

IMMUNOBIOCHEMICAL INDICES IN CLINICALLY HEALTHY HORSES AND HORSES WITH EQUINE INFECTIOUS ANEMIA

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Aim. Equine infectious anemia (EIA) remains an urgent problem for many countries. This article presents the initial comprehensive comparative analysis of the immunobiochemical profiles of clinically healthy horses and those afflicted with the EIA in the western region of Ukraine. To address this objective, the immunobiochemical indices in healthy horses in the western region of Ukraine, the seasonal and age-related dynamics of the content of circulating immune complexes (CIC), vitamin C, and lysozyme were investigated. A possible association between the increase in the CIC rate and the content of serum fractions of protein and immunocompetent cells in horses with asymptomatic EIA course was determined. **Methods.** The study encompassed 329 non-purebred male and female horses of various age groups, comprising 225 EIA-free horses and 104 horses with asymptomatic EIA. Common serological and biochemical methods were employed, and identification of horses with EIA was accomplished via agar gel immunodiffusion assay with diffuse precipitation. **Results.** The aspects of changes in the immunobiochemical status of the animals in terms of age and season were determined. The findings demonstrated that with the increase in CIC in the serum of healthy animals, there was an increase in the amount of total protein, albumins, α -globulins, γ -globulins, and the number of O-cells and subpopulations of Tr- and Ts-lymphocytes. The number of β -globulins and immunocompetent cells of T- and B-lymphocytes diminished. The animals with the asymptomatic EIA course demonstrated a reliable decrease in the fraction of albumins ($p < 0.05$), β -globulins ($p < 0.05$), and the increase in the fraction of γ -globulins ($p < 0.05$). In spring, healthy four-year-old and older horses had a considerable increase in the CIC rate (30.4–90.6 c.u.) as compared to other seasons (33.4–63.6 c.u.), which demonstrated the accumulation of antigen-antibody complexes in the organism during this period. Generally, the analysis showed that the older the horses were, the clearer the tendency towards higher CIC rate was, and the smaller the content of vitamin C with age, except for the group of 10-year-old and older horses where it was slightly increased, and there was the decrease in the lysozyme activity with age. The content of heterohemagglutinins in healthy horses increased from 6.25 to 12.02 c.u. with age, and in horses with the asymptomatic EIA course there was a decrease from 10.6 c.u. in 3-year-old horses to 6.26 in 4–9-year-old horses, and a slight increase up to 8.07 when horses were ten or more years old. The study of the seasonal state of non-specific factors of protection and humoral immunity in the selected group of horses with EIA demonstrated that the CIC rate in healthy and infected horses changed dynamically depending on the season – it was higher in spring and lower in all the other seasons. The maximal rate of CIC in infected horses was in spring (90.62 c.u.), and the minimal one – in summer (52.73 c.u.). The titer of specific antibodies to the EIA virus did not change considerably in different seasons. **Conclusions.** The study determined that a high CIC rate in the blood of horses with asymptomatic EIA indicated substantial alterations in their immunobiological status. The elevation in the rate of isoantibodies in horses with asymptomatic EIA, as compared to the healthy animals, highlighted the development of the autoimmune process in EIA-afflicted horses. This phenomenon occurred concurrently with an increase in other non-specific antibodies, contributing to an overall rise in CIC levels, while the levels of specific antibodies remained relatively stable. This study represents the first simultaneous investigation of both specific immunity (CIC) and non-specific immunity (lysozyme activity, content of vitamin C, protein fractions of blood, etc.) in the context of EIA.

Key words: horses, infectious anemia, humoral immunity, lysozyme activity, circulating immune complexes.

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INTRODUCTION

In modern conditions, the course of infectious diseases undergoes significant changes due to the impact of various factors. Intensive animal breeding, the administration of numerous substances for the prevention and treatment of animal diseases, the use of feed additives, the adaptation mechanisms of the organism, and other factors contribute to alterations in the primary clinical manifestations of infectious diseases in farm animals. Consequently, a typical course of any disease, as described in various sources, including EIA (Coggins et al, 1972; Sellon, 1993; Busol et al, 1996), is rarely observed. An asymptomatic (latent) disease course is more common, and it can only be diagnosed through laboratory testing. Under this course, the status of the immune system remains entirely unexamined. Such animals serve as carriers of the disease agent, and if clinical signs are absent, they pose a potential threat to infecting other animals. For instance, the study of the immune complexes during the asymptomatic course of bovine leukosis revealed that there were almost no differences in the content of immune complexes between the groups of healthy and diseased animals (Mandyhra & Rud', 2001). It is still unclear why the immune system of the organism responds to the presence of the disease agent while the animal remains a carrier of the virus for the rest of its life. There is no definitive answer to this question.

The physiological role of circulating immune complexes (CIC) is to eliminate unwanted antigens within the organism. Elevated CIC concentrations are observed in many systemic diseases, neoplasms conditions, and inflammatory states (Gołda et al, 2014). Immune complexes are a natural component of the immune response, and they serve as the triggering mechanism for disease development in only certain cases. Diseases associated with immune complexes encompass equine infectious anemia (EIA), rheumatoid arthritis, Aleutian disease of mink, serum disease, hepatitis, dengue hemorrhagic fever, and post-streptococcal glomerulonephritis. The composition of CIC arises from the recognition of both endogenous and exogenous antigens present in the organism. The most common exogenous antigens include bacterial, viral, and fungal proteins, toxins, as well as proteins from the cellular membrane of parasites. In contrast, endogenous antigens originate from neoplastic cells, membrane proteins, or products of cell degeneration, which occur due to inflammatory conditions or autoimmune phenomena (Gołda et al, 2014).

Equine infectious anemia (EIA) is a persistent viral infection affecting horses. The causative agent of this disease belongs to the *Lentivirus* genus within the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other representatives of *Lentivirus* genus are the bovine immunodeficiency virus, feline immunodeficiency virus, human immunodeficiency virus 1 and 2, simian immunodeficiency virus, and maedi/visna virus (equine infectious anaemia, 2015; Leroux et al, 2004). EIA was identified as a contagious virus disease of horses and other Equidae representatives at the beginning of the last century. Since then, the clinical signs and transmission route, the clinical pathology, and diagnostics have been described in detail (Sellon, 1993). Currently, EIA and its diagnostics pose an urgent problem for many countries (Morales et al, 2015; Malik et al, 2017), and it ranks as the top equine disease on the World Organization for Animal Health (OIE) list. In the USA alone, nearly one hundred cases of EIA were reported last year (Equine Infectious Anemia Report, 2022). Instances of EIA have also been documented in Africa, Asia, and Australia (A Code of Practice for Equine Infectious Anaemia, 2015), as well as in Europe, with reported cases in countries like Romania (Informe Commission European RASVE, 2012) and Poland (Gołda et al, 2014).

Under the asymptomatic disease course, the main mechanisms of pathological process development in the organism of sick animals are conditioned by immunological disorders previously stated in the publications of different researchers (McGuire et al, 1972; Kono et al, 1976). The pathological changes, occurring under this disease, are related to the immune complexes, which are among the components of a normal immune response, and only in some cases are they the trigger mechanism, launching the disease development.

Once a horse is infected with the EIA virus (EIAV), its blood remains infectious for the rest of its life, and a horse is a potential source of infection for other horses (Cheevers & McGuire, 1985). The virus is transmitted via blood or an infected secretion from the infected animal. In nature, the virus spreads from a clinically sick horse to susceptible horses via sanguivorous insects. The virus can also be passed by the iatrogenic pathway through the use of infected blood products, needles, syringes, IV sets or other equipment; there can also be a fetal prenatal infection (Kemen & Coggins, 1972).

The titer of the virus is higher in horses with clinical signs, and the risk of virus transmission from these animals is higher than the transmission from animal car-

riers with a lower titer of the virus. However, the studies on mules demonstrated that infected animals with positive results of a solid phase enzyme immunoassay (ELISA) and vague results of agar gel immunodiffusion (AGID) could have the same viral load as the one for the animals with a strong reaction of antibodies, so they may be a potential source of transmission (Sci-cluna et al, 2013).

It is known that immediately after infecting, the virus replicates to a high titer, mainly in macrophages of mature tissue of the liver, spleen, lymph nodes, lungs, kidney, and suprarenal glands (Sellon et al, 1992). The virions of the progeny enter the blood, and virus titers usually increase simultaneously with the rectal temperature; a high concentration of viral antigens in blood and tissues stimulates the production of antibodies in the host (Sellon et al, 1993). Specific antiviral antibodies are often detected by Western blotting as early as 7–10 days after infecting (Clabough et al, 1991). 45 days after infection, the antibody to bovine protein p26 is detected by the immunodiffusion testing in almost all the infected horses (Coggins et al, 1972). The obvious reaction of antibodies also extends onto external glycoproteins of the virus envelope gp90 and gp45. The final result is heterogeneous hypergammaglobulinemia with increased concentrations of Ig G, Ig G(T), and Ig M 60 days after the infection, especially in horses, which have numerous instances of fever (Henson et al, 1971).

Most antibodies, formed in the early stages of EIA infection, are directed against conservative epitopes of superficial glycoproteins gp90 and gp45. These antibodies are not neutralizing, i.e. they unite with the circulating virus, not depriving it of its infectious nature (Hussain et al, 1987; Payne et al, 1989). Thus, most viruses in the serum of feverish horses are in the form of circulating infectious immune complexes (CIC) (McGuire et al, 1972). These immune complexes may enhance the ability of the virus to penetrate the host cells and are likely to be involved in the development of most clinical signs of EIA, including fever, depression, thrombocytopenia, anemia, and glomerulonephritis. Therefore, being another manifestation of the enhanced infection, mediated by antibodies, platelets may serve as a mechanism of virus transmission in the form of infectious immune complexes to the host cells, which are most susceptible to virus replication (Clabough et al, 1991).

The disappearance of serum viremia and the related fever episode depend on the response mediated by specific B- and T-cells (Perryman et al, 1988). A fast ap-

pearance of high antibody levels in the blood serum of infected horses may demonstrate their participation in removing the virus from circulation. However, *in vitro* studies demonstrate that most antibodies, neutralizing the virus, are aimed at highly variable antigenic epitopes of superficial glycoproteins (Hussain et al, 1987; Payne et al, 1989). These antibodies are not detected in the serum of infected horses for several days or weeks after the fever passes, which means that neutralizing antibodies may not be significant for removing the virus from circulation. Most researchers believe that cell-mediated immune response should be critically involved in this important function.

As already stated, regardless of the development of a strong humoral and cell-mediated immune response to the EIA virus, the infected horses cannot remove the virus from their organism completely and remain infected all their life. Direct evidence of virus persistence is as follows: infection transmission from animals-carriers to susceptible animals via the transfusion of washed leukocytes or whole blood (Ishizaki et al, 1978; Sellon et al, 1993); the presence of infectious CIC (Morales et al, 2015); the induction of the disease after the administration of dexamethasone (Kono et al, 1976). A cell reservoir for the EIA virus in clinically healthy horses is unknown, but it is assumed to be some subpopulation of tissue macrophages.

The status of immunobiochemical indices of blood under the asymptomatic EIA course is yet to be studied, which is proven by the actual lack of scientific publications on this topic. At present, there are no studies on the ratio between the indices of specific (CIC) and non-specific immunity under EIA. This issue has not been paid much attention in Ukraine as well. It is known that the asymptomatic course is the absence of any clinical signs of the disease; the only one remaining is the presence of specific antibodies to the disease agent in the organism of a sick animal, which proves the presence of this agent in the body. Thus, it is reasonable to study the indices of non-specific immunity and the ratio between them and specific antibodies under the asymptomatic EIA course. The urgency of the problem is also conditioned by the fact that in recent years, there have been almost no testing for EIA in Ukraine, the diagnostics is absent, only some owners of purebred horses have contacted the veterinary service, and the disease is usually not registered in their farms. As for non-purebred horses, the processes of virus circulation are practically left unattended,

The aim of our study was to investigate immunobiochemical indices in healthy horses of the western

region of Ukraine and the horses with asymptomatic EIA course, to determine seasonal and age-related dynamics in the content of CIC, vitamin C, and lysozyme, to find probable connection between the increased CIC rate and the content of serum fractions of protein and immunocompetent cells.

MATERIALS AND METHODS

To solve this task, we studied the CIC concentration, the content of protein, protein fractions (of albumins, α -, β -, γ -globulins), vitamin C and lysozyme, the population of immunocompetent cells (T-, Tr-, Ts- and B-cells).

The study was conducted at the Experimental Station of Epizootiology at the Institute of Veterinary Medicine, NAAS of Ukraine (Rivne); the horses were from private farms in the western regions of Ukraine, contaminated with infectious anemia. A total of 329 non-purebred horses were examined; 225 of them were free from the EIA virus, and 104 had the asymptomatic EIA course. No other infectious diseases were detected in the horses under investigation. All horses were subjected to clinical and hematological examinations; a total of 453 biochemical studies and 762 studies in determining the content of T- and B-lymphocytes were conducted. Under the asymptomatic EIA course, the blood samples were taken from each animal in the experiment every 90–180 days at least for two years.

The data of district laboratories of veterinary medicine for 1995–2015 were also used. During this period, over two thousand horses from private farms in Rivne and Volyn regions of Ukraine were examined. These were healthy female and male horses of different age groups (from several months to 16 years) and horses with evident or asymptomatic EIA course. Non-purebred horses were examined; according to our data, the purebred horses did not happen to have EIA. Specific antibodies to the EIA virus in the blood serum of horses were determined by standard agar gel immunodiffusion involving a precipitation reaction (Coggins et al, 1972; Maslianko et al, 2007; Vlizlo et al, 2012; Malik et al, 2017). The rate of immune complexes in the blood of horses was studied by colorimetry using the extinction value (in conventional units) in blood serum at the wavelength of 450 nm (Gołda et al, 2014). We also used the method of Yu.A. Grinevich (Grinevich & Kamenets, 1986), based on the selection ability of precipitation of polyethylene glycol-6000.

Four age groups were formed out of 225 animals while determining the seasonal and age-related dynamics of the content of immune complexes, vitamin C, and

lysozyme in clinically healthy horses: I age group (up to one year) – 14 animals, II (1–3 years) – 60, III (4–9 years) – 90, IV (10 and older) – 61. While determining the change in biochemical and cellular indices of immunity in horses depending on the concentration of immune complexes in blood serum, the blood samples of 44 healthy horses, aged four years and older, were studied. While determining the dynamics in the changes in immunobiochemical indices of blood under EIA, 72 sick horses of different age groups were examined.

The amounts of albumins, fractions of globulins, immune complexes, immunoglobulins, homo- and heterohemagglutinins and heterohemagglutinins, vitamin C, and lysozyme were determined by common methods (McGuire et al, 1972; Sentsui and Kono, 1976; Busol et al, 1996). For instance, the lysozyme activity in blood serum was determined by the lysis rate of the reference culture *Micrococcus lysodecticus*, the number of T- and B-lymphocytes in blood was determined by calculating the rosette formation, the subpopulations of T-lymphocytes were determined using monoclonal antibodies, the photolorimetry method was used to determine the content of ascorbic acid (Murray et al, 1998; Popova and Polikarpova, 2012). The determination of total protein, albumins, fractions of globulins, and hemoglobin also involved the method of V. E. Chumachenko (Chumachenko et al, 1990).

RESULTS

1. *Immunobiochemical indices of healthy horses.* The seasonal and age-related dynamics of the content of immune complexes (CIC), vitamin C, and lysozyme in clinically healthy horses of different age-related groups was determined (**Table 1**).

The analysis demonstrated that the rate of immune complexes in age groups I and II was constant during the year, and in groups III and IV, their number was higher only in spring. For instance, their limits during this season were 30.4–90.6 c.u. of extinction (c.u.), and during all the other seasons – 33.4–63.6 c.u. A reliable difference between the CIC contents in age groups I, III, and IV in spring was determined ($p < 0.01$). As for the rate of immune complexes in terms of seasons, it is obvious that in spring, four-year-old and older horses demonstrated a considerable increase in the rate of immune complexes, which proved the accumulation of antigen-antibody complexes in the organism of horses during this period (**Figure**).

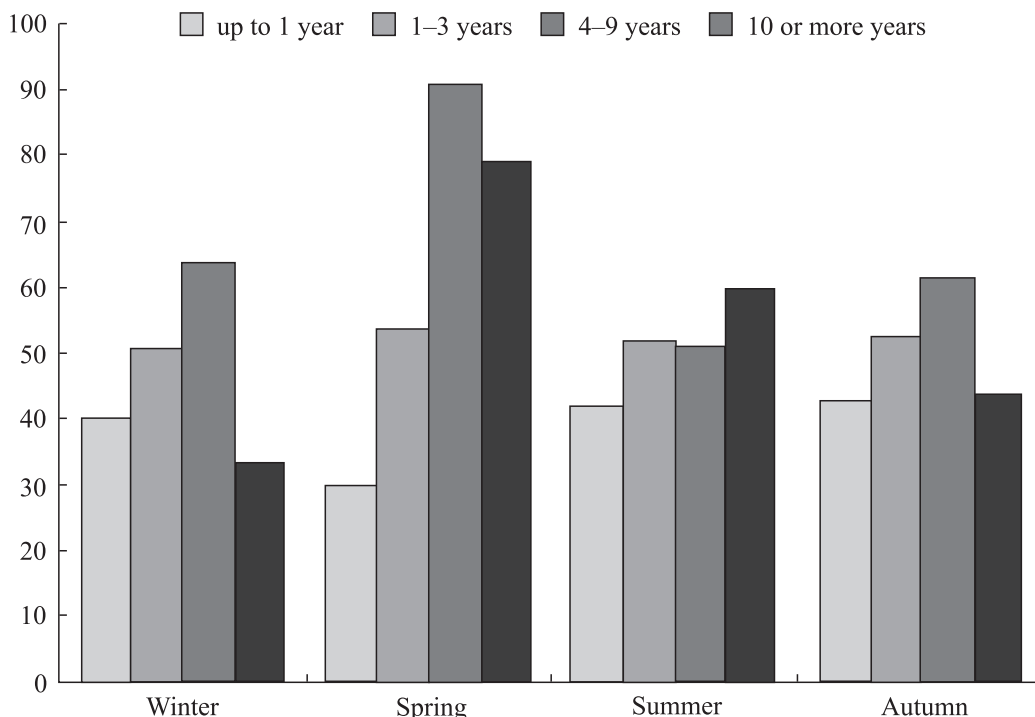
The content of lysozyme and vitamin C gives us an indirect idea about the general state of the immune system. The determination of the levels of lysozyme activi-

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Table 1. The seasonal and age-related changes in the CIC content, vitamin C, and lysozyme in clinically healthy horses

Number of animals	Age, years	CIC, c.u.	Vitamin C, mmol/l	Lysozyme activity, %
<i>WINTER</i>				
14	up to 1	40.0 ± 13.52	21.07 ± 1.36	42.67 ± 4.31
60	1–3	50.11 ± 17.33	17.62 ± 1.05	44.50 ± 3.51
90	4–9	63.63 ± 19.50	17.34 ± 1.73	47.59 ± 7.44
61	≥10	33.45 ± 14.39	20.41 ± 3.44	40.71 ± 7.12
<i>SPRING</i>				
14	up to 1	30.51 ± 4.62	20.53 ± 2.83	47.36 ± 2.51
60	1–3	53.66 ± 12.52	19.75 ± 2.27	32.55 ± 2.41
90	4–9	90.65 ± 17.33 **	18.34 ± 1.83	37.80 ± 6.44
61	≥10	79.18 ± 16.30 **	17.25 ± 3.06	35.99 ± 1.86
<i>SUMMER</i>				
14	up to 1	42.0 ± 14.61	21.07 ± 2.93	40.33 ± 5.50
60	1–3	52.0 ± 16.25	21.53 ± 1.37	37.20 ± 4.71
90	4–9	51.11 ± 17.46	20.46 ± 1.62	26.0 ± 5.34
61	≥10	59.67 ± 15.30	20.22 ± 2.03	30.61 ± 6.15
<i>AUTUMN</i>				
14	up to 1	42.61 ± 15.62	14.19 ± 1.73	33.13 ± 5.61
60	1–3	52.0 ± 13.63	18.17 ± 2.15	41.50 ± 4.35
90	4–9	61.63 ± 9.43	18.77 ± 1.53 *	31.65 ± 3.12
61	≥10	44.0 ± 15.24	23.47 ± 1.83 ***	29.0 ± 3.41

Note. The degree of probability in the age group of up to one year as compared to other age groups * p < 0.05, ** p < 0.01, *** p < 0.001.



The change in the rate of circulating immune complexes in different age-related groups of horses in terms of seasons

Table 2. The biochemical indices and immune complexes in different age groups of horses

Number of animals	Age, years	CIC, c.u.	Vitamin C, mmol/l	Lysozyme activity, %
14	up to 1	38.28 ± 9.51	19.07 ± 1.94	40.83 ± 4.52
60	1–3	51.94 ± 12.55	19.97 ± 2.27	38.90 ± 7.11
90	4–9	66.76 ± 14.36	18.76 ± 2.12	35.76 ± 3.34
61	≥10	58.67 ± 14.30	18.16 ± 2.54	34.01 ± 2.15

ty showed that its increase was observed in the winter-spring period and its decrease – in summer-autumn. The limits in winter-spring were 32.5–47.5 %, and in summer-autumn – 26.0–41.5 %.

The content of vitamin C in the seasonal dynamics was decreased in winter. The horses, aged under one year, had a reliable ($p < 0.001$, $p < 0.05$) decrease in the vitamin rate in autumn as compared to older age groups, which may be related to intense infection of helminths in young animals. Therefore, the content of immune complexes, lysozyme, and vitamin C in healthy horses changed rather dynamically depending on the age of the animals and the season.

In terms of the scientific aspect, noteworthy are not only the changes in these indices depending on the age of animals and a season, when the study was conducted, but also the question of how considerable the changes were in biochemical indices and immune complexes that occurred with age. The generalized results demonstrated that the older the horses were, the clearer the tendency towards the increase in the rate of immune complexes was (**Table 2**). The content of vitamin C decreased with age; it was only slightly increased in the group of horses aged 10 or more years. The lysozyme

activity also decreased with age. The data obtained indicated some changes in the physiological state related to the organism's aging and its transformation in maintaining internal homeostasis.

2. *The changes in biochemical and cellular indices of immunity in horses depending on the concentration of immune complexes in blood serum.* To determine a possible association between the increased rate of immune complexes and the content of serum fractions of protein and immunocompetent cells in horses, we investigated the CIC concentration, the content of protein, protein fractions (albumins, α -, β -, γ -globulins), the population of immunocompetent cells (T-lymphocytes, subpopulations of Tr- (or theophylline-resistant), Ts- (or theophylline-sensitive) lymphocytes and B-cells).

The blood samples of 44 healthy horses, aged 4 and more years, were studied. Two groups of examined horses were formed after determining the CIC content. Group I was comprised of healthy horses with the CIC content of 10–32 c.u., group II – also healthy horses with the CIC content of 63–83 c.u. The comparison of two groups of healthy horses demonstrated that the increase in the CIC in blood serum takes place simultaneously with the increase in the amount of total protein, albu-

Table 3. The immunobiochemical indices of healthy horses depending on the content of immune complexes

Number of animals under investigation	Content of CIC, c.u.	Total protein, g/l	Albumins, %	Fractions of globulins, %			Relative content of lymphocytes, %				
				α	β	γ	T	B	O	Tr	Ts
<i>1</i>											
n = 25	10–32	70.0 ± ±5.18	27.57 ± ± 3.54	17.82 ± ± 2.79	33.72 ± ± 3.41	21.73 ± ± 3.58	25.54 ± ± 3.41	10.31 ± ± 2.12	64.15 ± ± 7.72	12.85 ± ± 1.92	2.46 ± ± 0.36
<i>2</i>											
n = 19	63–83	73.16 ± ± 4.77	28.96 ± ± 3.25	18.04 ± ± 1.87	29.71 ± ± 4.0 *	23.88 ± ± 2.5	22.06 ± ± 2.74	8.38 ± ± 2.63	69.56 ± ± 8.81	15.38 ± ± 1.92	3.14 ± ± 0.41 *

Note. Degree of probability of differences between groups I and II, * $p < 0.05$.

mins, α -globulins, γ -globulins, as well as the number of O-cells and subpopulations of T-lymphocytes: Tr and Ts (**Table 3**). In its turn, against the background of the mentioned indices, the number of β -globulins and the immunocompetent cells, T- and B-lymphocytes was decreased, though no reliable difference between the indices under investigation was found. Therefore, a high rate of CIC in the blood of horses is an indicator of changes in the immunobiological status. No considerable changes in the state of immunity were found in healthy horses under a high rate of the CIC, though, as compared to the indices in horses under a low rate of the CIC; they demonstrated evident deviations towards the decrease in the immunity indices.

3. *The status of non-specific factors of protection and humoral immunity of horses under the EIA.* The dynamics of humoral indices and non-specific factors of immune protection was determined by the rate of

specific antibodies to the EIA virus, isoantibodies, heterohemagglutinins, the CIC, lysozyme activity, and vitamin C. The studies were conducted involving both healthy and infected horses, aged 4 to 9 years (Table 4–6). The results demonstrated that the titer of specific antibodies to the EIA virus did not change considerably in different seasons. The titer of antibodies in horses increases with age. The rate of immune complexes in healthy and infected horses changes dynamically depending on the season (**Table 4**). It is higher in spring and lower in all the other seasons. No statistically significant difference in the CIC content was found between the investigated groups, though there was some increase in their content in sick horses. For instance, the maximal rate was in spring (90.62 c.u.) and the minimal one – in summer (52.73 c.u.).

The lysozyme activity was somewhat lower in sick horses in spring and autumn and higher in winter and

Table 4. The indices of non-specific factors of protection and humoral immunity in terms of seasons

Groups of animals and their numbers	CIC, c.u.	Vitamin C, mmol/l	Lysozyme activity, %
<i>WINTER</i>			
Healthy, n = 90	63.63 ± 19.57	19.01 ± 7.31	47.51 ± 7.40
Sick, n = 49	66.51 ± 15.21	19.06 ± 9.67	52.74 ± 4.71
<i>SPRING</i>			
Healthy, n = 90	90.62 ± 17.30	20.05 ± 5.32	37.82 ± 6.41
Sick, n = 49	90.0 ± 13.71	14.25 ± 4.26	29.0 ± 5.61
<i>SUMMER</i>			
Healthy, n = 90	51.11 ± 17.41	28.68 ± 3.23	26.0 ± 5.38
Sick, n = 49	52.75 ± 12.51	22.39 ± 4.51	29.51 ± 3.47
<i>AUTUMN</i>			
Healthy, n = 90	61.63 ± 13.41	19.03 ± 6.31	31.62 ± 6.19
Sick, n = 49	72.67 ± 16.41	19.34 ± 5.21	28.41 ± 8.0

Table 5. The indices of non-specific factors of the protection and humoral immunity in different age groups of healthy horses and EIA-positive horses under the agar gel immunodiffusion

Age, years	Number of horses	Titers of normal antibodies, c.u.	Titers of isoantibodies, c.u.	CIC, c.u.	Lysozyme activity, %	Vitamin C, mmol/l
1–3	*n = 60	6.85 ± 2.29	3.95 ± 1.85	46.6 ± 7.0	33.6 ± 7.02	20.67 ± 2.74
	**n = 5	10.6 ± 4.70	4.51 ± 1.52	n/s	n/s	n/s
4–9	*n = 90	11.51 ± 2.16	3.45 ± 1.49	62.6 ± 16.0	40.0 ± 6.91	18.30 ± 8.32
	**n = 49	6.26 ± 3.87	3.38 ± 2.55	75.8 ± 16.0	38.3 ± 5.62	19.0 ± 7.66
≥ 10	*n = 61	12.02 ± 3.75	2.81 ± 0.53	58.0 ± 11.3	33.8 ± 8.43	21.19 ± 2.74
	**n = 50	8.07 ± 2.53	5.58 ± 2.83	65.0 ± 14.0	35.2 ± 6.72	17.25 ± 3.35

Note. * healthy horses, ** positive in agar gel immunodiffusion, n/s – not studied.

Table 6. The indices of non-specific factors of protection and humoral immunity in the agar gel diffuse precipitation reaction (AGDPR) of the EIA-positive horses depending on the titer of specific antibodies

Indices	Titers in AGDRP		
	native and titer 1:2, n = 28	1:4–1:8, n = 27	1:16–1:32, n = 17
Immune complexes, c.u.	54.80 ± 10.31	64.20 ± 13.40	77.65 ± 14.65
Lysozyme activity, %	43.01 ± 6.90	40.10 ± 7.70	33.50 ± 4.80
Vitamin C in blood, mmol/l	181.90 ± 16.80	178.70 ± 22.40	160.30 ± 15.60
Titers of normal antibodies, c.u.	1:5.40	1:5.72	1:4.42
Titers of isoantibodies, c.u.	1:7.5	1:9.2	1:10.5

Note. n – the number of examined animals in each group with the titer of antibodies, determined in AGDPR.

Table 7. The immunobiochemical indices of healthy horses and horses with the EIA depending on the content of immune complexes

Content of CIC, c.u.	Titer in AGDRP	Total protein, g/l	Albumins, %	Fractions of globulins, %			Relative content of lymphocytes, %				
				α	β	γ	T	B	O	Tr	Ts
<i>1</i>											
<i>Number of animals under investigation n = 25</i>											
10–32	–	70.0 ± ± 5.18	27.57 ± ± 3.54	17.82 ± ± 2.79	33.72 ± ± 3.41	21.73 ± ± 3.58	25.54 ± ± 3.41	10.31 ± ± 2.12	64.15 ± ± 7.72	12.85 ± ± 1.92	2.46 ± ± 0.36
<i>2</i>											
<i>Number of animals under investigation n = 19</i>											
63–83	–	73.16 ± ± 4.77	28.96 ± ± 3.25	18.04 ± ± 1.87 *	29.71 ± ± 4.0 **	23.88 ± ± 2.5 **	22.06 ± ± 2.74	8.38 ± ± 2.63	69.56 ± ± 8.81	15.38 ± ± 1.92	3.14 ± ± 0.41 *
<i>3</i>											
<i>Number of animals under investigation n = 15</i>											
61–147	1:8–1:64	78.34 ± ± 6.87	16.8 ± ± 3.15	27.25 ± ± 3.0 *	18.21 ± ± 2.5 *	37.63 ± ± 3.0 **	20.0 ± ± 1.74	8.0 ± ± 1.23	72.0 ± ± 8.19	14.6 ± ± 1.41	4.6 ± ± 1.0 *

Note. Degree of probability * <0.05 , ** $P < 0.01$.

summer. No statistically reliable difference was found between healthy and sick groups of horses. No considerable difference was found between the analyzed groups in terms of the content of vitamin C; there was only a slight decrease in the sick horses in spring and summer.

Noteworthy is also the dynamics of the indices under investigation depending on the age of horses (**Table 5**). With age, the rate of normal antibodies (heterohemagglutinins) in healthy horses increased from 6.25 to 12.02 c.u. At the same time, in horses with the asymptomatic EIA course, there was a decrease from 10.6 c.u. at the age of three years to 6.26 at the age of 4–9 years, and down to 8.07 when horses were 10 or more years

old. The results demonstrated a decrease in the number of normal antibodies in older horses under the asymptomatic course of the EIA and a simultaneous increase in the rate of antibodies to their own cells as compared to the healthy ones. It demonstrated the development of the autoimmune process under the EIA. The data of Table 4 (autumn-winter) and Table 5 also showed the increase in the CIC rate in the blood serum of horses with the asymptomatic EIA course, which was also a marker of the autoimmune process development under the EIA. No considerable changes in the content of vitamin C were found in the blood of horses with the asymptomatic EIA course.

The determination of the rate of humoral indices in horses depending on the degree of the infectious process development, caused by the EIA virus, involved the examination of 72 sick horses. The dependence of humoral indices of seropositive horses on the rate of the titers of specific antibodies to the EIA virus was found (**Table 6**).

The analysis of results demonstrated that with the development of the infectious process, there was an evident tendency towards the decrease in the titers of normal antibodies, vitamin C, lysozyme activity, and the increase in the rate of immune complexes and iso-antibodies.

A possible association between the increased CIC rate and the content of protein fractions in blood serum and the rate of immunocompetent cells was investigated in horses with asymptomatic EIA course. The CIC concentration, protein content, protein fractions (of albumins, α -, β -, γ -globulins), and the population of immunocompetent cells (T-, Tr-, Ts- and B-cells) were also determined for healthy horses.

The blood samples of 59 horses with asymptomatic EIA course, aged 4 and more years, were studied. The results of blood testing of healthy horses were used as the control. Three groups of examined horses were formed after determining the CIC content. Group I included healthy horses with the CIC content of 10–32 c.u., group II – healthy horses with the CIC content of 63–83 c.u., group III – sick horses with the CIC content of 61–147 c.u. and the titer of specific antibodies from 1:8 to 1:64 (**Table 7**).

The comparison of two groups of healthy horses demonstrated that the increase in the CIC in the blood serum of horses took place simultaneously with the increase in the amount of total protein, albumins, α -globulins, γ -globulins, as well as the number of O-cells and subpopulations of Tr- and Ts- lymphocytes. In turn, in the background of these indices, the number of β -globulins and the number of immunocompetent cells of T- and B-lymphocytes was somewhat smaller, though no statistically significant difference between the indices under investigation was found.

DISCUSSION

The analysis of immunological indices in the blood of healthy and sick horses with high CIC concentration, i.e. groups II and III, demonstrated that sick animals had a reliable decrease in the fraction of albumins ($p < 0.05$), β -globulins ($p < 0.05$) and an increase in the fraction of γ -globulins ($p < 0.05$). Therefore, a high rate

of CIC in the blood of horses with asymptomatic disease course was an indicator of considerable changes in their immunobiological status. No considerable changes were found in healthy horses under a high rate of the CIC, though, as compared to the indices in horses with a low rate of the CIC; they demonstrated some deviations towards the decrease in the immunity indices. While in healthy horses, a high rate of the CIC activated the increase in the rate of protein fractions – albumin and α -, γ -globulin (β -globulin – without changes), in sick ones, it was manifested with the increase in the content of α - and γ -globulins and a decrease in the content of albumins and β -globulins.

It was determined that the precipitate, formed using polyethylene glycol, contained immunoglobulins of different classes. The main part was formed by Ig γ (45–90%), this was the main class of Ig-antibodies. Our study determined that with the increase in γ -globulin fraction in the serum of sick horses, there was a simultaneous increase in the rate of immune complexes.

The results obtained confirm the thesis that a high rate of the CIC is a marker of the asymptomatic disease course, signaling about the deterioration of the total immune state of the organism. It should also be noted that under the asymptomatic course of infectious anemia, humoral indices of immunity reflect only some aspects of the immunobiological state. In general, the changes in the immunity functioning were noted in the organism of sick animals as compared to the animals, which were found not to have antibodies to the EIA virus, and it was also observed by other researchers (McGuire et al, 1972; Kono et al, 1976). It is rather a complicated task to determine the immunological changes in the organism under the asymptomatic course as it requires a deep analysis of the indices under investigation and the corresponding methodological approaches in the experiments. One of these approaches is the complex nature of the indices under investigation, which, considering the urgency of the EIA (Morales et al, 2015; Malik et al, 2017; Equine Infectious Anemia Report, 2022), allows for a more realistic evaluation of the immunity status of the animals.

For instance, the decrease in the lysozyme activity is associated with the inhibition of the phagocytic activity of neutrophils, which produce this enzyme. This factor was observed before for other pathologies (Skards, 1968). The increase in the CIC rate is closely related to the increase in the γ -globulin fraction, which demonstrates a possibly high content of this fraction in the immune complexes. The decrease in the content of

vitamin C, which is a component of many enzymes, is a consequence of the inhibition of lysozymosis and phagocytosis. The rate of normal antibodies and isoantibodies is interconnected with the inhibition of the immunoregulatory function of immunocompetent cells. These results confirm that similar to the tuberculosis infection, infecting with the EIA virus causes various changes in many systems of organs, many of which are immunologically mediated (Henson et al, 1971).

Our analysis demonstrates a close association between the above-described indices and shows that each specific index, characterizing the state of immunity, is interrelated with many others. It creates a more integral picture of the state of specific and non-specific immunity under the asymptomatic EIA course.

CONCLUSION

This is the first large-scale cross-section of the state of immunobiochemical indices of healthy horses and the ones with the EIA, kept at private farms in western Ukraine in 1995–2015. In particular, these are the Rivne and Volyn regions, this is the region of the Western Polissia of Ukraine, and partially the Forest-Steppe zone.

We noted the seasonal and age-related dynamics in the content of immune complexes (CIC), vitamin C, and lysozyme in healthy horses. The dynamic changes in these indices depending on the age of animals and the season were determined. The increase in the lysozyme activity is observed in winter-spring (32.5–47.5 %), and the decrease – in summer-autumn (26.0–41.5 %). The content of vitamin C in the seasonal dynamics is decreased in winter. The horses, aged under one year, had a reliable ($p < 0.001$, $p < 0.05$) decrease in the vitamin C rate in autumn as compared to older age groups, which may be related to intense infection of helminths in young animals. In spring, healthy four-year-old and older horses had a considerable increase in the CIC rate (30.4–90.6 c.u.) as compared to other seasons (33.4–63.6 c.u.), which demonstrated the accumulation of antigen-antibody complexes in the organism of horses during this period.

A high rate of CIC in the blood of healthy horses was an indicator of changes in the immunobiological status. The increase in CIC in the blood serum was simultaneous with the increase in the amount of total protein, albumins, α -globulins, γ -globulins, and the number of O-cells and subpopulations of Tr- and Ts-lymphocytes. In its turn, in the background of these indices, the amount of β -globulins and immunocompetent cells of T- and B-lymphocytes was somewhat smaller.

The study of the seasonal state of non-specific factors of protection and humoral immunity of horses with EIA demonstrated that the CIC rate in healthy and infected horses changed dynamically depending on the season – it was higher in spring and lower in all the other seasons. The maximal rate of CIC in sick horses was in spring (90.62 c.u.), and the minimal one – in summer (52.73 c.u.). The titer of specific antibodies to the EIA virus did not change considerably in different seasons.

The lysozyme activity was somewhat lower in sick horses in spring and autumn and higher in winter and summer; no statistically significant difference was found between the groups of healthy and sick horses. No considerable difference was found between the infected and healthy horses in terms of the content of vitamin C; there was only a slight decrease in the sick horses in spring and summer.

As for the age-related differences in the content of heterohemagglutinins, their rate in healthy horses increased from 6.25 to 12.02 c.u. with age, and in horses with the asymptomatic EIA course, there was a decrease from 10.6 c.u. in 3-year-old horses to 6.26 in 4–9-year-old horses, and a slight increase up to 8.07 when horses were 10 or more years old. The increase in the rate of isoantibodies in horses with asymptomatic EIA course as compared to healthy animals demonstrated the development of the autoimmune process under EIA, and there was an increase in the rate of CIC in blood serum. The content of vitamin C in the blood of horses with asymptomatic disease course did not change with age.

A possible association between the increased CIC rate (specific immunity) and the content of protein fractions in blood serum and the rate of immunocompetent cells (non-specific immunity) was determined. The findings demonstrated that with the increase in CIC in the serum of healthy animals, there was an increase in the amount of total protein, albumins, α -globulins, γ -globulins, and the number of O-cells and subpopulations of Tr- and Ts-lymphocytes. The number of β -globulins and the number of immunocompetent cells of T- and B-lymphocytes somewhat diminished. Sick animals demonstrated a reliable decrease in the fraction of albumins ($p < 0.05$), β -globulins ($p < 0.05$), and the increase in the fraction of γ -globulins ($p < 0.05$). Therefore, a high rate of CIC in the blood of horses with asymptomatic disease course was an indicator of considerable changes in their immunobiological status.

Over 40 years ago, the veterinary service of Ukraine (and that of the USSR) declared that the EIA was eli-

minated, and the cases of detecting horses with clinical signs of the disease have been rather a rare phenomenon in recent decades. The use of horses in biofactories with the purpose of obtaining immune sera under other infections in 1990–2000s and the transfer of non-purebred horses from collective farms to small private farms led to the uncontrolled spreading of asymptomatic forms of the EIA course during that period. Thus, in our opinion, it would be reasonable to resume selective state epizootic monitoring of this disease in Ukraine.

Adherence to ethical principles. We used animal biological material in accordance with the rules of biology ethics. Blood for the study of erythrocytes was taken from live animals, and internal organs for cell cultures were taken from slaughtered animals at specialized slaughterhouses and meat processing plants.

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Імунобіохімічні показники у клінічно здорових коней та при захворюванні на ІНАН

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Актуальність. Інфекційна анемія коней (ІНАН) залишається актуальною проблемою для багатьох країн світу. Вперше проведено масштабний порівняльний аналіз імунобіохімічного стану клінічно здорових коней та коней хворих на ІНАН для Західного регіону України. **Мета.** Дослідити імунобіохімічні показники у здорових коней Західного регіону України, сезонну та вікову динаміку вмісту циркулюючих імунних комплексів (ЦК), вітаміну С та лізоциму, визначити можливий зв'язок

між підвищенням рівня ЦК і вмістом сироваткових фракцій білка та імунокомпетентних клітин у коней з прихованим перебігом ІНАН. **Методи.** Для вирішення цієї задачі досліджували концентрацію ЦК, вміст білка, білкові фракції (альбумінів, α -, β -, γ -глобулінів), вітаміну С та лізоциму, популяцію імунокомпетентних клітин (Т-, Тр-, Тч- і В-клітин). Досліджено 329 непородних коней обох статей різних вікових груп. З них 225 тварин були вільні від вірусу інфекційної анемії та 104 з прихованим перебігом хвороби. Використано загально-визнані серологічні та біохімічні методи, ідентифікацію коней з ІНАН проводили методом імунодифузії в агаровому гелі за допомогою реакції дифузної преципітації. **Результати.** З'ясовано вікові та сезонні аспекти змін імунобіохімічного статусу тварин. Визначено, що у здорових коней зі збільшенням в сироватці ЦК збільшується і кількість загального білка, альбумінів, α -глобулінів, γ -глобулінів, а також кількість О-клітин та субпопуляцій Тр- і Тч-лімфоцитів. Кількість β -глобулінів, а також кількість імунокомпетентних клітин Т- і В-лімфоцитів дещо зменшується. У тварин з прихованим перебігом ІНАН спостерігається вірогідне зменшення фракції альбумінів ($p < 0,05$), β -глобулінів ($p < 0,05$) і збільшення фракції γ -глобулінів ($p < 0,05$). У здорових коней 4-х річного віку і старших, весною відмічається суттєве збільшення рівня ЦК (30,4–90,6 умовних одиниць) порівняно з іншими сезонами року (33,4–63,6 ум.од.), що свідчить про накопичення в цей період в організмі коней комплексів антиген-антитіло. В цілому, аналіз показує, що зі збільшенням віку коней є чітка тенденція до збільшення рівня ЦК, вміст вітаміну С з віком зменшується, лише в групі коней 10-ти і більше років він незначно підвищений, лізоцимна активність з віком також зменшується. Вміст гетерогемаглотинінів у здорових коней з віком зростає з 6,25 до 12,02 ум.од., а у коней з прихованим перебігом ІНАН відмічається зниження з 10,6 ум.од. в трирічному віці, до 6,26 у віці 4–9 років, і незначний ріст до 8,07 у 10 річному віці і старше. Дослідження сезонного стану неспецифічних чинників захисту та гуморального імунітету вибіркової групи коней при ІНАН показало, що рівень ЦК у здорових та інфікованих коней змінюється динамічно в залежності від сезону року, у весняний сезон він підвищений, а в усі інші знижений. У інфікованих коней максимальний рівень ЦК був у весняний сезон (90,62 ум.од.), а мінімальний в літній сезон (52,73 ум.од.). Титр специфічних антитіл до вірусу ІНАН в залежності від сезону року суттєво не змінюється. **Висновки.** Визначено, що високий рівень ЦК в крові у коней з прихованим перебігом хвороби є показником суттєвих змін в їх імунобіологічному стані. Збільшення рівня ізоантитіл у коней з прихованим перебігом ІНАН в порівнянні зі здоровими вказує на розвиток аутоімунного процесу при ІНАН, що не виключає зростання та накопичення інших неспецифічних антитіл на тлі

яких відмічається підвищення загального рівня ЦІК при відносно стабільному рівні специфічних антитіл. Вперше одночасно досліджено показники специфічного імунітету за ІНАН (ЦІК) і неспецифічного (лізоцимна активність, вміст вітаміну С, білкових фракцій крові та ін.).

Ключові слова: коні, інфекційна анемія, гуморальний імунітет, лізоцимна активність, циркулюючі імунні комплекси.

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