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COLLECTION OF MICROVOLUME BLOOD SAMPLES INTO GLASS CAPILLARIES FOR THE DETECTION OF ANTIBODY AGAINST AUJESZKY'S DISEASE VIRUS IN PIGS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND SOLID-PHASE RADIOIMMUNOASSAY (RIA)

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Abstract

Rodák L., B. Šmíd, L. Valíček, E. Jurák: *Collection of Microvolume Blood Samples into Glass Capillaries for the Detection of Antibody Against Aujeszky's Disease Virus in Pigs by Enzyme-Linked Immunosorbent Assay (ELISA) and Solid-Phase Radioimmunoassay (RIA).* Acta vet. Brno, 54, 1985: 207—216.

A description is given of a technique of microvolume blood sample collection into heparinized glass capillaries from which the samples are immediately transferred to diluting solution to obtain a basic 100-fold dilution. The samples are then ready for serological examination by ELISA and RIA without further processing at the laboratory, a distinct advantage over present methods of blood collection for serological examination. Moreover, the blood sampling itself is rapid and easy to perform with a minimum trauma to the animals and the samples can be stored at room temperature for as long as 78 days without substantial changes in their antiviral antibody titres.

The technique was tested and verified in pigs by comparing the sensitivity of the detection of antibody against Aujeszky's disease virus by ELISA, RIA and the virus neutralization (VN) test. The antiviral antibody titres demonstrated by ELISA and VN showed a high degree of correlation ($r = 0.881$, $n = 54$), which implies a highly significant ($P < 0.01$) agreement between the two methods.

The examination by ELISA was made using two peroxidase conjugates: (a) conjugate with specific antibody against swine IgG purified by affinity chromatography (HRP-RIgaSwIgG) and (b) conjugate with staphylococcal protein A (HRP-protein A). The antibody titres by ELISA using HRP-RIgaSwIgG and HRP-protein A were 51.9-fold and 10-fold higher, respectively, than those obtained by the VN test.

Further application of the technique for the detection of antibody by ELISA and RIA in veterinary and human medicine is discussed.

Aujeszky's disease, enzyme-linked immunosorbent assay (ELISA), solid-phase radioimmunoassay (RIA), antiviral antibodies, collection, microvolume blood samples.

Up to now, the routine practice of obtaining blood from animals for serological examination consists of venipuncture and subsequent centrifugation of clotted blood at the laboratory to separate blood serum which is then used for examination by various methods - agglutination, precipitation, haemagglutination, virus neutralization, etc.

An example of this is the procedure for serological diagnosis of Aujeszky's disease (AD) in pigs. In Czechoslovakia, about 280 000 blood serum samples are examined each year for antibodies against AD virus by virus neutralization (VN) test. Blood samples are drawn, for the most part, from the vena cava cranialis and, occasionally, from the ear vein. Animals blood-sampled from the vena cava cranialis are traumatized, which has adverse effects on rate of gain and, occasionally leads even to death. Moreover, the handling and restraining of animals under field conditions

presents certain difficulties and requires the assistance of a number of people. Therefore any modifications which make blood collections easier and more rapid to perform and produce less trauma to the animals would be of economic value. For serological diagnosis of AD by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (Briaire et al. 1979; Kelling et al. 1978; Moennig et al. 1981; Moutou et al. 1978; Rodák et al. 1981) microlitre volumes of blood serum will do because 0.2 to 0.4 ml of serum diluted 20- to 10-fold is sufficient for both screening and titration. The objective of the present study was to develop a simple method for collection of small blood volumes that could be stored at 20 °C for as long as one week before examination to obviate possible problems of blood sample transport and storage.

Two techniques of collecting blood serum and blood samples were tested, one using filter paper strips (Hamblin and Hedger 1982) and the other using heparinized glass capillaries. The samples thus obtained were examined for antibodies against AD virus by ELISA and RIA and the results were compared with those obtained by the VN test as well as with the results obtained with the three techniques on examination of fresh-prepared samples.

Materials and Methods

Experimental animals

Blood and blood serum samples from pigs, males and females, of different ages were examined. Samples in which virus-neutralizing antibodies against AD virus were demonstrated at titres of 1:2 to 1:4096 came from vaccinated or experimentally infected animals. Negative samples came from pigs of an AD-free herd.

Blood Sample Collection for VN Test

Blood samples were obtained from the ear vein or the vena cava cranialis and the blood sera were stored at —20 °C until use.

Blood Sample Collection on to Filter Paper

Ten μl of blood serum was applied on to one end of Whatman No. 1 paper strips, 70 × 9 mm' air-dried and then allowed to remain at 20° C for 7 days. For RIA, the serum-bearing parts of the strips (12 × 9 mm) were cut off with scissors and placed in test tubes containing phosphate buffered saline (PBS) (pH 7.2) plus 0.3 % Tween 20 (PBST) supplemented with 0.5 % bovine serum albumin (BSA) (SEVAC, Prague). After 1-hour elution, the solutions corresponding to serum dilutions of 1:100 were used directly for RIA.

Blood Sample Collection into Capillaries

Heparinized glass capillaries for haematological use were cut into capillaries of 25 mm in length. (In most cases we used capillaries produced by Kavalier Glass Works, Czechoslovakia, but capillaries obtained from other producers were used with equal results.) Five- to 10-ml test tubes were filled with 1-ml volumes of diluting and preserving solution of the following composition: NaCl, 8.5 g; KH_2PO_4 0.2 g; $Na_2HPO_4 \cdot 12 H_2O$, 2.9 g; Tween 20, 3.0 ml; Na_2 EDTA, 0.5 g; NaN_3, 0.2 g; BSA, 5.0 g; distilled water to make 1000 ml. The ear of the pig to be blood-sampled was disinfected with 70 % alcohol and the ear vein was punctured with a sterile injection needle. The first drop of blood was wiped off with cellulose cotton. To the second drop of blood, a glass capillary was applied upright. The blood in the capillary creeps up to 12.5 mm, a level corresponding approximately to a volume of 10 μl. (Capillaries produced by individual manufacturers have first to be tested.) Larger blood volumes are obtained by applying the capillary repeatedly to the same drop of blood or by sloping the drop to a slanting or horizontal position. The capillaries were then thrown into test tubes containing the diluting solution, and the blood was gently washed out. The tubes were labelled, stoppered and stored at 20 °C. The diluting solution prevented microbial growth and blood clotting, and haemolysed erythrocytes. A sample diluted thus to circa 1:100 could be used directly without further processing for ELISA or RIA.

Preparation and Purification of Antibodies and Protein A by Affinity Chromatography

Rabbit hyperimmune serum against swine IgG (RaSwIgG) was prepared by repeated immunization of rabbits with electrophoretically pure swine IgG in Freund's adjuvant. Specific antibody against swine immunoglobulin (RIgaSwIgG) was then isolated from the hyperimmune serum

by affinity chromatography on the column of the immunosorbent with covalently bound SwIgG. Activation of Sepharose 4B (Pharmacia, Uppsala, Sweden) and the binding of SwIgG (10 mg/ml gel) were carried out according to standard procedures (March et al. 1974). The same immunosorbent was used for isolation and purification of protein A from a 16-hour of *Staphylococcus aureus* strain Cowan 1 grown on nutrient agar supplemented with 0.2 % $CaCl_2$ (Hjelm et al. 1972; Lind 1974).

Peroxidase Conjugates

Staphylococcal protein A and rabbit antibody (RIgaSwIgG) purified by affinity chromatography were conjugated with horse-radish peroxidase (HRP) (Boehringer, Mannheim, grade I, RZ 3.0) by the natrium periodate method (Boorsma and Streefkerk 1979; Wilson and Nakane 1978). In brief, 10 mg RIgaSwIgG was conjugated with 5 mg HRP, and 10 mg protein A was conjugated with 10 mg HRP. Stock solutions of the conjugates containing 2 mg/ml of Ig and protein A were supplemented with 1 % rabbit serum albumin (RSA) and BSA, respectively, and stored either in sealed ampoules at —70 °C or at 4 °C after being mixed in equal volumes with a preservative solution consisting of 50 ml glycerol, 50 ml PBS and 0.02 g NaN_3. Before use, the two conjugates were diluted 1000-fold with PBST + 0.5 % RSA or BSA.

Viral and Control Antigen for ELISA and RIA

Primary pig kidney cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10 % (v/v) inactivated fetal bovine serum in Roux bottles. The monolayer was washed with MEM and the cells were infected with a virulent strain of AD virus and then transferred to Eagle's serum-free maintenance medium. At the peak of cytopathic effect, the cells and medium were homogenized by three freeze-thaw cycles. The virus titre was $1 \cdot 10^7$ plaque-forming units per ml. The same procedure was used to prepare homogenates of non-infected cells. All homogenates were centrifuged at 2000 g for 20 minutes to remove cellular debris and concentrated to 1/10 of the original volume by ultrafiltration (Amicon, Diaflo Ultrafilter PM 10). The homogenates of infected and non-infected cultures, hereafter referred to as viral (V) and control (C) antigens, were diluted 100- to 200-fold with 0.1 M carbonate-bicarbonate buffer, pH 9.6, prior to use.

Virus-Neutralization Test

Antibodies against AD virus in inactivated sera were determined by the VN test using rabbit kidney cell line RK 13. Serial twofold dilutions of the sera were mixed in equal volumes with AD virus suspension of 100 $TCID_{50}$. The resultant mixtures were shaken, incubated at 37 °C for 60 minutes, and 0.2 ml amounts of each serum dilution were inoculated into five test tubes. The tests were evaluated according to cytopathic effect observed after 5-day incubation at 37 °C. The VN titres were expressed as the reciprocal of the highest serum dilution that neutralized 100 $TCID_{50}$ of virus in at least 3 out of the 5 tubes.

RIA Procedure

The RIA procedure, antibody labelling with ^{125}I and evaluation of results were described in detail previously. In brief, polystyrene microtubes coated with V or C antigen were washed with PBS and then filled with 100 l volumes of pig serum to be examined. After incubation for 1 hour at 37° C or for 2 hours at 20° C in a humidified chamber they were incubated with 100 μl volumes of ^{125}I-RIgaSwIgG solution having an activity of 5 KBq/ml under the same conditions as described above. Between the incubations and at the end of the procedure the microtubes were washed three times with PBS. After the radioactivity bound in individual microtubes was measured (NE 1600, Nuclear Enterprises, Ltd., Edinburgh, U. K.), the radioactivities of microtubes coated with V or C antigen and incubated with the same dilution of serum were compared.

ELISA

The ELISA was performed in flat-bottom polystyrene microtitre plates (Koh-I-Noor, Czechoslovakia). Vertical rows of wells were coated alternately with 100 μl volumes of V or C antigen by incubation overnight at 20 °C. The next day the plates were washed three times with PBS. The samples were diluted threefold in the range of 1:100 to 1:218 700. Each sample dilution was added in 100 μl volumes to each of duplicate wells coated with V or C antigen. After 1-hour incubation at 37 °C in a humidified chamber, the plates were washed three times with PBS and incubated for an additional 60 minutes at 37 °C with 100 μl volumes of HRP-conjugates (HRP-RIgaSwIgG and HRP-protein A) diluted 1000-fold with PBST supplemented with 0.5 % RSA or BSA.

Table 1.

Comparison by solid-phase radioimmunoassay (RIA) of the effects of storage for 7 days at 20 °C on the antiviral antibody activity of blood serum samples collected into capillaries and on to filter paper strips.

The results are presented as averages of radioactivity measurements (counts per minute ± S. D.) in triplicate wells containing viral (V) or control (C) antigen. The V : C ratios at serum dilution of 1 : 100 are compared with the results obtained with fresh diluted sera by RIA and the virus neutralization (VN) test.

Serum sample No.	Freshly diluted sera			Capillaries RIA after 7 days at 20 °C			Filter paper strips RIA after 7 days at 20 °C			VN titre
	Average activity cpm V—Ag±SD	Average activity cpm C—Ag±SD	V : C ratio	Average activity cpm V—Ag±SD	Average activity cpm C—Ag±SD	V : C ratio	Average activity cpm V—Ag±SD	Average activity cpm C—Ag±SD	V : C ratio	
1	1364±189	694±192	1.96	1176±45	683±30	1.72	1019±158	885±134	1.15	1 : 4
2	1453±187	563±70	2.58	1209±124	523±12	2.31	1619±492	832±163	1.94	1 : 64
3	541±94	628±207	0.86	628±147	628±147	1.03	963±101	811±200	1.19	neg
4	623±147	646±143	0.96	459±60	482±69	0.95	662±236	626±159	1.06	neg
5	1818±348	571±23	3.18	1780±144	623±108	2.86	1677±316	953±85	1.76	1 : 1024
6	3116±460	708±70	4.40	2906±377	618±68	4.70	3043±402	1381±232	2.20	1 : 4096
7	2291±293	702±121	3.26	2886±257	1182±200	2.44	1754±317	906±188	1.93	1 : 1024
8	2165±311	631±100	3.42	2772±222	627±147	4.42	3126±116	1189±354	2.63	1 : 4096
9	2547±196	539±88	4.72	2203±541	489±28	4.51	2327±86	960±274	2.42	1 : 1024
10	2050±123	530±28	3.87	1756±52	507±31	3.46	1754±404	729±102	2.14	1 : 512
11	1150±123	702±43	1.64	1146±166	684±166	1.67	875±81	769±87	1.14	1 : 2
without serum	173±15	175±12	0.99	157±11	156±14	1.00	161±9	165±24	0.98	—

After washing the plates, a 100 μl amount of substrate was added to each well. (Preparation of the substrate: 8 mg of 5-aminosalicylic acid were dissolved in 10 ml H_2O, the pH was adjusted to 5.0 with 0.1 M NaOH and 1 ml of 0.05 % H_2O_2 was added.) After one hour, the reaction was read at 492 nm in a Titertek Multiskan (Flow Laboratories) and the values for the wells coated with V or C antigen and incubated with the same dilution of serum were compared. Sera were considered positive, i.e. containing antiviral antibody, if the V:C ratio was greater than 2.0. The V:C ratio obtained after incubation without serum or with negative serum was 1.0 $\pm$ 0.2.

Results

Comparison of Blood Serum Samples Collected on to Filter Paper and into Capillaries

Blood serum samples of known VN titres were pipetted on to filter paper and the same volumes of the same samples were collected into heparinized capillaries and transferred immediately to 1 ml volumes of the diluting solution. After storage of both sample types for 7 days at 20 °C, blood serum spots on filter paper were each eluted into 1 ml of PBST + 0.5 % BSA. The effect of storage on the stability of the antiviral antibody was assessed by RIA, using freshly prepared solutions of the test sera in PBST + 0.5 % BSA as controls. Triplicate microtubes coated with V or C antigen were incubated with 100-fold diluted test samples. From the values obtained for each serum, the average activity, standard deviation and V : C ratio of triplicate wells coated with V or C antigen were calculated. The results show that air-drying of the serum samples and their storage for 7 days at 20 °C had an adverse effect on the sensitivity of the detection of antiviral antibodies (Table 1). The decrease in the sensitivity of antibody detection was due mainly to increased non-specific binding activity, in consequence of which the V : C ratio which is essential for evaluation of the results was adversely affected. On the other hand, blood samples collection into capillaries and immediate transfer of the samples to diluting solution proved very useful. The V : C ratios found in samples collected into microcapillaries and stored for 7 days at 20 °C did not differ substantially from the results obtained on examination of freshly diluted sera.

Table 2.
Antiviral antibody titres by ELISA of blood samples collected into capillaries and stored at 20 °C for 7 and 78 days.

Blood sample No.	ELISA titres	
	7 days at 20 °C	78 days at 20 °C
1	1 : 2700	1 : 2700
2	1 : 8100	1 : 8100
3	1 : 8100	1 : 8100
4	1 : 8100	1 : 2700
5	1 : 2700	1 : 2700
6	1 : 8100	1 : 2700
7	1 : 24300	1 : 24300
8	1 : 8100	1 : 8100
9	1 : 8100	1 : 24300
10	1 : 8100	1 : 8100
11	1 : 8100	1 : 24300
12	1 : 2700	1 : 900
13	1 : 2700	1 : 2700
14	1 : 8100	1 : 2700
15	1 : 8100	1 : 8100
16	1 : 2700	1 : 8100
17	1 : 2700	1 : 8100
18	1 : 8100	1 : 2700
Average titre	1 : 7200	1 : 8200

Prolonged Storage of Samples Collected into Capillaries

Blood samples from 18 sows vaccinated with inactivated vaccine against AD (Bioveta Ivanovice) were collected into capillaries and immediately transferred to the diluting solution and then stored at room temperature. Seven and 78 days after collection they were titrated for antibodies against AD by ELISA using HRP-RIgaSwIgG conjugate. The highest sample dilution giving a V:C ratio $>$ 2.0 was considered positive. As can be seen from Table 2, no substantial difference was found in antiviral antibody titres between the samples examined 7 and 78 days after collection: the differences in titre were by one dilution at the most and the average antibody titres at 7 and 78 days were 1:7200 and 1:8300, respectively.

Comparison of the Sensitivity of Antiviral Antibody Detection by ELISA and VN

Sixty blood samples collected into capillaries and immediately transferred to the diluting solution were examined by ELISA using two peroxidase conjugates: HRP-RIgaSwIgG and HRP-protein A. Antibody titres obtained with the two conjugates were compared with the results of VN tests performed on sera obtained by simulta-

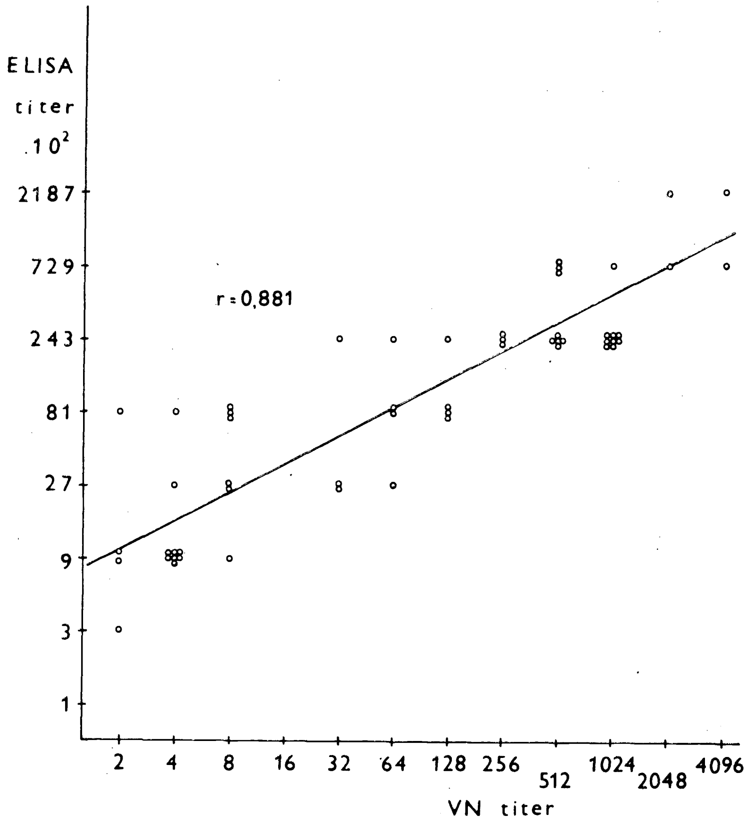

Fig. 1
Comparison of antibody titres against Aujeszky's disease virus as determined in the blood by enzyme-linked immunosorbent assay (ELISA) and in the blood serum of pigs by the virus neutralization (VN) test.

neous collection of larger blood volumes. All 54 samples positive by VN were also positive by ELISA and the remaining 6 samples were negative by both methods. There were only differences in titre. The average VN antibody titre was 1:506, while the average antibody titre obtained by ELISA with HRP-RIgaSwIgC conjugate was 1:26 272, i. e. 51.9 times higher. The degree of correlation between the VN and ELISA titres was determined by comparing the natural logarithms of these titres. The correlation coefficient ($r = 0.881$, $n = 54$) implies a highly significant ($P < 0.01$) agreement between the results obtained by the two methods (Fig. 1).

Similar results were obtained by ELISA using HRP-protein A conjugate although the sensitivity of detection in this case was lower than with the previous conjugate. The average ELISA titre of 1:5618 was only 10 times higher than the average VN titre and 5 times lower than the average ELISA titre obtained with HRP-RIgaSwIgG conjugate.

Discussion

The technique of blood sample collection for ELISA and RIA into capillaries described in the present study has been previously verified by examination of several thousands of samples. It has a number of advantages over present methods of blood sampling for serological examination. It incurs less time and effort (a blood sample from a restrained animal is withdrawn within 10 seconds) with a minimum of trauma to the animals. It obviates the necessity of further processing of the samples (centrifugation, serum withdrawal) at the laboratory. Blood samples diluted 100-fold by transfer to the diluting solution immediately after collection into capillaries can be used directly for ELISA or RIA. Storage of these diluted blood samples at room temperature for as long as 78 days causes no substantial changes in their antiviral antibody titres by ELISA. The technique of blood sample collection into capillaries is not limited to the pig but may resolve some blood collection problems also in other species. In large animals in general where restraining is a problem it makes blood sampling substantially more rapid and easier to perform.

In small and laboratory animals the new technique of blood sampling may replace tedious blood collections from small or difficult-to-reach blood vessels. In view of its aforementioned merits the new technique may find application not only in veterinary medicine but also in human medicine where facilities for examination by ELISA or RIA are available.

To our knowledge, collection of blood samples into heparinized capillaries and their immediate transfer to the specified diluting solution has not been reported before. In a similar study (Hamblin and Hedger 1982) blood samples were collected from pigs on to filter paper (Whatman No. 1) or white blotting paper, air-dried at room temperature, dissolved and examined by ELISA for antibody against swine vesicular disease virus; the results were in agreement with those obtained by VN tests.

Our study also included experiments on collection of blood and blood serum samples on to filter paper. When filter paper strips were placed in the diluting solution with the blood serum spots still wet, the ELISA results were similar to those obtained for samples collected into capillaries. However, blood samples stored in the dry state at 20 °C for several days showed a marked rise in non-specific binding activity of the antibody, due presumably to its partial denaturation. In our view, blood sample collection into capillaries (and possibly on to filter paper without drying) is therefore more suitable and easier to perform than conventional blood collection techniques because it avoids further processing of the sample.

Comparison of the ELISA and VN titres of serum samples from the same animals obtained by collection into capillaries showed a high degree of correlation (r = 0.881) between the results obtained by the two methods, which is in agreement with the findings reported by other writers (Moenning et al. 1981). Antiviral antibody titres by ELISA were higher than those obtained by VN tests. However, there were differences in ELISA titres obtained for the same samples depending on which of the two peroxidase conjugates was used. With HRP-RIgaSwIgG conjugate, the titres were about two dilutions higher than with HRP-protein A conjugate. Compared with VN results, the ELISA titres obtained with the two peroxidase conjugates were 51.9-fold and 10-fold higher.

The use of peroxidase-conjugated protein A has been described and verified by a number of writers. It is very suitable for ELISA diagnosis of AD in pigs because it reacts very well with the Fc fragment of swine IgG (Bommeli et al. 1980). It also reacts well with Fc fragments of rabbit, dog, human and other IgG's. In ELISA procedures one HRP-protein A conjugate can therefore replace a number of species-specific peroxidase conjugates. However, one disadvantage of HRP-protein A conjugate is evident from the present study: it is less sensitive than conjugates of antibodies purified by affinity chromatography. In our view, the choice of conjugate for ELISA in individual laboratories will depend on whether a universally applicable or a particularly sensitive method is required.

Mikrokapilární odběr krve k diagnostice protilátek proti viru Aujeszkyho choroby u prasat metodami ELISA (enzyme-linked immunosorbent assay) a RIA (solid-phase radioimmunoassay)

Je popsán mikrokapilární způsob odběru krve pro ELISA a RIA metody, který má ve srovnání s dosavadním způsobem odběru pro sérologické vyšetření řadu výhod. Odběr je méně pracný, časově kratší a s minimální traumatizací zvířat a rovněž odpadá další zpracování vzorků v laboratoři. I po více než dvouměsíčním uchovávání naředěných vzorků krve při pokojové teplotě se titry antivirových protilátek v podstatě nemění.

Mikrokapilární odběr krve byl prakticky ověřen u prasat srovnáním citlivosti průkazu protilátek proti viru Aujeszkyho choroby metodami ELISA, RIA a VN. Mezi titry antivirových protilátek zjištěnými metodami ELISA a VN byl prokázán vysoký stupeň korelace (r = 0,881).

Byly srovnány dva peroxidázové konjugáty pro ELISA metodu:

a) konjugát se specifickými protilátkami proti prasečímu IgG purifikovanými afinitní chromatografií (POD-RIgASwIgG),
b) konjugát se stafylokokovým proteinem A (POD-protein A).

ELISA metodou s použitím prvého konjugátu byly zjištěny 51,9× a s použitím druhého konjugátu 10× vyšší titry protilátek než VN testem. Je diskutována možnost využití mikrokapilárního odběru krve pro ELISA a RIA diagnostiku ve veterinární a humánní medicíně.

Микрокапиллярный отбор крови для диагностики антител против вируса болезни Ауйески и поросят методами ELISA (enzyme-linked immunosorbent assay) и RIA (solid-phase radioimmunoassay)

Приводится описание микрокапиллярного способа отбора крови для методов ELISA и RIA, который по сравнению с существующим способом отбора для серологических исследований отличается множеством положительных сторон. Отбор отличается небольшой трудоемкостью, меньшей продолжительностью, минимально травмирует животные и нет необходимости дальнейшей лабораторной обработки проб. Даже после чем двухмесячного хранения разбавленных образцов крови при комнатной температуре титры антивирусных антител по существу не меняются.

Микрокапиллярный отбор крови проверяли на практике у поросят сравнением чувствительности идентификации антител против вируса болезни Ауйески методами ELISA, RIA и VN. Между титрами антивирусных антител, установленных методами ELISA и VN, была обнаружена высокая степень корреляции (=0,881).

Проводились сравнения двух пероксидазных конъюгатов для метода ELISA:

а) конъюгат со специфическими антителами против IgG поросят, очищенными аффинной хроматографией (POD-RIgASwIgG),

б) конъюгат со стафилококковым протеином А (POD-protein A). Мето-

б) конъюгат со стафилококковым протеином А (POD-protein A).

Методом ELISA с применением первого конъюгата были установлены в 51,9 раз и с использование второго конъюгата в 10 раз больше титры чем методом VN. Обсуждается возможность использования микрокапиллярного отбора крови для ELISA и RIA диагностики в ветеринарной и общей медицине.

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