

## METHODS

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### APPLICATION OF FLUORESCHEIN COPOLYMER TO IMPROVE THE EFFICIENCY OF COUNTER-IMMUNOELECTROPHORESIS FOR DIAGNOSTICS OF ANIMAL INFECTIOUS DISEASES

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*The counter-immunoelectrophoresis (CIE) is a highly specific, simple and rapid method, which does not require expensive equipment, input materials and long-term staff training as compared with other express methods of viral infections diagnostic. Despite the CIE high informativeness, the results in practice can be doubtful at times. Therefore, the purpose of the study was to improve CIE method by strengthening the antigen-antibody interaction with the use of synthesized amphiphilic fluorescein-containing copolymer. It was demonstrated that the use of the fluorescein copolymer -antigen complex enhanced the interaction with serum/plasma antibodies of rabbits immunized against hemorrhagic disease and increased protein content in the area of precipitation. This effect was more pronounced when more hydrophobic copolymer containing 5.87% fluorescein was introduced into the complex with antigen. The ability of the amphiphilic fluorescein copolymer to enhance the antigen-antibody interaction and the ability to visualize the fluorescent precipitation zone may increase CIE effectiveness for express diagnostic of infectious diseases.*

*Keywords:* counter-immunoelectrophoresis, amphiphilic fluorescein copolymer, rapid diagnostics, rabbit hemorrhagic disease.

The concentration of a large number of animals in a confined space due to industrial technologies for animal housing causes problems of rapid development and spread of various infectious diseases of both bacterial and viral origin. In case of infectious diseases it is important to conduct urgent rapid diagnostics with the aim of establishing the pathogen, and immediate development of effective measures of infection elimination and prevention.

The counter-immunoelectrophoresis (CIE), immunofluorescence (IF), solid-phase hemadsorption

(SPHA) and others tests are used for rapid diagnostics of viral infections. Among these methods, the CIE does not require expensive equipment and long-term staff training; it is simple and rapid to perform [1, 2]. CIE is based on the precipitation reaction for the detection of specific antibodies against the pathogen. It is known that precipitating antibodies, most of which belong to class G immunoglobulins (IgG), under certain conditions of electrophoresis in an electric field move to the cathode. The antigen has a positive charge and moves to the anode. Since the

antigen and antibody move in opposite directions, due to the electric field, their migration occurs towards each other. Visible precipitates appear after 30-60 minutes from the beginning of CIE [3].

This assay is used for the diagnostics of infectious diseases in veterinary and human medicine [4-7]. In particular, in the diagnosis of Aleutian mink disease of viral origin, CIE is characterized by high specificity and allows the detection of the disease on 6-15 days after infection. After experimental infection of animals, the disease is detected on 6-7 days when CIE is used [1, 8]. For suckling puppies, the reaction becomes positive within the first hour after suckling the sick females' milk, which has maternal antibodies. The diagnostic efficiency of Aleutian mink disease is 98-100%. CIE assay is also highly informative for the diagnosis of coronavirus infection for cattle [9].

Sometimes, the results of CIE can be doubtful. In such cases, for antigen-antibody precipitate detection, the agar gel is immersed in 12.5% NaCl solution for 7-10 min or stained (1% Amido black 10-B or 1% tannin solution), and then immunoelectrophoregrams are washed. If the result is questionable, the study should be repeated. These additional procedures delay the diagnosis of the disease, which is undesirable because of the rapid development of viral infection.

Our previous studies [10, 11] showed that one of the ways to increase the antigen-antibody interaction during immunoelectrophoresis is the appliance of an amphiphilic fluorescein-containing copolymer. This copolymer is able to enhance protein molecule binding and facilitate visualization due to visible fluorescence. During the study of an interaction between the obtained polymers and albumin, their ability to bind up to 70% of blood proteins was established [12-14].

The aim of the research was to improve the specificity of the CIE reaction due to the synthesized amphiphilic fluorescein-containing copolymer by enhancing the antigen + antibody interaction, which can be used for rapid diagnosis of infectious diseases.

## Materials and Methods

Fluorescein copolymers obtained by Steglich reaction were used for the studies [13].

*Antigens.* For immunization of rabbits were used the antigens of two strains: BG-04  $\geq$  640 hemagglutinating units and GBK-2 of  $\geq$  640 hemag-

glutinating units. These strains are the basis of the inactivated vaccine against hemorrhagic disease of rabbits (Lapimun GEM-2). The pathogen of rabbit hemorrhagic disease is a virus that contains RNA and possess a very high virulence. The vaccine administration protects rabbits against hemorrhagic disease for 12 months, and protective antibodies are produced on 4-14 days after vaccination [15].

*Animals and immunization procedure.* All animals were maintained in a specific pathogen-free animal facility with water and commercial food provided ad libitum. The protocol for animal experiments was approved by the Ethical Committee of the Institute of Animal Biology of NAAS of Ukraine and the experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986). 1-month-old rabbits of the Termonic White breed ( $n = 9$ ) were selected for the experiments. To obtain specific antibodies, the rabbits were immunized with the Lapimun GEM-2 vaccine, which was administered subcutaneously under shoulder-blade. Dosing was based on manufacturer's recommendation - 1.0 ml per head. The experiments were conducted in two stages: for 21 days ( $n = 6$ ) and, repeatedly for 28 days ( $n = 3$ ). Before vaccination (0 days) and after immunization on 14, 21 and 28 days a blood samples were taken into heparin tubes from the marginal auricular vein of rabbits.

*Preparation of agar gel.* To carry out immunoelectrophoresis, a 1% agar solution was prepared on 1:1 diluted tris-glycine buffer (pH 8.8). The heated agar solution (temperature  $\sim 60^\circ\text{C}$ ) was poured by 20-25 ml onto glass plates (6x11 cm) placed on a table to prepare gel samples for horizontal electrophoresis. After the agar was solidified (after 15-20 min), two parallel rows of 10-15  $\mu\text{l}$  holes were produced by the punch, with a distance of 1 cm between the rows.

*Sample processing.* Antigen samples were prepared as follows: 5  $\mu\text{l}$  of 1.0% solutions of fluorescein copolymer in two modifications (MF-37 and MF-47) were added to 1 ml of vaccine against hemorrhagic rabbit disease (Lapimun GEM-2). The samples of amphiphilic fluorescein-containing copolymer differed in the fluorescein content.

10  $\mu\text{l}$  of antigen (Lapimun GEM-2 vaccine) were introduced into the holes situated closer to the cathode: control one (without adding fluorescein copolymer; sample No. 1) and two experimental ones with the addition of MF-37 (sample No. 2) and MF-

47 (sample No. 3). Each blood sample was divided into 3 parts and 10  $\mu$ l of blood plasma were introduced into the holes closer to the anode.

**Counter-immunoelectrophoresis.** CIE was performed in a BIO-RAD chamber (200 V; 10 mA) for 25–30 min. The movement of fluorescein and the appearance of precipitation zones were visually controlled. After immunoelectrophoresis, the agar gel plates were covered with a transparent film and the results of the studies were recorded by direct scanning of the immunoelectrophoregrams (HP Scanjet G2410, United Kingdom). Protein content in the precipitation zones was determined using TotalLab TL120 software.

**Statistical analysis.** Statistical calculations of results ( $M \pm m$ ) were performed using the Microsoft Excel 2007. The probability of differences was determined by the Student's *t*-test with  $P < 0.05$  accepted as statistically significant.

## Results and Discussion

The appearance of new strains of viruses pre-determines both the improvement of existing and the search for new rapid diagnostic tests for infectious diseases. To improve the effectiveness of CEI for rapid diagnostics of viral diseases, we combined a precipitation reaction (precipitation of a certain antigen from a colloidal solution under the influence of serum specific antibodies with the formation of the antigen + antibody complex and sedimentation) with fluorescence using new fluorescein copolyesters. A generalized scheme of the elemental unit structure of fluorescein-containing copolyesters is shown in Fig. 1.

Polymeric products based on lipophilic 2-(octadecanoylamino) pentanedioic acid, hydrophilic polyoxyethylene diol with a molecular weight of 1000 or 1500, dipropylene glycol and fluorescein were obtained according to the procedure and their characteristics are shown in Table 1.

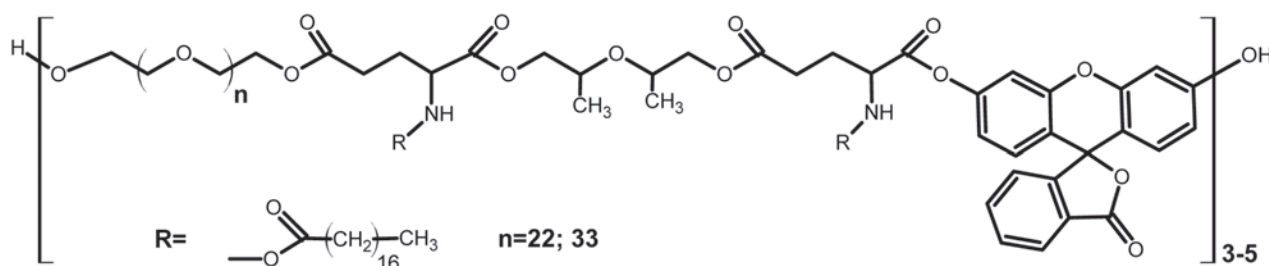


Fig. 1. Generalized scheme of the copolyester elemental unit structure

The synthesized copolyesters exhibit amphiphilic properties and are capable of forming self-stabilized dispersed media in water [17]. Moreover, they contain a dye (fluorescein) capable of fluorescence at pH 7 and higher pH. Due to this, the new copolyesters are used as fluorogenic markers to obtain images from the biological objects environment [18].

After immunoelectrophoresis, the precipitation zones between the antigen (inactivated vaccine viruses) and serum antibodies were formed on the gel plates. The zones were formed in response to the introduced vaccine and characterize the reaction-response of the immune system of the rabbit organism (Fig. 2).

Precipitation zones and, respectively, the formation of antibodies on the 14<sup>th</sup> day of research are clearly observed. At the same time, before the vaccination, the protein content of the antigen + antibody precipitation zones (both without and with the use of the fluorescein copolymer in the antigen composition) was in the range of 31.3–36.1% (Fig. 3, Table 2).

On the 14<sup>th</sup> day after vaccination, the antigen-antibody complex content in the precipitation zone changed: it was by 15.1–23.6% ( $P < 0.01$ – $0.001$ ) higher in CIE with complex between fluorescein copolymer and the antigen than in CIE without polymer. The established differences were maintained during the study of the content of complexes between antigen and antibody after 21 days. The difference between the use of a fluorescein copolymer in the complex with the antigen, compared to pure antigen, was smaller (5.8–8.7%), but remained statistically significant ( $P < 0.05$ – $0.01$ ).

It should be noted that for some animals the antigen-antibody precipitation zones were detected before vaccination. Apparently, their presence was caused by passive immunity provided by immunized female rabbits. Thus, the presence of antibodies against hemorrhagic disease agents in the blood of young rabbits (up to 1 month) can indirectly charac-

Table 1. Characteristics of fluorescein copolyesters

Characteristics of new fluorescein copolyesters	Generalized structural formula	
	GluSt-PEG1000-DPG-F; MF-37	GluSt-PEG1500-DPG-F; MF-47
Monomers ratio in copolyester, mole parts:		
Acid	0.5	0.50
PEG	0.27	0.30
DPG	0.09	0.09
Fluorescein (F)	0.14	0.11
Fluorescein content in the polymer, %	5.87	2.40
MM, Da	6150	7750
CMC, %	0.0052	0.007
Hydrophylic-lipophylic balance (HLB)	6.30	7.70
$\zeta$ - potential, mV	-25.60	-22.30

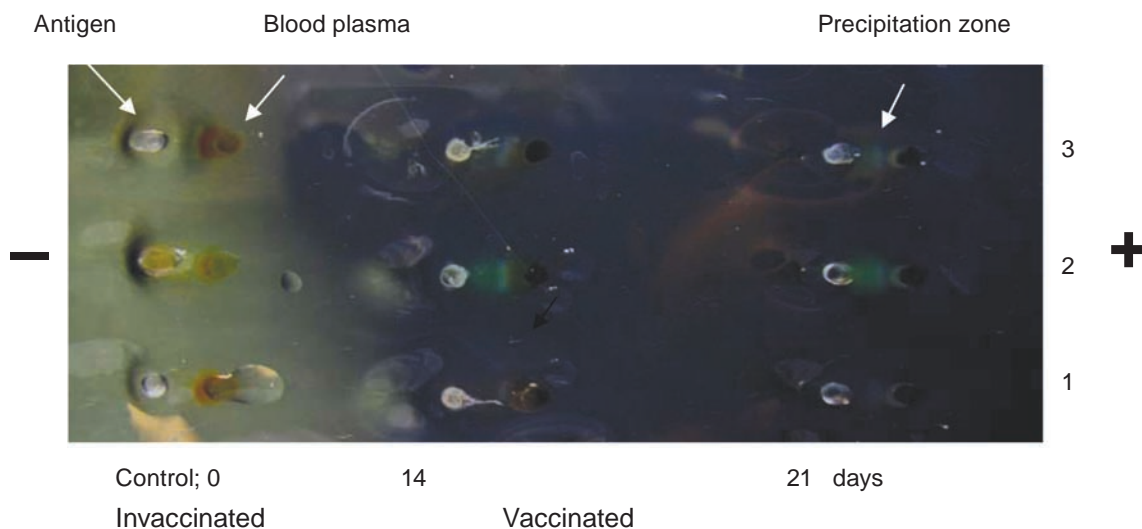


Fig. 2. Results of immunization of young rabbits with vaccine against hemorrhagic disease on the 14<sup>th</sup> and 21<sup>st</sup> days: sample 1 – antigen without fluorescein copolymer; sample 2 – antigen with modifications of fluorescein copolymer MF-37; sample 3 – antigen with modifications of fluorescein copolymer MF-47

terize the immunity state of female rabbits from which they are originated.

Repeated studies of the antigen-antibody complex content in the fluorescein copolymer complex confirm that fluorescein copolymer addition to the antigen makes the appearance of precipitation zones more intense (Figs. 4, 5).

Particularly, before vaccination, the concentration of the antigen-antibody complex in precipitation zones (without and with the use of the fluorescein copolymer in the antigen composition) was in the range of 31.3–36.2% (Table 3).

After 14 days of vaccination the highest protein content in the precipitation zone ( $41.1 \pm 4.86\%$ ) was observed for the samples using the fluorescein copolymer MF-37; the lower value ( $32.5 \pm 4.15\%$ ) was observed using the polymer MF-47 and the lowest one (6.1–14.7%,  $P < 0.05$ ) was determined for the samples without polymers. The established relation between the samples with and without fluorescein copolymer was also preserved after 21 and 28 days of immunization (9.2–16.7%,  $P < 0.05$ ) and 1.7–2.7%, respectively.

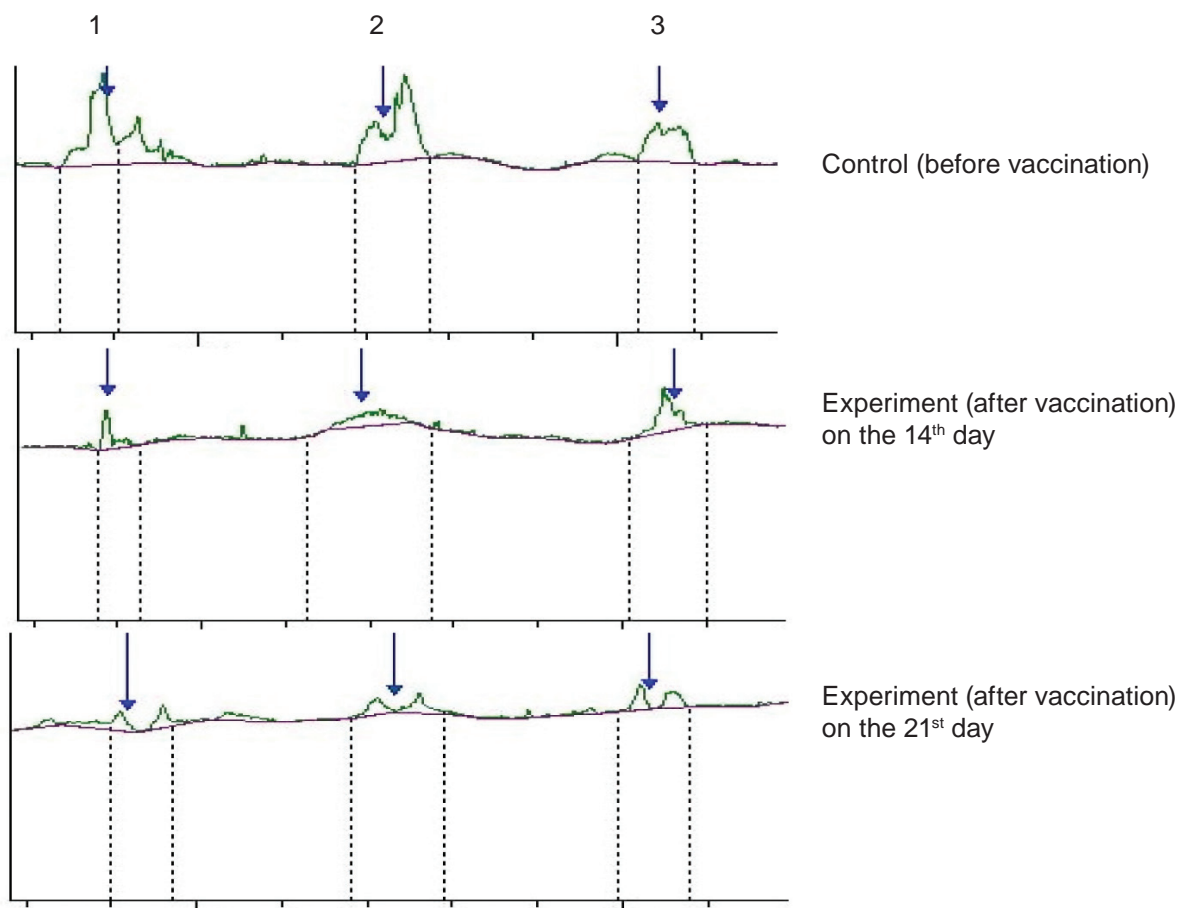


Fig. 3. Immunoelectrophoresis densitograms of precipitation reaction between antigen and antibody: 1 – antigen without fluorescein copolymer; 2 – antigen with modifications of fluorescein copolymer MF-37; 3 – antigen with modifications of fluorescein copolymer MF-47. The arrow indicates precipitation zone

Table 2. The concentration of the antigen-antibody complex in the precipitation zones, ( $M \pm m$ ,  $n = 6$ )

Experimental conditions, days	The ratio between the content of antigen-antibody complexes in the samples, %		
	Control (without polymer)	Polymer MF-37	Polymer MF-47
0 (before vaccination)	$32.60 \pm 1.63$	$36.10 \pm 2.38$	$31.30 \pm 2.24$
14	$20.50 \pm 1.57$	$44.10 \pm 1.89^{***}$	$35.60 \pm 2.95^{**}$
21	$28.50 \pm 1.30$	$37.20 \pm 1.78^{**}$	$34.30 \pm 1.70^*$

Note. Data were compared to the control. The difference is judged to be statistically significant when:  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$

Table 3. The concentration of the antigen-antibody complex in the precipitation zones, ( $M \pm m$ ,  $n = 3$ )

Experimental conditions, days	The ratio between the content of antigen-antibody complexes in the samples, %		
	Control (without polymer)	Polymer MF-37	Polymer MF-47
0 (before vaccination)	$32.50 \pm 1.27$	$36.20 \pm 2.38$	$31.30 \pm 2.24$
14	$26.40 \pm 0.86$	$41.10 \pm 4.86^*$	$32.50 \pm 4.15$
21	$27.20 \pm 2.91$	$43.90 \pm 4.45^*$	$28.90 \pm 3.42$
28	$29.30 \pm 2.51$	$38.60 \pm 1.69^*$	$32.10 \pm 1.24$

Note. Data were compared to the control. The difference is judged to be statistically significant when:  $*P < 0.05$

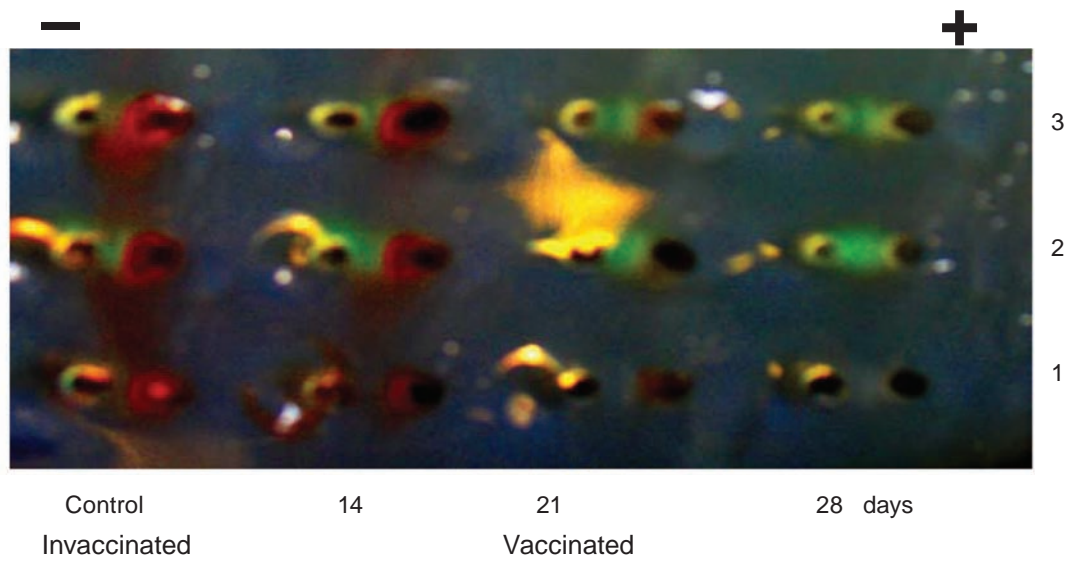


Fig. 4. Results of immunization of young rabbits with a vaccine against hemorrhagic disease using the fluorescein copolymer for 28 days: sample 1 – antigen without fluorescein copolymer; sample 2 – antigen with modifications of fluorescein copolymer MF-37; sample 3 – antigen with modifications of fluorescein copolymer MF-47

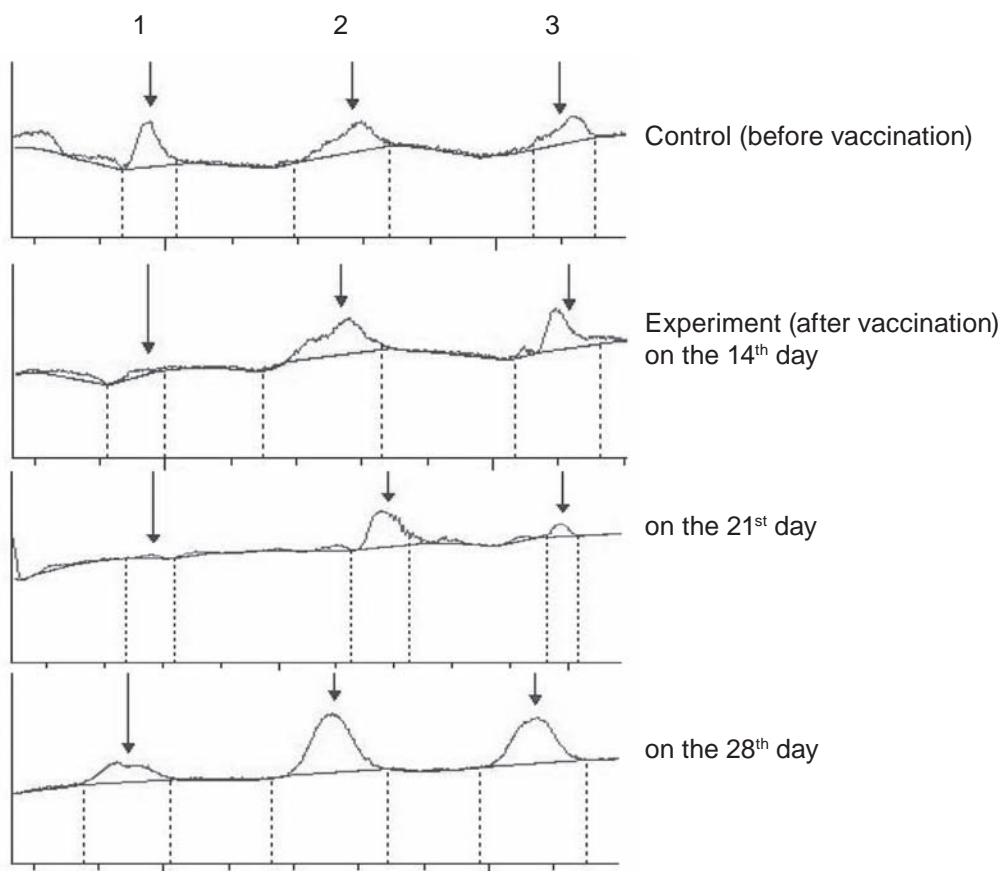


Fig. 5. Immunoelectrophoresis densitograms of precipitation reaction between the antigen and antibody: 1 – antigen without fluorescein copolymer; 2 – antigen with modifications of fluorescein copolymer MF-37; 3 – antigen with modifications of fluorescein copolymer MF-47. The arrow indicates precipitation zone

Table 4. The dynamics of complex formation between antigen (with fluorescein copolymer) and antibody after 28 days from the immunization of young rabbits against hemorrhagic disease, ( $M \pm m$ )

Experimental conditions, days	The ratio between the content of antigen-antibody complexes in the samples, %		
	Control (without polymer), $n = 6$	Polymer MF-37, $n = 3$	Polymer MF-47, $n = 3$
0 (before vaccination)	13.10 $\pm$ 1.43	11.90 $\pm$ 1.28	12.70 $\pm$ 1.59
14	19.70 $\pm$ 1.71*	18.60 $\pm$ 0.78**	21.20 $\pm$ 1.68*
21	26.20 $\pm$ 1.76***	26.70 $\pm$ 1.34***	28.20 $\pm$ 2.56**
28	41.00 $\pm$ 2.97***	42.80 $\pm$ 1.42***	37.90 $\pm$ 2.68***

Note. Data were compared to the control. The difference is judged to be statistically significant when: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

The efficacy of using fluorescein copolymer to improve the specificity of the antigen-antibody binding reaction during CEI was confirmed by changes in the formation of antigen-antibody complexes for 28 days (Table 4).

Hence, the percentage content of antigen-antibody complexes increased from 11.9–13.1% (before vaccination) to 25.2–30.9% ( $P < 0.05$ – $0.001$ ) on 28<sup>th</sup> day after vaccination. Moreover, in each period of study, the difference between the values of protein content in the precipitation zone without and with the use of the fluorescein copolymer was within the error of the arithmetic mean (0.3–4.9%). The experimental results indicate the formation of antibodies in the animal body in response to the introduced pathogen and the suitability of the fluorescein copolymer for diagnostic studies. At the same time, a strong correlation ( $\eta = 0.983$ ) between the increase of protein content in the precipitate zone and the day of study characterizes the advantage of the fluorescein copolymer MF-37 for diagnostic study. This copolymer enhances the antigen + antibody interaction for detection of specific antibodies (or pathogene(s) if specific antibodies are present) during the ascertainment of the strength of the immune response and infectious disease diagnostic by CEI.

In this manner, during diagnostic studies using CEI the fluorescein copolymer complex in the antigen composition intensifies the precipitation with

serum (plasma) antibodies for rabbits immunized against hemorrhagic disease. The content of antigen-antibody complexes in the precipitation zone increases by 1.7–16.7% ( $P < 0.05$ ). During CIE the appearance of precipitation zones is the most intense when more hydrophobic (HLB = 6.3) copolymer MF-37 containing 5.87% of fluorescein is used in the antigen complex with antigen. The ability of fluorescein amphiphilic copolymer to enhance the antigen-antibody interaction together with the fluorescence possibility to visualize the precipitation zone can be used to improve the efficiency of the CIE rapid diagnostics for detecting antibodies (or antigen if specific antibodies are present) during the ascertainment of the strength of the immune response and infectious diseases diagnosis in animals. CEI using the fluorescein-containing amphiphilic copolymer is rapid and easy to perform assay which provides visual control and result recording.

*Conflict of interest.* Authors have completed the Unified Conflicts of Interest form at [http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi\\_disclosure.pdf](http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf) and declare no conflict of interest.

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## ЗАСТОСУВАННЯ ФЛУОРЕСЦЕНТНОГО КОПОЛІМЕРУ ДЛЯ ПІДВИЩЕННЯ ЕФЕКТИВНОСТІ ЗУСТРІЧНОГО ІМУНОЕЛЕКТРОФЕРЕЗУ ЗА ДІАГНОСТИКИ ІНФЕКЦІЙНИХ ХВОРОБ ТВАРИН

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Метод зустрічного імуноелектрофорезу (ЗІЕФ) характеризується високою специфічністю, простотою і швидкістю проведення діагностичних досліджень без використання дорогого обладнання, витратних матеріалів та тривалого навчання персоналу. Попри високу інформативність (до 98–100%) результати застосування в експресдіагностиці можуть бути сумнівними. Тому метою досліджень було підвищити ефективність ЗІЕФ шляхом посилення взаємодії антигену з антитілом за використання синтезованого амфіфільного флуоресцеїновмісного кополімеру. Встановлено, що комплекс кополімер флуоресцеїн–антиген посилював взаємодію з антитілами сироватки/плазми крові імунізованих проти геморагічної хвороби кролів, що виявлялось у підвищенні вмісту протеїнів у зоні преципітації в разі проведення ЗІЕФ. Цей ефект був виразнішим за введення до складу комплексу з антигеном більш гідрофобного кополімеру, що містив 5,87% флуоресцеїну. Здатність амфіфільного флуоресцентного кополімеру посилювати взаємодію антиген-антитіло та можливість візуалізувати флуоресцентні зони преципітації можуть підвищити ефективність ЗІЕФ як експрес-методу для виявлення антитіл за оцінки напруженості імунітету під час діагностики інфекційних хвороб.

Ключові слова: експрес-діагностика, зустрічний імуноелектрофорез, амфіфільний кополімер флуоресцеїну, геморагічна хвороба кролів.

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