Characterization of an IgY polyclonal antibodies directed against the canine distemper virus

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Abstract
Canine distemper is a contagious, incurable, often fatal, multisystemic viral disease that affects the respiratory, gastrointestinal, and central nervous systems. Distemper is caused by the canine distemper virus (CDV). The development of antibodies to use in therapy and diagnosis is essential for the control of some diseases. Immunization of chickens with CDV results in the production of antibodies specific. However, there is relatively limited information available concerning immune response of CDV in this species. In the present study, immune responses were examined in serum and egg yolk from laying hens injected with CDV. The results demonstrated that the increase of antibody activity occurs first in the serum, and then in egg yolk with a lag in time of 1 to 3 week in the chickens. However, the time of elevated levels of antibody activity was much shorter in serum than the egg yolk.

Keywords: *Canine distemper virus – Egg yolk antibody – ELISA, indirect.*

INTRODUCTION

The virus is widespread and mortality in juveniles is higher than in adults (APPEL, 1969), is classified as morbilliviruses in the paramyxoviridae family. The virus is enveloped and contains a negative sense single stranded RNA and a RNA polymerase. The lipoprotein envelope is readily destroyed by lipid solvents wich renders the virus-non infectious (APPEL; SUMMERS, 1999). The CDV is very resistant to cold and the majority of distemper cases in domestic dogs are seen in the fall and winter (GREENE; APPEL, 1998). Transmission occurs via an aerosol-droplet route, direct contact, or possibly by contact with contaminated objects (APPEL; SHEK; SUMMERS, 1982). The CDV is shed in the feces and urine of infected individuals and some evidence exists for transplacental transmission. The usual route of infection is through the upper respiratory tract, following inhalation of infective virus (KRAKOWKA; OLSEN; CONFER, 1985).

Occasionally infection occurs from ingestion of infective material. Following entry into the upper

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respiratory tract, the virus is spread to the tonsils and lymph nodes, where viral replication occurs (APPEL, 1987). The virus then enters the blood stream where it is transported to epithelial cells throughout the body, including the intestinal and respiratory tract (SUMMERS; GREISEN; APPEL, 1984). Typical signs of canine distemper seen in the domestic dog include respiratory and intestinal problems such as coughing, diarrhea, vomiting, nasal and ocular discharge, anorexia, and hyperkeratosis of the nasal planum and footpads. Central nervous system signs may follow the above clinical signs (VANDEVELDE et al., 1982). In wild carnivores, signs of abnormal behavior and apparent lack of fear, suggestive of rabies, may be the only signs grossly visible. In many countries the diagnosis is based on clinical signs.

The clinical diagnosis, made on the basis of physical examination, history and examinations complementary, at times, is inconclusive because the same pattern can also be found at other infectious and parasitic diseases of dogs (AMUDE; ALFIERI, A.A.; ALFIERI, A.F., 2007). To achieve the diagnosis of CDV several diagnostic methods were developed, especially the search for inclusion in the corpuscle cells present in secretions and neutrophils circulating, the fluorescent antibody, Polymerase Chain Reaction (PCR),Cerebrospinal (CSF) Analysis, the immunohistochemistry and in the isolation of CDV in cell culture (APPEL; SUMMERS, 1999). However, all methods have disadvantages that may prevent the use in routine laboratory such as low sensitivity or specificity, laborious steps of processing of biological material and time required for completion of the outcome (MORITZ; FRISK; BAUMGÄRTNER, 2000).

Antibodies presently available for research, diagnostic and therapies are mostly mammalian monoclonal or polyclonal antibodies. Traditionally, bigger animals such as horses, sheep, pigs and also rabbits and guinea pigs, were used for the production of polyclonal antibodies, while mice and rats were used as source of spleen for the production of monoclonal antibodies (NARAT, 2003). The major problem of monoclonal antibody production is that some antigens are weakly or not at all immunogenic for mice, moreover, both monoclonal and polyclonal antibodies technologies involve some injury of animals involved, such as the immunization, collecting of blood samples and bleeding (or sacrifying for spleen removal) (CHILOW et al., 2000). Therefore, the development of alternative methods for the production of antibodies is necessary. In this case, the use of chickens for antibody production represents a new way for the animal and human industry. This work showed the alternative way for antibody produce against the canine distemper virus by using chicken egg yolk antibody and standardization the ELISA to detect the virus.

MATERIAL AND METHODS

Canine distemper virus

The canine distemper virus (CDV) strain Snyder Hill (ATCC® Number: VR-526™) was obtained from the ATCC (The Global Bioresource Center). The viral amount was determined by protein concentration with Bio-Rad protein assay (Bio-Rad Laboratories) and then samples were frozen at –70ºC.

Antibodies using as positive and negative control

Positive control

The antibody using as positive control was mouse anti-CDV IgG obtained from Acris Antibodies Gmbh (Germany).

· *Negative control*

The negative control used was made with IgY anti-Staphylococcus aureus in the serum and egg yolk produced in our laboratory.

Preparation of immunogens

The viral suspension composed of 300 ng of CDV in 500 ìl de PBS at pH 7.2 was mixed with 500 µL of the alhydrogel (aluminum hydroxide $Al(OH)_3$) as adjuvant (Accurate Chemical & Scientific Co, Westbury, New York) for the first injection. The others three inoculations were made of the same way.

Immunization of hens

Six White Leghorn laying hens of twenty five-week-old from the North Fluminense State University were used for this immunization. The chickens were keeping in individual cages with water

and food ad libitum. The first immunization was conducted by injecting 1.0 mL of the mix of viral suspension and adjuvant into the pectoral muscle. The second and third inoculation was carried out 1 and 3 weeks after the first inoculation. The last inoculation was made at 6 weeks after the first.

Eggs and blood collection

The blood and eggs were collected all week after the first immunization. The collected eggs were stored at 4°C for extraction of IgY. The blood was collected with 0.1% sodium citrate as anticoagulant, after this the blood was centrifuge at 1500 rpm to 15 min for separate the plasma. Both were analyzed by indirect ELISA.

Extraction of egg yolk antibody

Eggs were collected from hens immunized all days after the first inoculations and then sorted by week. Egg yolk antibodies were extracted using chloroform (SHIN, N.R. et al., 2001; SHIN; KIM; YOO, 2001). For this, the egg yolk was separated from the egg white and homogenized after mixing with an equal volume of PBS. The homogenized egg yolk was then mixed with two volumes of chloroform and incubated for 2 hours at room temperature. After this incubation, the supernatant was collected and stored at –70°C (SHIN, J.H. et al., 2002).

ELISA assay

The chicken egg yolk antibodies (IgY) titers were measured by indirect ELISA. For the IgY titers, was used the original canine distemper virus (CDV) strain Snyder Hill of the ATCC.

For the IgY titers, was used the original CDV strain Snyder Hill of the ATCC. Ninety-six well microplates (Dynatech laboratories, Inc.) were coated with 100 μL of CDV (200 ng/mL) and incubated at 4°C. After the incubation, the plates were washed with 150 μL PBS/0.05% Tween 20 (wash buffer) three times, blocked with 0.5% gelatine in PBS (pH 7.2), 200 μL per well, 1 h at room temperature, and washed with wash buffer three times. Then, was added 50 μL IgY anti-CDV (diluted in PBST 1:1000) suitably diluted to each well and incubated at room temperature for 1 h, washed the plate with wash buffer three times. After the washing, 50 μL (diluted in PBST 1:5000) rabbit anti-chicken IgG conjugated with horseradish peroxidase (Biomeda Corporation) suitably diluted was applied to each well of the plate and incubated at room temperature for 1 h. Substrate, 2,2-azino-bis (3-ethylbenz-thiazoline-6- sulfonic acid) (Sigma Co.) was added into each well of the plate. Optical densities at 490 nm were measured with a microplate ELISA reader after stopping of the reaction with 2 M HCl solution.

Western blotting

The western blotting assay was used to confirm the IgY antibody reactivity against CDV. The CDV proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) and transferred to polyvinyledineflouride (PVDF) membranes by electroblotting at 100 V for hours. The membrane was treated with 50% v/v methanol immediately prior to and after the electroblotting. The membrane was then blocked with 0.5% BSA in PBS at 37°C for 60 min. After blocking, the membrane was washed with PBS (3 × 10 min), probed with IgY anti-CDV (1:2000) and incubated at 37°C for 60 min. The membrane was then washed with PBS, followed by addition of 1:10,000 diluted affinity-purified rabbit anti-chicken IgG conjugated with horseradish peroxidase (Biomeda Corporation) and incubated at 37°C for 60 min. After incubation, the membrane was washed extensively with PBS followed by addition of tetramethylbenzidine-hydrogen peroxide (TMB/H2O2), which enabled the antibody reaction to be visualized (data not show). In the case of blotting using the solution of IgY as antigen, the membrane was incubated with a solution of rabbit IgG anti-chicken, and then revealed as described.

Validation

The cut-off was calculated using the average of negative samples plus three times the standard deviation of these samples, where the number of standard deviations used in the formula guarantees the level of confidence of the result. For validation were calculated the sensitivity, specificity, positive predictive value and negative predictive value of the test.

RESULTS

Antibody extraction

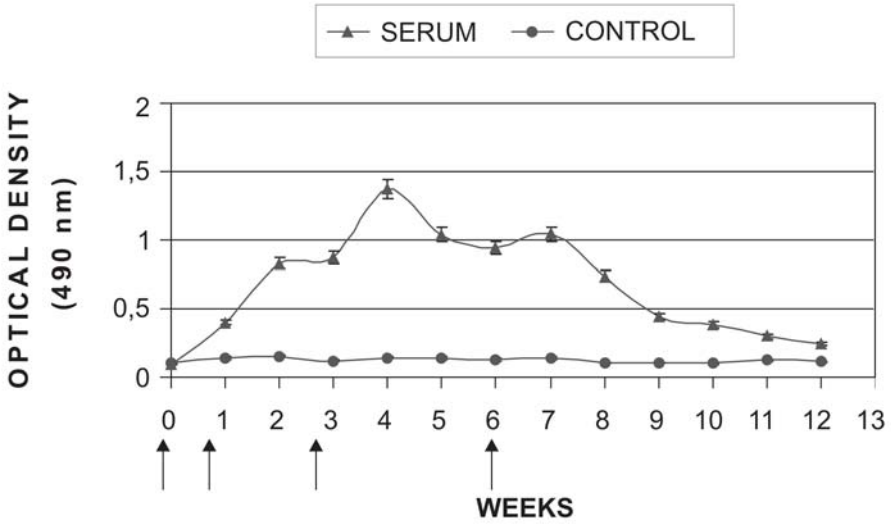
The method of IgY extraction anti CDV using chloroform was effective in extracting. It was not used none method for purifying the IgY, therefore the concentration of total protein measured by Bradford (1976), arrived at a concentration of 6.5 mg of total protein per mL of egg yolk. The Figure 1,A shows polyacrylamide gel of which 4 samples were used with solutions containing the IgY antibody the solution of yolk in decreasing concentrations of 19.5 mg to 1.3 mg. As the gel and samples were placed in a position denaturants, the antibody is presented in two bands, one showing the heavy chain of about 67 - 70KDa and the other light chain around 25KDa. The other bands are certainly related to other proteins found in the yolk of the egg, since the samples were not subjected to purification, only to extraction. Beside the gel, in Figure 1,B, is the picture of a nitrocellulose membrane that was obtained from the rush of a polyacrylamide gel (SDS-PAGE), which were applied 24.7 mg and 19.5 mg of total protein containing IgY and transferred to nitrocellulose membrane, where the rabbit IgG anti-chicken peroxidase conjugate (Sigma-Aldrich) was used.

It was possible to observe the heavy chain (67 - 70 kD) and light chain (~25kD) of the antibody IGY extracted from egg yolk, both in a polyacrylamide gel, as the nitrocellulose membrane.

Specific activity of IgY against CDV

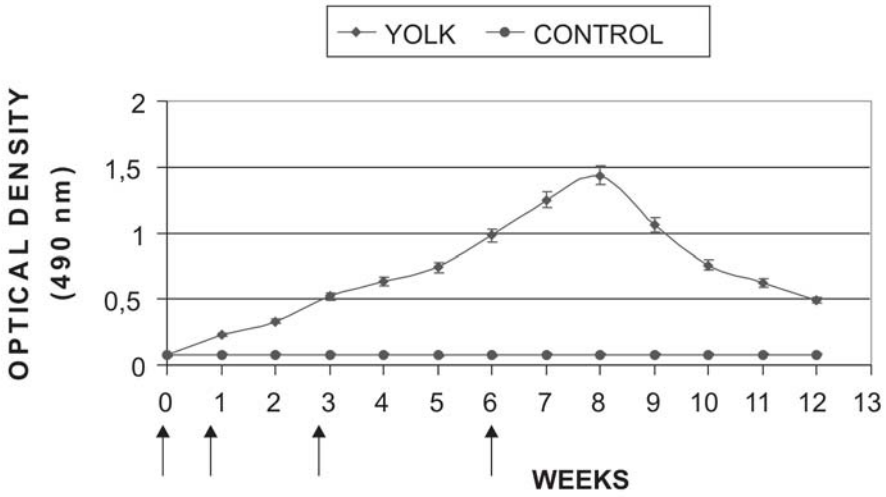
The immune response of laying hens against CDV was monitored by measuring antibody activities in serum and egg yolk by indirect ELISA. In serum the level of activity of anti-CDV (Graphic 1) sharply increased after the first immunization, became highest at 28 day. The level was maintained up to 7 days and decreased therefore. The short time which the level of IgY in the serum remained high could be due adjuvant, antigen concentration and genetic of animals (ALLISON; BYARS; WATERS, 1986). However, the transport of IgY from maternal serum to the offspring is other determinant factor (ROSE; ORLANS; BUTTERS, 1974). For this reason, the level of antibody IgY activity in yolk stared to increased 10 days after first immunization.

The lag in time between serum and egg yolk antibody was approximately 1 week. The antibody activity became highest at 63 day, and remained relatively constant up to 7 days (Graphic 2). This suggests that the activity of anti-CDV IgY can be



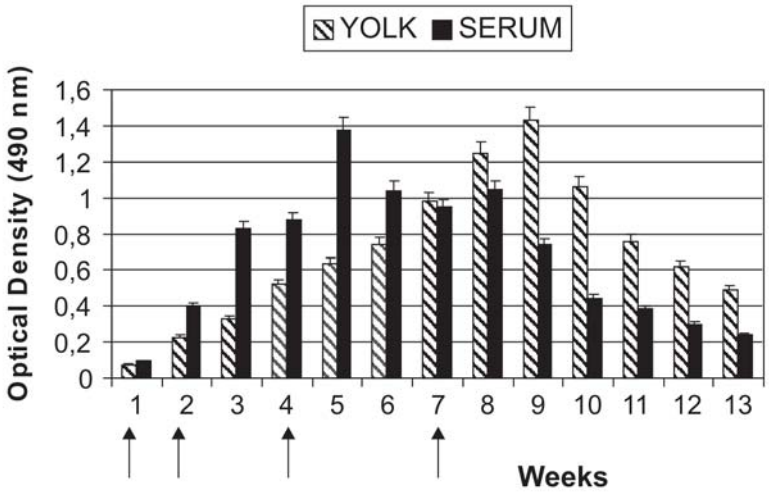
Graphic 1- Antibodies titres against canine distemper vírus in the serum of laying hens immunized.

Notes: - the values are means of four samples; - the vertical bars indicate the standard deviation; - arrows indicates the time of booster injection of CDV.



Graphic 2 - Antibodies titres against canine distemper virus in the egg yolk of laying hens immunized.

Notes: - the vertical bars indicate the standard deviation;- arrows indicates the time of booster injection of CDV.



Graphic 3- Changes of antibody activity in chicken serum and egg yolk during the immunization period.

Notes: - anti-CDV antibody activities in serum and egg yolk were measured by indirect ELISA using the CDV as an antigen and are expressed as ELISA abosorbance at 490 nm for serum and egg yolk at 1:1000 dilution;- the values are means of four samples; - vertical bars indicate the standard deviation; - arrows indicates the time of booster injection of CDV.

maintained much longer in the yolk than in the serum. Similarly, Shimizu, Fitzsimmons and Nakai (1988), and Sunwoo et al. (1996) in a study of immunized hens with lipopolysaccharide (LPS) showed that the maximum level of anti-LPS antibody activity in the yolk persisted longer than in the serum (Graphic 3).

Validation of ELISA

The evaluation of ELISA tests was performed based mainly on negative and positive controls. In all the assays the positive control was more than negative control, and the samples test were considered positive.

DISCUSSION

Polyclonal antibodies are used extensively in research, diagnostics and immunotherapies and are conventionally taken from the blood of mammals, especially rabbits. The fact of being able to detect antibodies in the egg yolk of hens immunized led to the development of antibody technology of IgY as an alternative that is less stressful for the animal and has many other advantages that arouses interest in various groups to protect animal, search and commercial production. In this study looking produce, extract and identify IgY antibodies against canine distemper virus and show the various applications that these antibodies may have, such as immunotherapies and diagnostic kit. With these purposes, chickens immunized with a suspension of canine distemper virus and antibodies were extracted from egg yolk, using chloroform methodology, which has advantages as a methodology for relatively low cost, requiring few steps and is efficient in extraction of antibodies, in addition to not interfere with the activity of the antibody. It was possible to detect and characterize the IgY antibodies anti-CDV through a polyacrylamide gel that showed in his eletrophoresis wider bands of heavy chains of the antibody between 67 and 70kDa and thinner bands of light chains of approximately 25 kDa, other bands were also detected which are certainly of other proteins that were present in the egg yolk. To confirm the location of heavy and light chains in the gel and to differentiate more clearly contamination has also been done a electrotransfer of IgY antibodies anti-CDV to a nitrocellulose membrane and marked with rabbit IgG anti-chicken peroxidase, bands of heavy chains and light emerged clearly, it was possible to observe that there is a contaminant protein very close to the heavy chain of approximately 66 kDa. Its detection was possible through the difference between the thickness of the band of 66 kDa of the gel and the membrane, as shown in Figure 1. The activity of the serum and egg yolk IgY antibody is represented in the graphics 1 and 2 obtained by the indirect ELISA test with successive dilutions in log 2, showing through the optical density, which the antibody is able to effectively recognize the antigen adhered to the bottom of the plate. It can be observed that initially the curve of the antibody level in the serum is higher than the yolk and, over time, there is a change in these levels. There was the relationship of these antibodies in serum title with successive immunizations. In the week after immunization there is an increase in the form of antibodies in circulation. The serum antibodies to remain high until the fourth eightieth day after the first immunization, showing that the IgY shows the ability to remain high in debt for a long period (WARR; MAGOR; HIGGINS, 1995).

CONCLUSION

In conclusion, immune response of chickens was studied with canine distemper virus as antigen. The chickens produced antibodies and were analyzed by ELISA. The results showed the importance of create the alternative way for the diagnosis and therapeutic use.

Caracterização de anticorpos policlonais IgY dirigidos contra o vírus da cinomose canina

Resumo

A cinomose é uma doença contagiosa, incurável, muitas vezes fatal, sistêmica, que afeta os sistemas respiratório, gastrointestinal e nervoso central. A enfermidade é causada pelo vírus da cinomose (CDV). O desenvolvimento de anticorpos para uso em diagnóstico e terapia é essencial para o controle de algumas doenças. Imunização de galinhas com CDV resulta na produção de anticorpos específicos. No entanto, há relativamente poucas informações disponíveis relativas à resposta imunológica do CDV nesta espécie. No presente estudo foi analisado a resposta imunitária em soro e gema de ovo de galinhas poedeiras injetadas com CDV. Os resultados demonstraram que o aumento

da atividade ocorre primeiro anticorpos no soro e, em seguida, na gema de ovo com uma defasagem no tempo de 1 a 3 semanas nas galinhas. No entanto, o tempo de níveis elevados de atividade anticorpos no soro foi muito mais curto do que a gema de ovo.

Palavras-chave: Cinomose canina, virus da – IgY anticorpos – ELISA indireto.

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