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Immunobiological properties of inactivated anti-highly pathogenic avian influenza vaccines based on antigens of A/H5N1 avian influenza virus strains of different virulence

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SUMMARY
Antigen of H5N1 low pathogenic avian influenza virus Yamal strain included in the inactivated emulsion vaccine is able to induce strong immunity against highly pathogenic avian influenza in chickens. Inactivated emulsion vaccines based on antigen of H5N1 low pathogenic avian influenza virus Yamal strain and antigen of H5N1 highly pathogenic avian influenza virus A/chicken/Primorsky/85/08 strain are capable of inducing dose-dependent cross immunity against current H5N1 and H5N8 highly pathogenic avian influenza viruses. Thus, inoculation dose of H5N1 low pathogenic avian influenza virus Yamal strain antigen required for protection of 95% of chickens against H5N1 highly pathogenic avian influenza virus A/chicken/Primorsky/85/08 strain and against H5N8 highly pathogenic avian influenza virus A/duck/KChR/1590-20/20 in the vaccine inoculation volume shall be at least 609 HAU and 255 HAU, respectively. The inoculation dose of H5N1 highly pathogenic avian influenza virus A/chicken/Primorsky/85/08 strain antigen for protection from H5N8 highly pathogenic avian influenza virus A/duck/KChR/1590-20/20 strain shall be at least 294 HAU. Protective effect of the tested inactivated vaccines was associated with humoral immunity level in poultry. Predicted titre of antibodies to homologous virus antigens conferring expected 95% protection of vaccinated poultry was 1:538 or 9.1 $\log_2$. Inactivated vaccine based on H5N1 low pathogenic avian influenza virus Yamal strain antigen demonstrates its high immunogenicity in chickens infected with H5N1 and H5N8 highly pathogenic avian influenza influenza virus.

Keywords: inactivated vaccine, low pathogenic avian influenza (LPAI) virus antigen, immunity, protective antibody titre, H5 highly pathogenic avian influenza (HPAI)

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Иммунобиологические свойства инактивированных вакцин против высокопатогенного гриппа птиц, изготовленных на основе антигенов штаммов вируса гриппа подтипа A/H5N1 различной вирулентности

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РЕЗЮМЕ

Антиген вируса низкопатогенного гриппа птиц подтипа H5N1 штамма «Ямал» в составе инактивированной эмульгированной вакцины способен индуцировать выработку напряженного иммунитета у кур против высокопатогенного гриппа птиц. Инактивированные эмульсионные вакцины на основе антигена вируса низкопатогенного гриппа птиц подтипа H5N1 штамма «Ямал» и антигена вируса высокопатогенного гриппа птиц подтипа H5N1 штамма A/chicken/Primorsky/85/08 способны вызывать дозозависимый перекрестный иммунитет в отношении актуальных вирусов высокопатогенного гриппа птиц подтипов H5N1 и H5N8. Так, для защиты 95% вакцинированной птицы прививная доза антигена вируса низкопатогенного гриппа птиц подтипа H5N1 штамма «Ямал» против вируса высокопатогенного гриппа птиц подтипа H5N1 штамма A/chicken/Primorsky/85/08 в прививном объеме вакцины должна составлять не менее 609 ГАЕ и против вируса высокопатогенного гриппа птиц подтипа H5N8 штамма A/duck/KChR/1590-20/20 – 255 ГАЕ. Аналогичный показатель, установленный для антигена вируса высокопатогенного гриппа птиц подтипа H5N1 штамма A/chicken/Primorsky/85/08 против вируса высокопатогенного гриппа птиц подтипа H5N8 штамма A/duck/KChR/1590-20/20, должен составлять не менее 294 ГАЕ. Протективный эффект испытанных инактивированных вакцин связан с напряженностью гуморального иммунитета птицы. Прогнозируемый титр антител к гомологичным антигенам, при котором ожидаемая защита вакцинированной птицы достигнет 95%, составил величину 1:538, или 9,1 $\log_2$. Инактивированная вакцина против гриппа птиц H5 на основе антигена вируса низкопатогенного гриппа птиц подтипа H5N1 штамма «Ямал» обладает высокой иммуногенной активностью и защищает птиц при заражении вирусом высокопатогенного гриппа птиц подтипов H5N1 и H5N8.

Ключевые слова: инактивированная вакцина, антиген вируса низкопатогенного гриппа птиц, иммунитет, протективный титр антител, высокопатогенный грипп птиц подтипа H5

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INTRODUCTION

Highly pathogenic avian influenza (HPAI) is caused by RNA virus of H5 and H7 serotypes regardless of their pathogenicity belonging to *Alphainfluenzavirus* genus, *Orthomyxoviridae* family. The virus has fragmented genome and is capable of diverse genetic rearrangements that at a phenotype level result in changes in its antigenic and virulent properties. Low pathogenic avian influenza (LPAI) that is non-notifiable to the World Organization for Animal Health (WOAH) is caused by other virus subtypes [1].

Type A avian influenza virus is characterized with high variability especially in virion surface glycoproteins: HA and NA. Host antibodies against HA and NA are shown to confer the basis of humoral immunity thereat protective antibodies to HA play the leading role. There are 18 known HA subtypes (H1–H18) and 11 known NA subtypes (N1–N11). The avian influenza virus genome is segmented and consists of 8 segments having high reassortment ability [2, 3].

Various HA and NA combinations could result in emerging of highly virulent strains causing high mortality. HPAI caused by type A H5 avian influenza virus currently spreads across Eurasia territory. The said subtype is believed to pose the greatest pandemic threat [4].

H5 LPAI viruses demonstrate almost similar low pathogenicity under experimental conditions when they are tested based on intravenous pathogenicity index (IVPI), however, they often cause moderate to high morbidity and mortality [5].

Comparison of the recovered avian influenza virus isolates show their high genetic and antigenic variability. This forces to carry out continuous searching for new avian influenza virus isolates suitable for preparation of diagnostics and vaccines [5, 6].

Highly pathogenic avian influenza is described as acute and subacute disease causing 100% mortality of birds in a flock and having epidemic potential.

The significant economic damage caused by the disease necessitates the development of tools for specific disease prevention, and inactivated whole-virion vaccines have been so far the most effective ones [7].

Tests of inactivated vaccines based on both highly and low pathogenic viruses for their effectiveness show that their protective ability depends on two (complementary)

factors: antigenic matching between infecting virus and vaccine virus [8–10] and inoculation dose of the antigen in vaccine [10–13]. At the same time, the use of HPAI virus strains for inactivated vaccine production is not recommended by the WOAH due to their epidemic threat [1] and due to the low accumulation of viral antigen in the cultivation system [14].

Most scientists prove that effective immunization against avian influenza requires vaccines that are based on antigenically related viruses [7, 15, 16].

The data given in scientific publications on the effectiveness of vaccines against heterologous viruses (belonging to different genetic lines within the same subtype) are contradictory. Thus, the US researchers found with a variety of methods that inactivated vaccines based on H5 whole virus conferred 100% protection from death to the chickens infected with heterologous virulent H5N8 and H5N2 clade 2.3.4.4 viruses [15].

Other researchers have found based on the cross-hemagglutination test results, that vaccines based on highly virulent H5N1 viruses have different protective activity against the virulent virus [16]. Russian researchers pointed out that homologous subtype virus antigen concentration plays a crucial role in the vaccine effectiveness, and highly virulent H5 viruses are not always effective as vaccine strains due to their low accumulation in the cultivation system [14].

The WOAH lays down relevant requirements to immunogenicity of vaccine against avian influenza the main requirements of them are antigen concentration in inoculation dose and postvaccinal antibody titres. Thus, recommended antigen concentration in an inoculation dose should be 50 PD_{50} (50% protective dose) or 3μg of hemagglutinin [17]. Minimum hemagglutination inhibition antibody titre in chickens in the field should be 1:32 to protect the birds from death, and not less than 1:128 to provide reduction in virulent virus replication and shedding for antigenically related vaccines and virulent virus [1].

The study was aimed at examination of vaccine against avian influenza based on various antigens against currently circulating highly virulent H5 avian influenza virus variants for their immunogenic properties.

MATERIALS AND METHODS

Highly virulent avian influenza virus strains: A/chicken/Primorsky/85/08 of H5N1 subtype, clade 2.3.2 (in the paper – H5N1 HPAIV Primorsky strain); A/duck/KChR/1590-20/20 of H5N8 subtype, clade 2.3.4.4 (in the paper – H5N8 HPAIV KChR strain).

Low virulent avian influenza virus strains: production Yamal strain of H5N1 subtype (in the paper – H5N1 LPAIV Yamal strain).

Virus-containing materials. Samples of the virus-containing extraembryonic fluid cultivated in SPF chicken embryonated eggs and kept at minus 70 °C were used. The extraembryonic fluid was clarified by centrifugation at 1,000 g before use in the experiments.

Chicken embryonated eggs. Nine – eleven day-old SPF chicken embryonated eggs (VALO BioMedia GmbH, Germany) were used.

Virus infectious titre determination. Method of serial multiple dilutions of the virus-containing material was used. SPF chicken embryonated eggs were used as a susceptible test objects. Infectious material was injected in allantoic cavity. Infectious titre was determined using Karber method and expressed as EID_{50}.

Virus antigens. Clarified H5N1 HPAIV Primorsky strain and H5N1 LPAIV Yamal strain-containing materials cultivated in chicken embryonated eggs inactivated with aminoethylethanolamine (0.25%) at 22 °C for 24 hours were used for preparations of the antigens. The virus was tested for its inactivation completeness by two serial passages in SPF chicken embryonated eggs. Antigen titre was determined with hemagglutination test (HA test) and expressed in hemagglutinating units (HAU).

Vaccines. Emulsion vaccines were prepared based on the viral antigens and oil adjuvant. Each antigen was prepared in several dilutions. Montanide ISA 70 (SEPPIC, France) with antigen-containing phase / oil ratio of 30:70 (w/w) was used as an oil adjuvant. During vaccine preparation the active component (antigen) was combined with oil adjuvant and emulsified in Silverson laboratory high-speed mixer (Great Britain) at 6,000 rpm for 5 minutes. As a result, specimens of emulsion vaccine with a given antigen concentration were prepared.

Poultry. Egg-type Lohmann Brown chicks at the age of 3–4 weeks seronegative to AI virus from the holding free from acute infectious diseases were used for the experiment.

All experiments in animals were carried out in strict compliance with the international standard on laboratory animal keeping and handling, GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification as well as according to the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Immunization of chickens. Each vaccine specimen (with specified antigen concentration) was injected intramuscularly to chickens of a separate group: 0.5 cm^3 per chicken in thigh. Besides, control groups of non-vaccinated chickens were formed.

Test for postvaccinal humoral immunity level. Blood samples were taken from immunized and control chickens 28 days after vaccination to prepare sera. Anti-AIV antibody titres were determined with hemagglutination inhibition test in the said sera.

Infection of chickens. Vaccinated and control chickens were infected with HPAI virus at a dose of at least 6.0 lg in volume of 0.5 cm^3 in thigh.

Assessment of the vaccine protective effect. Chickens of all groups were infected 28 days after vaccination and observed to record their clinical state and the virus lethal effect.

Hemagglutination test. Standard hemagglutination test was used. Hemagglutination titre in the tested materials were determined.

Hemagglutination inhibition test. Standard hemagglutination inhibition test was used. The test was carried out in accordance with the Instruction on detection of antibodies against H5 avian influenza virus with hemagglutination inhibition test (FGBI "ARRIAH", Russia). Titre of anti-AIV antibodies blocking hemagglutinating effect was determined in tested sera.

Solutions. The virus-containing materials and antigens are diluted with 0.9% sodium chloride solution (saline

solution). Solutions indicated in the relevant instructions are used for hemagglitunation test and hemagglutination inhibition test.

Data processing. Standard methods of statistical processing of selected varying variables [18], as well as correlation and regression analysis [19] elements were used. Computations and graph plotting were carried out with Microsoft Excel.

Test design. Two inactivated emulsion vaccines were tested for their immunobiological properties:

– vaccine (LP) based on H5N1 LPAIV Yamal strain antigen. Virus material infectivity before inactivation was 9.6 lg EID_{50}/cm^3. Inactivated material contained 10 log_2 HAU/0.1 cm^3 (10,240 HAU/cm^3). Specimens with the following relative antigen concentrations (D) were tested: 1, 1/25, 1/50 and 1/100;

– vaccine (HP) based on H5N1 HPAIV Primorsky strain antigen. Virus material infectivity before inactivation was 9.2 lg EID_{50}/cm^3. Inactivated material contained 9 log_2 HAU/0.1 cm^3 (5,120 HAU/cm^3). The specimens were prepared similar to that ones of LP vaccine and tested.

Two experiments (I, II) were carried out during which different combinations of the vaccines and challenge viruses were tested.

Experiment I. Four groups of chickens (20 chickens per group) were immunized with LP vaccine specimens with the specified antigen concentration. A group of 10 chickens was remained non-vaccinated and served as control. Blood samples were taken from all chickens 28 days after vaccination and then the chickens were challenged with H5N1 HPAIV Primorsky strain at dose of 6.0 lg EID_{50} (challenge virus). The chickens were observed for clinical signs for 10 days after challenge. Avian influenza clinical signs and deaths of diseased chickens were recorded.

Experiment II. Four groups of chickens (9 chickens per group) were immunized with LP vaccine specimens. A group of 10 chickens was remained non-vaccinated and served as control. Blood samples were taken from all chickens 28 days after vaccination and then the chickens were challenged with H5N8 HPAIV KChR strain at dose of 6.7 lg EID_{50} (challenge virus). The following steps were similar to that ones described for Experiment I.

Four groups of chickens (10 chickens per group) were immunized with HP vaccine specimens with the specified antigen concentrations. A group of 10 chickens was remained non-vaccinated and served as control. Blood samples were taken from all chickens 28 days after vaccination and then the chickens were challenged with H5N8 HPAIV KChR strain at dose of 6.7 lg EID_{50} (challenge virus). The following steps were similar to that ones described for Experiment I.

Immunizing (inoculation) dose of antigen. Antigen inoculation dose (A) was calculated in HAUs based on proportion of the antigen (1/3) in vaccine inoculation volume (0.5 cm^3), its content in initial material volume (A_0, HAU/0.1 cm^3) and specified relative concentration (D). The following formula was used for calculations: $A = (0.5/3) \times (A_0/D)$, HAU/0.5 cm^3.

Protective index. Protective indices were determined after challenge of test and control groups of chickens as follows: C = pr/n, where C – protective index; pr – number of protected (survived) chickens; n – total number of chickens in the group.

Table 1
Results of challenge test of poultry immunized with the vaccines based on H5N1 LPAI virus Yamal strain antigen and based on H5N1 HPAI virus Primorsky strain antigen using H5N1 and H5N8 HPAI virus

Protective indices and their linear equivalents determined for tested inoculation doses of immunizing antigen in vaccine specimen				
Challenge strain	Immunizing antigen	Antigen dilution (D)	Protective index (C = pr/n)*	Protective index equivalent f = log (C/(1 – C)
H5N1 HPAIV Primorsky strain	H5N1 LPAIV Yamal strain	1	20/20 ('C_1 = 0.99)#	1.996
		1/25	6/20 (C = 0.3)	-0.368
		1/50	5/20 (C = 0.25)	-0.477
	Control	–	0/10 (C = 0)	n/d
H5N8 HPAIV KChR strain	H5N1 LPAIV Yamal strain	1	9/9 (C = 0.98)	1.690
		1/25	9/9 (C = 0.98)	1.690
		1/50	6/9 (C = 0.67)	0.308
		1/100	5/9 (C = 0.56)	0.105
	Control	–	0/10 (C = 0)	n/d
H5N8 HPAIV KChR strain	H5N1 HPAIV Primorsky strain	1	10/10 ('C = 0.98)#	1.690
		1/25	8/10 (C = 0.80)	0.602
		1/50	4/10 (C = 0.40)	-0.176
		1/100	2/10 (C = 0.20)	-0.602
	Control	–	0/10 (C = 0)	n/d

* C = pr/n, where pr – number of protected (survived) poultry; n – total number of poultry in group;
# estimate: 'C_1 = (1 – 1/5n); n/d - non detected.

Conversion of protective indices. For given "dose – effect" system, the 50% protective effect (C = 0.5) was assumed to be the point of symmetry of protective indices distribution based on the antigen dose. On that ground, Berkson logit transformation was applied to approximate the depended variables to linear equivalents [20, p. 267], which allows us to obtain the following linear equivalents of the protective indices: f = log (C/(1 – C). For C = 1 set for the lowest tested dose of antigen and C = 0 set for the highest dose of antigen, the following estimates were used: 'C_1 = (1 – 1/5n) and 'C_0 = 1/5n [21, p. 246]. The inverse conversion of equivalents was performed according to the formula:

C = 1 – 1/(1 + (antilg f)).

RESULTS AND DISCUSSION

Primary data. The results, protectivity indices and relevant linear equivalents, obtained during the experiments are consolidated in Table 1.

Analysis of dependence of chicken immune protection on antigen dilution. Relationship between logarithmic values of tested antigen dilutions (lgD) included in the vaccines and protective index equivalents (f) was examined. Regression analysis resulting in constructing the model allowing prediction of "f value for the specified lgD was carried out. Calculations and graph plotting were performed with Excel.

Regression equations given in Figure 1 were used for calculation of the antigen concentrations (D_0 dilutions), that confer protection to 50% of vaccinated poultry under the said test conditions (lgD_{50} = B/k) and 95% (lgD_{95} = (B – f_{95})/-k). The relevant doses of HAU in the vaccine inoculation volume (A_{50} and A_{95}) were determined for specified D_{50} and D_{95} values. Results of the calculations are given in Table 2.

Analysis of dependence of protective index equivalents on postvaccinal humoral immunity levels. Logarithmic antibody titre (log_2 T) determined based on f-value in

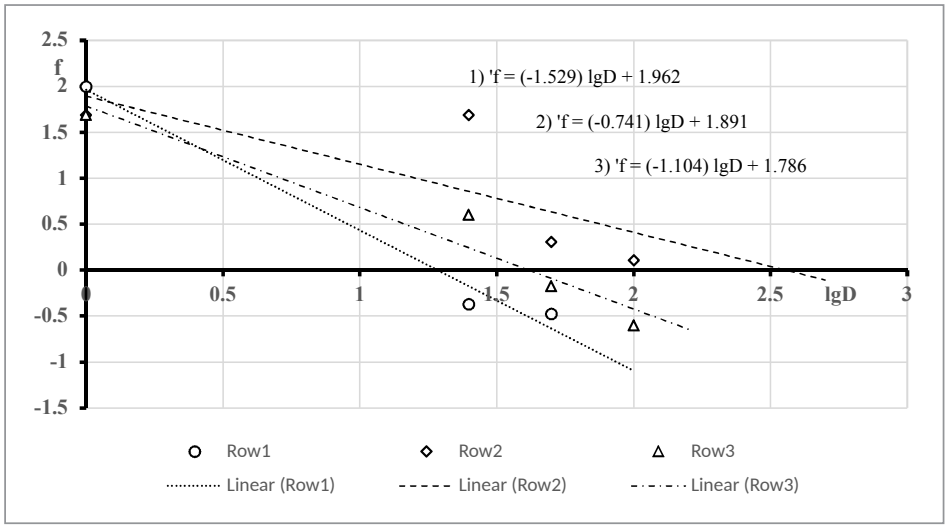

Fig. 1. Relationship between tested antigen dilutions (lgD) included in the vaccines and protection index equivalents (f) of immunized poultry determined after challenge
The ordinate axis is crossed at the point f = 0, corresponding to C = 0.5, i.e., 50% protection. The positions of experimental f estimates by lgD are shown. Regression equations of the form 'f = k (lgD) – B are given, where 'f is the expected equivalent of the protection index for given lgD; k and B are regression coefficients. The following combinations are presented:
1) H5N1 LPAI virus Yamal strain antigen × H5N1 HPAI virus Primorsky strain;
2) H5N1 LPAI virus Yamal strain antigen × H5N8 HPAI virus KChR strain;
3) H5N1 HPAI virus Primorsky strain antigen × H5N8 HPAI KChR strain.

Table 2
Antigen concentration conferring protection to 50% (D_{50}) and 95% (D_{95}) of vaccinated poultry and corresponding numbers of HAU (A_{50} and A_{95}), determined under given testing conditions: immunizing antigen × challenge virus strain

Immunizing antigen × challenge virus strain	lgD_{50}*	D_{50}	A_{50}**	lgD_{95}#	D_{95}	A_{95}
H5N1 LPAIV Yamal strain × H5N1 HPAIV Primorsky strain	1.283	D_0/19.2	89	0.447	D_0/2.8	609
H5N1 LPAIV Yamal strain × H5N8 HPAIV KChR strain	2.555	D_0/359	5	0.828	D_0/6.7	255
H5N1 HPAIV Primorsky strain × H5N8 HPAIV KChR strain	1.617	D_0/41.4	21	0.459	D_0/2.9	294

The antigen concentration was calculated using the following equation: 'f = k (lgD) – B,
where 'f – expected equivalent of protection index for given lgD; k and B – regression coefficients.
* logarithm of undiluted antigen dilution for f_{50} = 0 (lgD_{50} = B/-k);
** A = (0.5/3) × (A_0/D), where A – number of HAU in the vaccine inoculation volume for given antigen dilution (D) and HAU number in undiluted antigen (A_0);
# logarithm of undiluted antigen dilution for f_{95} = 1.279 (lgD_{95} = (B – 1.279)/-k).

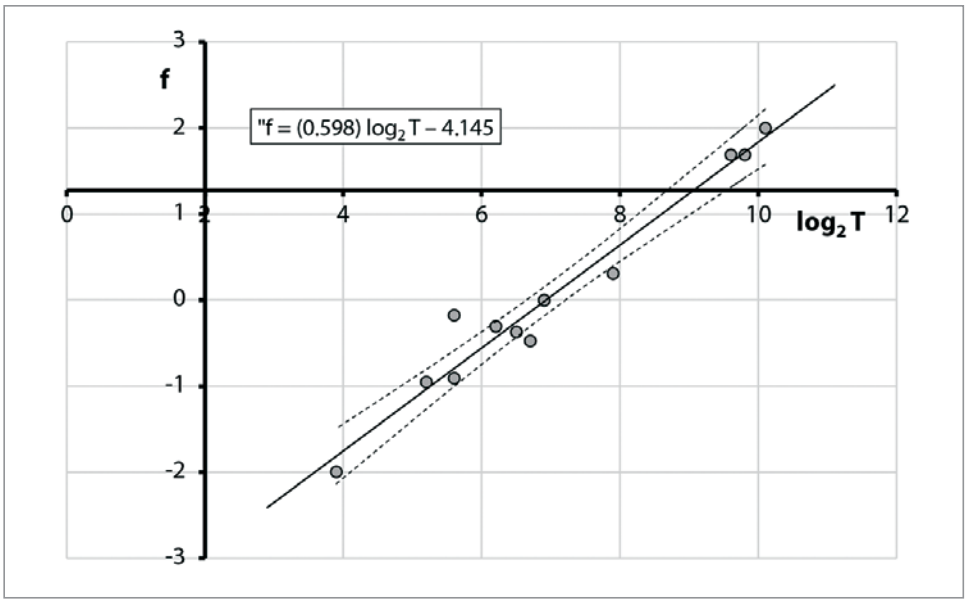

Fig. 2. Relationship between logarithmic mean antibody titre (log_2 T) and equivalent of protective index (f) in poultry immunized with vaccine based on H5N1 LPAI Yamal strain antigen and H5N1 HPAI Primorsky strain antigen
The ordinate axis is crossed at the point f = 1.279, corresponding to C = 0.95, i.e., 95% protection. Regression equation of the form "f = (0.598) log_2 T – 4.145 is given, where "f – expected equivalent for given log_2 T. The dashed line shows the boundaries of the confidence interval (p = 0.05) of the regression

the group of vaccinated chickens 28 days after their immunization with the vaccines containing different antigen dilutions was considered to be postvaccinal humoral immunity level. Values determined under the said conditions are given in Table 3.

Table 3
Hemagglutination inhibition tests of sera collected from poultry immunized with the vaccines based on H5N1 LPAI virus Yamal strain antigen and H5N1 HPAI virus Primorsky strain antigen against homologous virus antigens

Mean antibody titres (log_2 T, $n \geq 3$) against tested antigens and based on protective index equivalents (f) determined in chickens after their challenge		
Antigen	log_2 T	f
H5N1 LPAIV Yamal strain	10.1 ± 0.4	1.996
	6.9 ± 0.4	-0.368
	6.7 ± 0.5	-0.477
	3.9 ± 0.6	-1.996
	9.6 ± 0.5	1.690
	7.9 ± 0.6	0.308
	6.2 ± 0.4	-0.308
	5.6 ± 0.4	-0.908
H5N1 HPAIV Primorsky strain	9.8 ± 0.4	1.690
	6.5 ± 0.5	0.000
	5.6 ± 0.5	-0.176
	5.2 ± 0.5	-0.954

Relationship between humoral immunity responses (log_2 T) and protective index equivalents (f) were examined. Regression method was used for construction of relationship model for the "log_2 T – f" system. The results are given in Figure 2.

The following equation

$$"f = (0.598) \log_2 T - 4.145$$

allowed us to determine predicted antibody titre at which the expected level of vaccinated poultry protection will reach 95% (f_{95} = 1.279). The said titre was log_2 "T_{95} = (4.145 + 1.279)/0.598 = 9.07, or "T = 538. Confidence interval limits (p = 0.05) for the said estimation were as follows: 8.67 ≤ log_2 "T_{95} ≤ 9.60, or 407 ≤ "T_{95} ≤ 776.

CONCLUSION

H5N1 Yamal strain antigen of low pathogenic avian influenza virus included in the inactivated emulsion vaccine is able to induce strong immunity in chickens against highly pathogenic avian influenza. The following conclusions were made based on the experiment results:

– inactivated vaccine based on H5N1 LPAIV Yamal strain antigen is capable of inducing cross immunity against H5N1 HPAIV Primorsky strain. During the experiment, the vaccine specimen was able to confer 100% protection to the vaccinated poultry. The antigen included in the vaccine specimen contained 19.2 D_{50} against heterologous virus. Predicted dose of the antigen in the vaccine inoculation volume should be at least 609 HAU for 95% protection of immunized poultry;

– inactivated vaccine based on H5N1 LPAIV Yamal strain antigen is capable of inducing cross immunity against H5N8 HPAIV KChR strain. During the experiment, the vaccine specimen was able to confer 100% protection to the vaccinated poultry. The antigen included in the vaccine specimen contained 359 D_{50} against heterologous

virus. Predicted dose of the antigen in the vaccine inoculation volume should be at least 255 HAU for 95% protection of immunized poultry;

– inactivated vaccine based on H5N1 HPAIV Primorsky strain antigen is capable of inducing cross immunity against H5N8 HPAIV KChR strain. During the experiment, the vaccine specimen was able to confer 100% protection to the vaccinated poultry. The antigen included in the vaccine specimen contained 41.4 D_{50} against heterologous virus. Predicted dose of the antigen in the vaccine inoculation volume should be at least 294 HAU for 95% protection of immunized poultry;

– protective effect of tested inactivated vaccines is associated with humoral immunity level in the poultry. Predicted antibody titre at which expected protection of vaccinated poultry would reach 95% was "T_{95} = 538. Confidence interval range (p = 0.05) of the said estimate was as follows 407 ≤ "T_{95} ≤ 776. "T_{95} value could be useful for indirect assessment of postvaccinal immunity level.

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