;  
\( D \) - the region optical density at wave-length of 648 nm, used as its thickness;  
\( \Omega \) - uranium glass luminescence intensity at wave-length of 548 nm, used as a reference.

Biological object has peculiar properties due to the unequal organic matter content, located in different parts of the structural zones of cells, which characterize the functional status. Therefore, it is have to calculate an average of same organic matter data from different sites, all other things being equal. In our studies, we gave consideration to the mean value of three our results. The mean value of Prs content (RU) in the epithelium of mucous membrane of alveolar glands of poultry proventriculus was calculated by the following formula:

\[ B_c = \frac{1}{5} \sum_{n=1}^{5} B_n, \text{ where} \]

\( B_c \) - the mean value of Prs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus;  
\( B_n \) - the total content of Prs in the photometric sites;  
\( n \) - number of the tested arias.

The mean value of NAs content (RU) in the epithelium of mucous membrane of alveolar glands of poultry proventriculus was calculated by the following formula:

\[ N_c = \frac{1}{5} \sum_{n=1}^{5} N_n, \text{ where} \]

\( N_c \) - the mean value of nucleic acids content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus;  
\( N_n \) - the total NAs content in the photometric sites;  
\( n \) - photometric sites number.

The mean values of Prs and NAs content in the control end experimental groups chickens were used to estimate the functional status of epithelial cells during pharmacotherapy.

The evaluation of the proventriculus functional status by Prs content in cells of the experimental group chickens was determined by the following formula:

\[ K_b = B_c - B_z, \text{ where} \]

\( K_b \) – marker of the functional status of the epithelium of mucous membrane of alveolar glands of poultry proventriculus of the experimental group chickens;  
\( B_c \) - the mean value of Prs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus of control group chickens;  
\( B_z \) - the mean value of Prs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus of experimental group chickens.

The evaluation of the proventriculus functional status by NAs content in cells of the experimental group chickens was determined by the following formula:

\[ K_n = N_c - N_z, \text{ where} \]

\( K_n \) – marker of the functional status of the epithelium of mucous membrane of alveolar glands of poultry proventriculus of the experimental group chickens;  
\( N_c \) - the mean value of NAs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus of control group chickens;  
\( N_z \) - the mean value of NAs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus of experimental group chickens.
The mean value of NAs content in the epithelium of mucous membrane of alveolar glands of poultry proventriculus of experimental group chickens.

Experimental chickens were divided into 2 groups according to analogy principle: experimental (n=165) and control (n=165). The division of poultry into groups was made randomly by the method of “Random numbers”. A criterion was body weight (±10%).

Infection of poultry with 24-hour culture of *Klebsiella pneumoniae* spp *rhinoscleromatis* was carried out by oral inoculation of 2-day-old chickens with 0.4 ml of 2.5 x 10^9 colony forming units (CFU) / ml of *K. pneumoniae*. The control group chickens were inoculated with saline by oral route. Treatment of the experimental group chickens was carried out after the clinical signs appearance and bacteriological confirmation of the diagnosis “Klebsiellosis” on the 5th day after infection (7th day of life) using the “Enroflon” antibiotic according to the instruction.

The chickens were decapitated on the 6-8, 10, 11, 13 days (n=15 each group) with subsequent dissection and registration of the results. Pieces of proventriculus 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 [5].

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 and DTAF, followed by fluorescence microscopy with registration of absorption and luminescence 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 the scan point diameter was 10^-4 mm at 480x magnification (12x40).

### 3. Results and discussions

The fluorescence intensity was recorded in a blue-green (I528) and red (I648) spectrum regions. The functional status of cells was assessed by the quantitative NAs (*Kb*) and Prs (*Kn*) content according to the developed method. The data were statistically processed.

Obtained indicators of functional status of the epithelium of mucous membrane of alveolar glands of poultry proventriculus in chickens of experimental group, determined by NAs (*Kn*) and Prs (*Kb*) content are presented in table 1.

| Table 1. The indicators of functional status of mucous membrane epithelial. |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| Age, days           | Treatment, days     | *Kn* (n = 90)       | *Kb* (n = 90)       |
| 6                   | -                   | 1.10±0.12           | 1.00±0.10           |
| 7                   | 1                   | 1.07±0.14           | 1.04±0.18           |
| 8                   | 2                   | 0.81±0.10           | 0.76±0.14           |
| 10                  | 4                   | 0.55±0.24           | 0.36±0.09           |
| 11                  | 5                   | 0.17±0.20           | 0.24±0.10           |
| 13                  | 7                   | 0.17±0.12           | 0.14±0.11           |

As it follows from the presented in table 1 data, in experimental group chickens, the values of *Kb* and *Kn* markers gradually decreased from the 2nd day of the treatment initiation. It indicates restoration of the cells functional status under the antibiotic therapy.

Our findings correlate with the other researchers’ results. Luminescence spectral assay is often used to estimate the results of a scientific experiment [6]. In antibiotic amoxicillin treated birds the expression
of numerous genes changes, which potentially leads to changes in biological activities of the small intestinal mucosa [7]. Schwerdtfeger L A et all. (2019) established that histochemical research methods are highly informative to study epithelial proliferation rates after antibiotic treatment [8]. Algorithms of histochemical research analysis can be used as useful tools of quantitative evaluation of biological reaction in both normal and pathology tissues [9].

4. Conclusion
The results allow us to conclude that the developed luminescence spectral assay with EtBr and DTAF is capable of revealing the NAs and Prs distribution features in histologic specimens of proventriculus mucous membrane epithelium in chickens and determining their quantitative content in normal and pathological conditions.

The indicators of functional status of the epithelium of mucous membrane of alveolar glands, determined by NAs ($K_n$) and Prs ($K_b$) content, can be considered as a biological marker of the poultry gastrointestinal tract status in addition to already known biomarkers.

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