OPTIMIZING IMMUNOENZYMATIC REACTIONS (ELISA) FOR THE DETECTION OF ANTIBODY AGAINST NDV VIRUS

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abstract: The aim of this paper is to develop an ELISA diagnostic kit for antibody detection against NDV virus and to optimize the involved reactions. Therefore, we selected the optimal concentrations of coated antigen, antibodies (from sera) and peroxidase labeled conjugate. Our choice was made in agreement with ELISA technique requirements, which state that the absorbance for positive control serum should be higher than 1.300 and for the negative control serum lower than 0.300. We chose the incubation times for antigen (over night, 4^0C), sera and conjugate (30 minutes, 37^0C) and for substrate-choromogen mixture (10 minutes). Taking into account these data we elaborated a kit very useful for diseases control and diagnostic.

Introduction

The binding of antibodies to specific antigens is based on complementarity between the antibody combining sites and the antigenic determinants, similarly to enzyme – substrat reaction.

The immunoenzymatic assays are based on an enzymatic marker fixed on a reagent which participates to antigen-antibody interaction on an adsorbant, in a solid phase, emphasing this interaction. Thus, ELISA (Enzyme Linked Immunosorbent Assay) – indirect variant – is a technique consisting in an antigen-antibody reaction, the formed complex being evidentiated by an enzymatic reaction, with an antispecies enzyme labeled conjugate and a chromogen.

The ELISA techniques have proved to be very useful for the control and diagnostic of avian diseases, among which also Newcastle disease [1,2]. The aim of this paper is to develop an in house ELISA diagnostic kit for detection of antibody against Newcastle disease virus (NDV), optimizing the conditions of the involved reactions.

Material and methods

The antigen is represented by Newcastle disease virus (NDV) and it was obtained from NS Pasteur Institute from Bucharest.

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The antibodies are represented by sera from poultry vaccinated against this virus (positive sera).

The working procedure was described in detail [3] and it is summarized in Fig. 1 where one can distinguish the following steps:
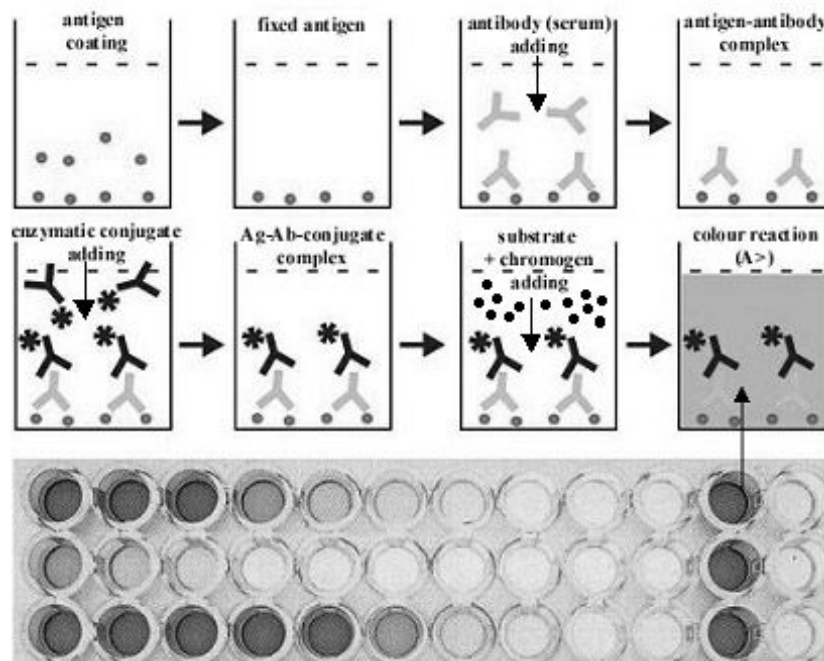

Fig. 1: *ELISA technique - indirect variant scheme:*

1) Antigen coating on a solid phase – microplate wells (Nunc MaxiSorp F96, Denmark) by adding 50μl/well NDV antigen diluted in coating buffer (NaOH 0.1M, pH 13) followed by 16-18 hours incubation at 4^0C; 2) Remove any unadsorbed antigen by washing with 300μl/well wash solution (0.5M NaCl, 0.05% Tween 20) in wells remaining only the adsorbed antigen (WellWash Ascent Labsystems automatic washer); 3) Adding 50μl/well of antibody (serum), 1/400 diluted in the appropriate buffer (1mM EDTA, 50 mM TrisBase, 0.5M NaCl, 0.05% Tween 20, pH 7.4); 4) Incubation at 37^0C for 30 minutes, when the antigen-antibody complex is formed (iEMS Labsystems thermostate); plate washing in order to remove any unreacted and unspecific antibody; 5) Adding 50μl/well of peroxidase labeled conjugate (Sigma) at suitable dilution; 6) Incubation at 37^0C for 30 minutes, when the conjugate specifically recognizes the antibody from the formed complex; plate wash in order to remove any unreacted conjugate molecule and remaining antigen-antibody-conjugate complex; 7) Adding of enzymatic substrate – chromogen mixture (50μl/well) represented by 0.0054% H_2O_2 - 0.054% ABTS- 2-2'-azino-di(3-ethyl benzthiazoline sulfonic acid solution in 0.1M citrate buffer pH 4.0 and incubation at room temperature for 10 minutes, in the dark; 8) Stopping the enzymatic coloured reaction with 50μl/well 1,5%NaF and reading of absorbances at λ = 405 nm (Multiskan EX Labsystems spectrophotometer).

For a serum that contains specific antibodies to coated antigen (pozitive serum), a colour reaction (green-blue) takes place, whose intensity is proportional to antibody concentration. For a negative serum (without specific antibody) the reactions described above do not occur, because unspecific antibodies and unreacted conjugate are removed in the washing steps.

In our experiments to optimize the NDV antigen, we performed a study of pH effect on coating process. Therefore we tested ELISA plates coated with NDV antigen (overnight, 4^0C), diluted in the following solutions and buffers: 0.1M acetate pH 5.0; 0.1M acetate pH 6.0; 0.1M saline phosphate pH 7.0; 0.1M saline phosphate pH 8.0; 0.1M carbonate bicarbonate pH 9.0; 0.1M carbonate bicarbonate pH 9.6 (the most used in ELISA); 0.1M carbonate bicarbonate pH 10.0; 0.1M carbonate-NaOH, pH 11.0,;0.1M carbonate-NaOH, pH 12.0; NaOH pH 12.7, 13 and 13.4. In these experiments we used the positive and negative control sera diluted 1/400, incubated at 37^0C for 1 hour. The anti chicken IgG conjugate labeled with peroxidase was used in diluted form 1/1000 (1 hour of incubation at 37^0C). The reaction was put in evidence by substrate-chromogene mixture after 15 minutes incubation in the dark. We also used a control for conjugate represented by a well which contained only conjugate and substrate-chromogen mixture (in order to show the presence or absence of unspecific interactions with solid phase).

Results and discussions

As we have mentioned, the aim of this paper is to develop a diagnostic kit for detection of anti NDV antibody by ELISA technique described above.

To develop such kit, one of the most important step is to obtain the antigen used for plate coating. We prepared two NDV antigenic variants applying two procedures. The first variant (coded NDV-M) was obtained by precipitation with organic solvents (methanol), following a procedure from literature and adapted in Pasteur Institute, Bucharest [2,4]. The second variant was obtained by high purification (centrifugation in cesium chloride gradient), resulting two fraction NDV-F1 and NDV-F2.

Developing ELISA kit for anti NDV antibody detection consisted in optimizing the reagents concentrations, incubation temperature and incubation time. All these factors were experimentaly determined by testing pozitive and negative control sera, in order to obtain absorbances higher than 1.300 for pozitive control and lower than 0.300 for negative one [2]. For the rapidity of the diagnostic tests, the overall time of this procedure is recommended to be short enough.

Optimizing the conditions for NDV antigen coating

A limitative factor for ELISA reactions is represented by the amount of antigen which can be fixed on the solid phase, this limiting factor leading to the sensitivity of this test. Determination of optimal antigen concentration (or dilution) is one of the mandatory steps in developing an ELISA kit.

The optimal concentration of our NDV antigenic variants was determined by their titration. The plates were coated with these antigens diluted from 1/25, 1/50, 1/100...1/6400.

Plotting the absorbances at λ=405 nm against the reciprocal value of antigen dilutions we obtained for each NDV antigen type the curves presented in Fig. 2.

The optimal concentration for antigen was in the dilution range of 1/100-1/400, for which the ratio between the absorbances of negative control serum and the positive one (N/P) has the lowest value. This ratio is a measure of the antigen capacity to diferentiate between

negative and pozitive sera. At low antigen concentrations (high dilutions), N/P ratio has high values suggesting that the conjugate unspecifically binds to the plate (solid phase). In this case there are unoccupied sites on the solid phase surface which is able to fix the molecules of other reagents. Comparing the response of the control sera for these antigens, we observed that only NDV-M antigen is in agreement to the recommended absorbance limits ($A_{positive\ control\ serum}$ > 1.300). Therefore we chose this type of NDV antigen for our experiments, by coating the antigen over night (16-18 hours) at 4^0C. We tried to shorten the time of this process by incubating the plates for 1 and 2 hours at higher temperature (37^0C), in order to increase the adsorbtion rate of viral particles.
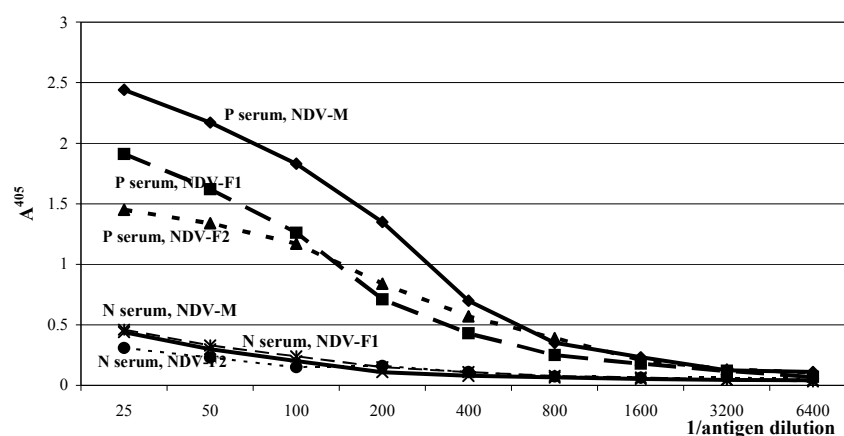

Fig. 2: *The absorbance variation with NDV antigen dilution*

Analysing the N/P ratio (Table 1), one can remark that for the incubation time of 16-18 hours there is not any unspecific interaction with the solid phase. This long incubation permits a better coating of free sites on the solid phase. At higher temperature and therefore shorter incubation time, the viral particles are adsorbed faster, but with unspecific binding. For this reason, we opted for the longer incubation time and for 1/400 serum dilution which presents the lowest N/P ratio.

Table 1. The determination of coating time for NDV-M antigen

Negative/Positive Ratio	Serum dilutions						
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
N/P (1 hour, 37^0C)	0.224	0.188	0.170	0.183	0.207	0.243	0.224
N/P (2 hours, 37^0C)	0.244	0.187	0.161	0.153	0.197	0.180	0.171
N/P (16 hours, 4^0C)	0.114	0.094	0.067	*0.058*	0.082	0.104	0.160

The described experiments were performed with NDV antigen prepared in 0.5 M NaOH solution, *p*H 13, according to the protocol described by Miers [4]. The literature indicates also other suitable buffers with various *p*H values, for sensitizing ELISA plates [5,6]. But, they did not investigate the influence of *p*H on coating process. Therefore, we carried out

experiments in which we sistematically varied the buffer to work at different pH, as one can see in Fig. 3.
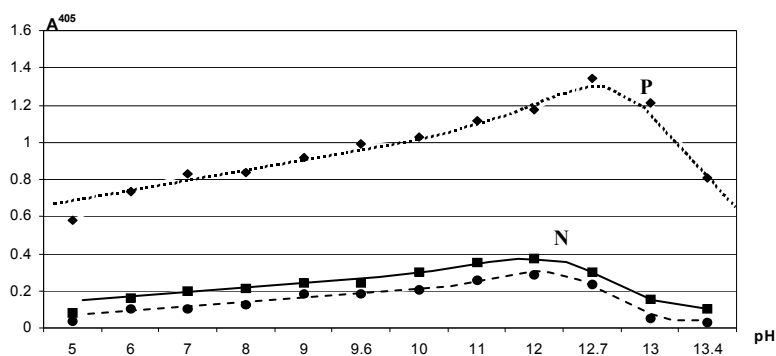

Fig. 3: *pH influence on coating process*

One observes that the positive control serum has the highest absorbance at pH=12.7, in agreement to ELISA requirements (A>1.3). But, at this pH, the negative and conjugate controls have absorbance values higher than 0.300 due to some unspecific interactions, because of the conjugate that fixed on solid phase. This suggests that the high absorbances of the positive control serum are related to these unspecific interactions of the conjugate, so this pH is not suitable for our experiments. In exchange, at pH=13 near of the first one, the negative control serum as well as the conjugate control present absorbances much lower than 0.3, so there is not unspecific binding. The absorbance for the positive control (~1.300) corrected by the conjugate contribution (0.050) is still in agreement with ELISA limits. For this reason, we selected 0.1M NaOH pH 13 as coating solution for the NDV antigen.

Optimizing the reaction conditions for antibodies in control sera and conjugate

The ELISA sensitivity and specificity depend on the quality of used sera which contain the antibodies specific to the coated antigen. Ideally, the positive control serum should have a high concentration of antibodies which have to react specifically only with the coated antigen. These antibodies should not interact with other reagents or even with the solid phase (plastic material).

The suitable concentrations of control sera and peroxidase conjugate were concomitantly determined. The working dilutions were 1/5, 1/10, 1/100...1/25600 for control sera and 1/1000, 1/2000...1/6000 for conjugate.

The experimental data obtained for each used conjugate concentration were represented in Fig. 4 as absorbance vs. the reciprocal serum dilution.

At high conjugate concentration (1/2000), there is a plateau ranging from 1/5 to 1/200 serum dilutions (high antibody concentrations) because all antibody sites are occupied by antigen. But, the absorbances decrease for higher dilutions of positive control (1/400-

1/1600), because it forms lower amounts of antigen-antibody complex. One can observe that at lower conjugate concentrations (1/3000) the plateau shortens (1/5-1/100 serum dilutions) and the curve appears as a sigmoid with a linear portion between 1/200 and 1/1600. At much lower conjugate concentrations (dilutions of 1/4000-1/6000) we did not remark any plateau, so that the absorbances decrease just from the beginning of the curves. From the experimental curves we selected the optimal conjugate concentration at dilution 1/3000 (sigmoidal curve). We chose this conjugate dilution because our experiments carried out with the negative control presented absorbances situated over 0.300 (not shown in this figure). So, for this reason, although the positive control presented absorbances over 1.300, we used only this sigmoidic curve in choosing the positive serum concentration. On the linear segment we selected the dilution 1/400 for positive serum ($\log 1/\text{dil} = 2.602$) which corresponds better to the ELISA requirements.
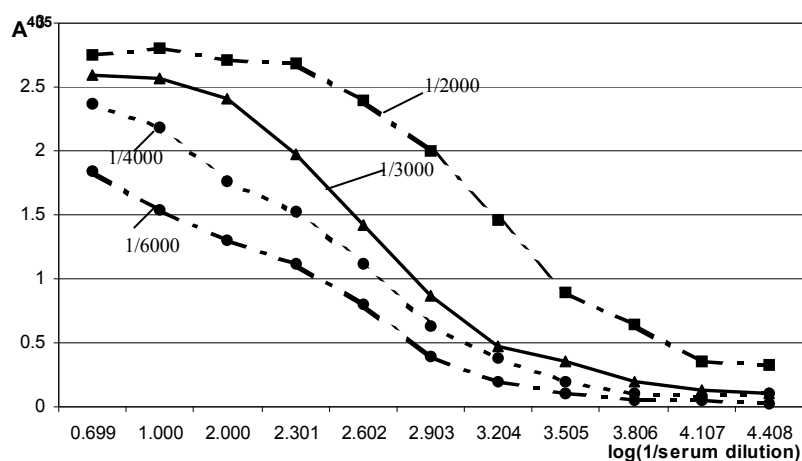

Fig. 4: *Selection of the optimal concentrations for positive control serum and peroxidase conjugate*

In order to determine the suitable time for reaction between antibody and antigen and also between the formed complex and enzymatic conjugate, we performed tests at different incubation periods: 15, 30 and 60 minutes at 37^0C, for the control sera as well as for the conjugate.

The experimental data for incubation times of the control sera showed that all these periods give a low N/P ratio with o good diferentiation between positive and negative controls. From Table 2 one can observe that the lowest N/P value is obtained for an incubation time of 30 minutes and therefore we chose it for our experiments.

Table 2. Determination of sera incubation time

|	Time (min)		
	15	30	60
A^{405} serum P	0.867	1.410	1.567
A^{405} serum N	0.089	0.134	0.244
N/P	0.102	0.095	0.155

Regarding the conjugate labeled with peroxidase, under the same experimental conditions we determined an incubation time of 30 minutes, convenient for the test rapidity too.

The determination of incubation time for substrate-chromogen mixture

The oxidizing reaction of ABTS chromogen to its cationic radical form, in the presence of peroxidase, takes place as follows:

$$H_2O_2 \xrightarrow{\text{peroxidase}} 2\overset{\bullet}{O}H$$

$$\overset{\bullet}{O}H + ABTS \longrightarrow ABTS^{+\bullet} + OH^-$$
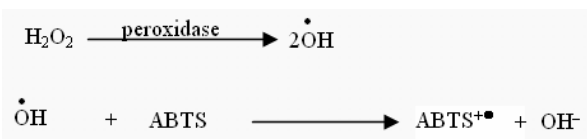
So, a H_2O_2 molecule will oxidize two ABTS molecules according to the reaction scheme:
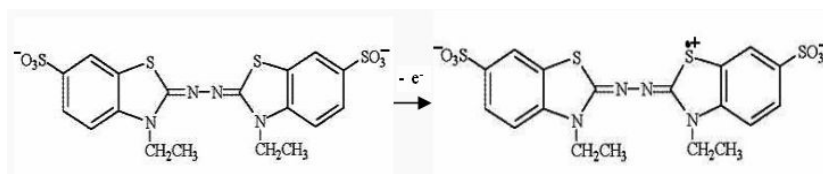
ABTS is colourless or very light green but it transforms into a green-blue product, with various intensities depending on the enzyme concentration.

It was proved that the oxidized chromogen is unstabilized by pH increase, so it is recommended to use it in an acetate or citric acid buffer at low pH (pH=4) [7]. The optimal reaction rate is reached at pH 4.2 for a 2mM ABTS concentration [8]. It is known that the peroxidase affinity for its substrate decreases as the pH increases. Higher ABTS concentrations than 2 mM shift the optimal reaction pH values to neutrality.

To determine the incubation time for substrate and chormogen, we carried out experiments at different times: 1, 5, 10, 15, 20, 30 and 60 minutes at room temperature, in the dark.
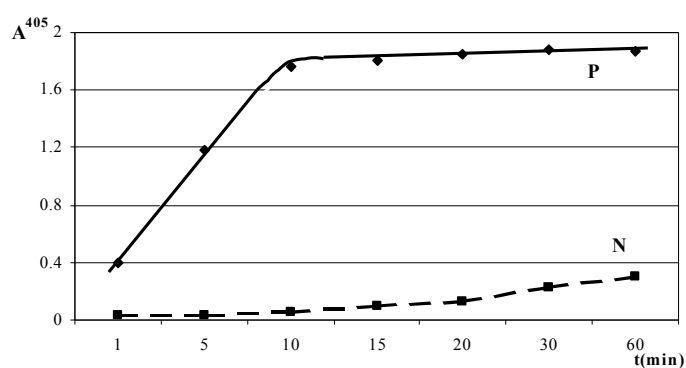

Fig. 5: *The selection of optimum incubation time for substrate-chromogen (H_2O_2-ABTS)*

In Fig. 5 we plotted the absorbances versus time. For positive control serum one can observe that the enzymatic transformation of substrate, H_2O_2, increases proportional in the

first 10 minutes. After that, a plateau is reached, suggesting that the maximum amounts of antigen-antibody complex is already formed.

The plot in Fig. 5 is in fact the kinetic curve for the followed reaction. In a previous study [9] we evaluated the rate constant for the antigen-antibody complex formation. Although the complex formation is a second order reaction, because in our experimental conditions the antigen was in excess, it follows a pseudo first order kinetics which is verified for the first 10 minutes of the reaction according to the equation : $\left(\log(A_\infty - A_t) = \log(A_\infty) - k_1 t \right)$. In this manner, the tests for the establishing the incubation time for substrate-chromogen mixture, which followed the antigen-antibody complex formation, take a theoretic, kinetic support, that we pointed out for the first time.

In the case of the negative control serum, one observes that for the first 10 minutes the absorbances have very low values according to ELISA technique. Therefore, we chose this incubation time (10 minutes) for which both the absorbances for positive and negative control sera are into optimal limits of experiments.

Within immunoenzymatic techniques, the chemical reactions join to immune processes making an assembly based on interaction between proteic molecules involved in antigen-antibody complex. The use of such interaction in the medical field by means of diagnostic techniques are very helpfull for the diseases prophilaxy and control. The test presented in this paper permits the detection of antibody specific to NDV virus in poultry sera, with direct applications in vaccination monitoring.

In addition, we made similar tests to create two other kits to test another antigen-antibody complexes (Egg Drop Syndrome - EDS and Infectious Bovine Rhinotracheitis viruses - IBR) [10], all three representing a premiere in Romania.

Conclusions

– Experiments employing the various steps and reagents used in Enzyme Linked Immunosorbent Assay (ELISA) were conducted to develop an ELISA kit for detecting antibody against Newcastle disease virus, with high sensitivity and specificity.

– Of the three NDV antigenic variants, we chose for our testing the one obtained by methanol precipitation, with an optimal concentration/dilution for coating of 1/200, with long incubation time (16-18 hours) at 4^0C.

– An important factor to obtain the maximum specific binding of the antigen to the microplate, is the pH of coating buffer for which we found an optimal value of 13.

– We optimized the reaction conditions for antibodies in control sera and peroxidase conjugate, choosing as optimal concentrations the 1/400 dilution for control sera and 1/3000 dilution for conjugate, with incubations for 30 minutes at 37^0C.

– For the enzymatic transformation of substrate-chromogen mixture (H_2O_2-ABTS) we selected an incubation time of 10 minute in the dark, which actually follows a pseudo-first order reaction of antigen-antibody complex formation, its kinetics being evidentiated for the first time by us.

− All these experiments permitted to develop an ELISA kit with direct applications in medical field, for three antigen-antibody complexes.

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