Production and use of egg-yolk antibody for detection of canine parvovirus in feces

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Abstract
Canine parvovirus (CPV), cause an intestinal disease characterized by bloody diarrhea, is often fatal in puppies. The virus is transmitted by contact with infected dogs or their feces. The virus is very stable in the environment and may survive for several months in contaminated areas. The CPV attacks the rapidly diving cells of the bone marrow and the small intestine. Several laboratory tests have been developed and are available for specific viral diagnosis. Where facilities are available, rapid diagnosis can be made by electron microscopy (EM) of fecal material from cases with typical signs of disease. The virus also can be isolated in several feline and canine cell lines such as canine and feline kidney cells, but isolation is seldom used in practice since cell cultures are required and at least 1 week for results is required. Fecal hemagglutination-hemagglutination (HA-HI) tests have provided a simple and rapid method for detecting virus in fecal and tissue samples and are employed by several diagnostic laboratories, however the HA test is less sensitive than EM or enzyme-linked immunoassays (ELISA). In this work, the laying chickens are immunized with the canine parvovirus strain Cornell 780916-80 and the egg yolk antibody (IgY) isolated and characterized by indirect ELISA to detect canine parvovirus in feces.

Keywords: *Canine parvovirus - Diagnosis - Egg yolk antibody- ELISA, indirect; Canine parvovirus - Diagnosis - HA-HI*

INTRODUCTION

Canine parvovirus is a highly contagious and serious disease caused by a virus that attacks the gastrointestinal tract of puppies, dogs, and wild canids[1]*. This family consists of small non-enveloped DNA virus*[2]*. Two distinct parvovirus (CPV)*[3] *are now known to infect dogs, the pathogenic CPV-2, cause several enteritis, which was recognizes in 1978, and the CPV-1 (MVC)*[4]*. The MVC, a completely different parvovirus, had not been associated with natural disease*[5]*. MVC may cause*

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pneumonia, myocarditis and enteritis in young pups, or transplacental infections in pregnant dams, with embryo resorptions and fetal death[6]. CPV-2 infects dogs and other canidae. There are two types of CPV-2 (CPV-2a and CPV-2b).

Because the infection is easily spread among susceptible animals, rapid diagnosis is essential for disease control and proper treatment[7]. CPV replicates in several lymphoid tissues and the intestinal epithelium of dogs, therefore, great amounts of parvovirus are found in the feces of infected dogs[8]. The diagnostic this pathology can be made by electron microscopy (EM) of fecal material from cases with typical signs of disease, fecal hemagglutination-hemagglutination inhibition (HA-HI) tests have provided a simple and rapid method for detecting virus in fecal and tissue samples[9], however the HA test is less sensitive than EM or enzyme-linked immunoassays (ELISA). The HA assay is recommended test for detection of parvovirus in feces[10,11,12], however, can be affected by certain factors in the fecal material resulting in nonspecific agglutination[13]. The specificity of the agglutination should be confirmed by an HI assay[11]. In general, these ELISA are made with monoclonal (mAbs) or polyclonal antibodies of mammals. Chicken egg yolk antibody (IgY), has been extensively studied to substantiate its effective use in diagnosis[14] and for passive immunization[15,16,17,18,19,20]. There are several advantages of using IgY. IgY can be easily isolated from egg yolk by the water-dilution method on a large scale without using any chemicals or organic solvents[21,22,23]. It is relatively stable under various conditions, including heat, pressure, alkalinity and acidity[24,25]. In this work we used the IgY for prepare a indirect ELISA for to detect the canine parvovirus in fecal material of dogs clinically sick. The ELISA results are compared with HA.

MATERIAL AND METHODS

Fecal samples

Canine fecal samples were collected of the suspect animals in veterinary clinics. To the viral extraction was used the approximately 10% suspensions of fecal material were made by mixing the fecal samples in Hank's medium with $NaHCO_3$ on a vortex apparatus. The debris was allowed to settle for 10 min, and the supernatant fluid was tested in the ELISA indirect or treated further. Additional treatments included testing the supernatant fraction after extraction with 0.1 volume of chloroform followed by centrifugation at 1500 x g for 10 min[26].

Canine parvovirus

The canine parvovirus strain Cornell 780916-80 (ATCC® Number: VR-2017™) was obtained from the Global Bioresource Center (ATCC). The viral amount was determined by protein concentration with Bio-Rad protein assay (Bio-Rad Laboratories) and then samples were frozen at –70ºC.

Preparation of Immunogens

The viral suspension composed of 300 ng of parvovirus in 500 ìl de PBS at pH 7.2 was mixed with 500 ìl 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.

Extraction of Egg Yolk Antibody

Eggs were collected from hens immunized all days after the first inoculations and then sorted by day. Egg yolk antibodies were extracted using chloroform[27]. 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 –70ºC[28]. The protein

concentration was determined with Bio-Rad protein assay (Bio-Rad Laboratories) and then samples were frozen at –70°C.

Antibodies

IgG monoclonal antibody anti-CPV (MCA2589-AbDSerotec) used as positive control.1:200.

Hemagglutination (HA)

The plate was coated with 50 ìl of fecal samples diluted 1:10 in phosphate-buffered saline (PBS) pH 7.2 in the first well, and then serial twofold dilutions was made. After this, 50 ìl of 0.5% porcine erythrocytes in virus adjusting diluent (VAD) was added. The plate was incubated at room temperature for 40 min. The hemagglutinant titer of antigen is the higher dilution which the hemagglutination was complete. Samples considered positives by HA test were confirmed by HI test.[29]

ELISA Assay

The chicken egg yolk antibodies (IgY) titers and the canine parvovirus in fecal material were measured by indirect ELISA. For the IgY titers, was used the original canine parvovirus of the ATCC and the canine parvovirus in fecal material. Ninety-six well microplates (Dynatech laboratories, Inc.) were coated with 100 ìl of canine parvovirus (200 ng/ml) isolated of fecal material diluted in coating buffer by overnight incubation at 4°C. After the incubation, the plates were washed with 150 ìl PBS/ 0.05% Tween 20 (wash buffer) three times, blocked with 0.5% gelatine in PBS (pH 7.2), 200 ìl per well, 1 h at room temperature, and washed with wash buffer three times. Then, was added 50 ìl IgY anti-CPV(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 ìl (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 405 nm were measured with a microplate ELISA reader after stopping of the reaction with 2 M HCl solution.

Electrophoresis and Western blotting analysis

To determine the specificity of produced egg yolk antibodies against CPV, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Preparations of CPV strain Cornell 780916-80 (ATCC® Number: VR-2017™) used in this study was suspended at a protein concentration of 1mg/mL in SDS-PAGE lysis buffer (50mM Tris-HCl (pH 6.8), 2% SDS, 10% (vol/ vol), 2% beta-mercaptoethanol, 0.1% bromophenol blue), heated in a boiling water bath for 3 min, and stored at -20°C until used. CPV proteins were separated by SDS-10% PAGE and the gel was electrophoretically transferred to nitrocellulose membrane filters (0.45 ìm pore size; Sigma-Aldrich, USA). Western blot was blocked with blocking buffer (5% w/v instant nonfat milk powder in PBS) and incubated for 1h with the polyclonal anti-CPV primary IgY in blocking buffer. The membranes were washed three times with TBS (pH 7.4) for 5 minutes and subsequently incubated with alkaline phosphatase-coupled rabbit anti-chicken antibodies for 2 h. After thorough washing, the membrane was placed in peroxidase chromogenic substrate solution (0.01M PBS, pH 7.4 with 0.05% 3131-diaminobenzidine, 0.03% nickel chloride and 0.03% H_2O_2) for 15 s, and then immersed in excess distilled water to stop the color development. Finally, the western blot membrane was digitally recorded using a gel documentation system (UVItec, Cambridge, England).

RESULTS

Immune response of hens to canine parvovirus strain Cornell 780916-80

Specific activities of IgY extracted of the egg yolk using chloroform were measured by indirect ELISA (GRAPHIC 1). All the chickens used in these experiments showed a relatively strong immune response against CPV. The chickens were immunized four times with aluminum hydroxide as adjuvant, which were sufficiently immunogenic to induce an immune response. The kinetics of IgY followed the principle of immunity, where the animal response with antibody production after stimulation.[30]

In the first immunization the hens initiate the primary response characterized by the log growth. After this, the response of the second immunization occurred quickly, but doesn't reach a greater magnitude, perhaps due the adjuvant or antigen concentration. The antibody level remains growing after others immunizations. This activity can be visualized in the Graphic 1 where after the first immunization the poultry initiated the antibody production with 0,49 OD and reaching the maximum level at the fortieth ninety day with 1,92 OD. The animals kept these levels for 10 days approximately. After this the curve decline slowly and if the animals were booster again the antibody production grow up. The kinetics of the IgY anti-canine parvovirus strain Cornell 780916-80 was compared with the control.

Hemagglutination test (HA)

The hemmaglutination is method is the clumping of red blood cells that can be caused by some viruses. This process is easily visualized and so has been turned into a method for identifying antibodies in the blood[29]. The canine parvovirus clumped (hemagglutinate) the porcine erythrocyte and this reaction was visible, all the samples were positives.

Indirect ELISA to detect the canine parvovirus in the fecal material

The IgY antibody extracted from egg yolk of immunized hens with canine parvovirus strain Cornell 780916-80 was used for to detect the canine parvovirus in the fecal material. Excrements of eight dogs with characteristic acute signal of the disease were collected and the virus was isolated utilizing a method for dilutions and centrifugation. The control used was of normal dog. Before the ELISA was made the HA test to confirm the presence of canine parvovirus in the fecal material. Eight samples of the eight different dogs were used and in all the samples the canine parvovirus was marked with IgY and the reaction detected by addition of the anti-chicken peroxidase. The reaction was measured by ELISA reader at 405 nm and all the results were greater than the control (GRAPHIC 2). The numbers showed that all the suspected dogs had optical density more than 0.8, while the optical density of the control was less than 0.2. The variation observed between animals can be related with the amount of parvovirus in the enteric tract.

Immunoreactivity of IgY by western blotting

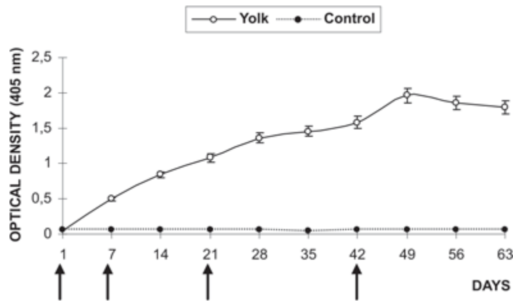
Our objective was to evaluate the potential efficacy of IgY antibody in the diagnostic of CPV. Immunoreactivity of IgY derived from chicken immunized with CPV was determined by western blotting. SDS-PAGE showed differences on protein banding due to viral fragmentation, evidencing the VP1, VP2 and VP3 with 79, 66 and 63 kDa respectively (FIGURE 1). IgY from immunized chickens recognized multiples bands with molecular sizes 79, 66 and 63 kDa (FIGURE 2). This result corroborates that IgY was a valuable tool for the identification of immunogenic antigens of CPV strain Cornell 780916-80 (ATCC® Number: VR-2017™).

DISCUSSION

Development of effective immunization strategies to obtain specific antibodies in chickens is done efficiently by several research groups in the world. However, its application as a diagnostic tool needs to be further studied.

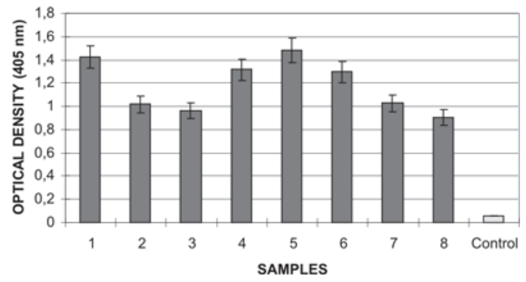
Since 1978, some works demonstrated techniques that developed the usual methods of diagnosis for detection of parvovirus[9,12,31,32]. In 1984, Teramoto and others[9] showed results of a double-antibody sandwich ELISA are compared with DNA hybridization, hemagglutination assay and electron microscopy results. All the works that use serological or fluorescence methods for detect parvovirus, as well commercial diagnostic kits are made with monoclonal antibodies.

The main hypothesis tested in this study was that CPV specific egg yolk-derived immunoglobulins (IgY) are efficient to diagnosis of disease in dogs. Chickens were immunized with CPV strain Cornell 780916-80 to produce IgY in levels easily quantifiable, isolated and stored. So, we development a rapid, simple to perform and sensitive ELISA assay for detect the canine parvovirus in feces. The chicken egg yolk antibody used in the ELISA provided excellent specificity. The results obtained from ELISA were compared with the HA test (TABLE 1).



Graphic 1-The kinetics of IgY titers in hens immunized with canine parvovirus strain Cornell 780916-80 (CPV).

Notes: -The level of IgY activity in a 1000-fold dilution was measured by indirect ELISA using CPV as an antigen and expressed as ELISA value (OD at 405 nm); Values are the mean of six samples (animals); Vertical bars indicate the standard deviation; the arrows indicate the time of immunization.



Graphic 2-Determination of canine parvovirus in fecal material.

Notes: -The presence of the canine parvovirus was determinated by indirect ELISA using as antigen the virus isolated at the dogs with clinical disease and expressed as ELISA value (OD at 405 nm); Values are the mean of four repetitions; Vertical bars indicate the standard deviation.

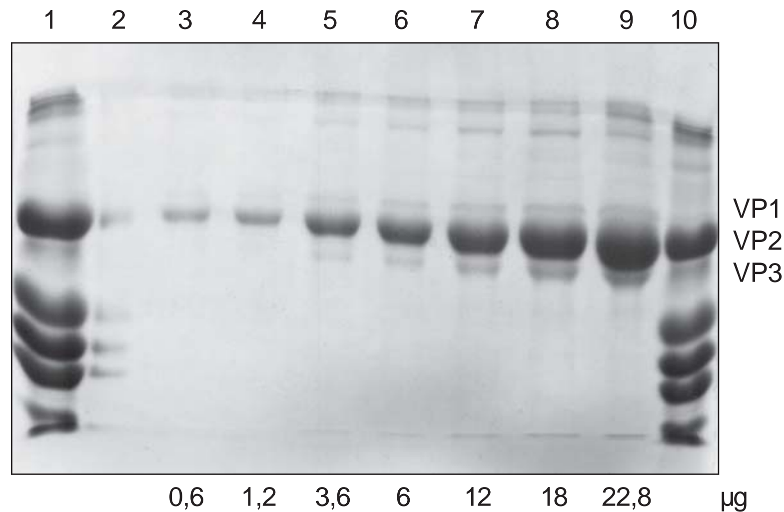


Figure 1- SDS-PAGE analysis of CPV strain.

Notes: - Lane 1, molecular weight marker; Lane 2-10, different dillutions of purified CPV.

Table1-Comparisons between ELISA and HA used for detection of CPV in canine fecal samples.

Results	ELISA	HA	Control (ELISA) (1)	Control (HA) (2)
Positive	8	8	0	0
Negative	0	0	1	1

(1) Negative control, feces of healthy dog; (2) Negative control, feces of healthy dog.

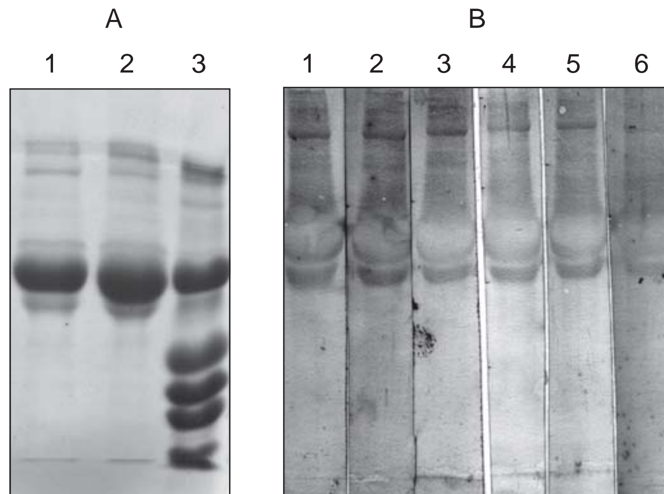


Figure 2 - Detection of viral suspension and confirmation of the specific activity of the IgY.

Notes: - A) SDS-PAGE with viral suspension and weight marker; B) Western blot detection of CPV with anti-CPV IgY.

The use of chicken egg yolk antibody in this work is justified by wide range of advantages above the similar IgG in mammals. We decided use these antibodies because IgY technology is an alternative method concerning animal welfare. In addition hens can be easily kept as laboratory animals and, compared to mammal IgG, chicken IgY is very economic either for diagnosis and therapeutic applications. Furthermore, hens produce a high yield of antibodies comparable to big mammals.[14]

The development the immunological assays with chicken egg yolk antibodies are increasing due some aspects. Several studies have demonstrated that chicken IgY has similar affinity and sensitive to mammal IgG. Moreover, due to phylogenetic distance between birds and mammals, chicken IgY is able to recognize highly conserved mammals proteins and peptides.[14]

Our group believes that IgY technology will offer in few years alternatives and solution to therapy and diagnosis science.

CONCLUSIONS

In all these experiments, the use of polyclonal IgY antibody was determinant, demonstrating its applicability in the diagnosis of infectious diseases. However, despite its general applicability, IgY technology has scarcely been used to date. It seems therefore of great importance to publicize your inherent advantages. Immunization of hens represents an excellent alternative to generate polyclonal antibodies. We believe that we need to increase the application of the tests, but even so, the results corroborate what the literature shows about the IgY. Considering the benefits of IgY application in research and medicine, it is expected that in near future, IgY technology will play an increasing role in research, diagnostics and immunotherapy.

Produção e aplicação da gema do ovo de galinhas para detecção de parvovírus canino em fezes

Resumo

O parvovírus canino (CPV) pode causar uma doença intestinal caracterizada por diarréia sangüinolenta e muitas vezes fatal em filhotes. O vírus é transmitido pelo contato com cães infectados

através das fezes. O vírus é muito estável no ambiente e pode sobreviver por vários meses em áreas contaminadas. O CPV infecta rapidamente as células da medula óssea e intestino delgado. Vários testes laboratoriais foram desenvolvidos e estão disponíveis para diagnóstico viral específico. O diagnóstico rápido pode ser feito por microscopia eletrônica (EM) do material fecal de casos com sinais típicos da doença. O vírus pode também ser isolado de caninos e felino e desenvolvidos em várias linhagens celulares, como células renais canina e felina, mas raramente é usada na prática, já que para culturas de células são necessárias pelo menos uma semana para obtenção dos resultados. A Hemaglutinação e inibição da hemaglutinação (HI-HA) são métodos simples e rápidos para a detecção do vírus em amostras fecais e de tecidos e são contratados por vários laboratórios de diagnóstico, porém, o teste de HA é menos sensível do que EM ou ensaios imunoenzimáticos (ELISA). Neste trabalho, imunizamos galinhas com a cepa Cornell 780916-80 do CPV, isolamos anticorpos policlonais IgY da gema de ovo, caracterizamos sua atividade biológica por ELISA indireto e western blotting. Posteriormente, foram detectados a presença de CPV nas fezes de animais suspeitos através do teste de ELISA indireto e com o teste de HA.

Palavras-chave: Parvovírus canino- Diagnóstico -Anticorpos IgY - ELISA indireto; Parvovírus canino- Diagnóstico- HA-HI.

REFERENCES

1 *POLLOCK, R.V.H. The parvoviruses. I. Feline panleukopenia virus and mink enteritis virus.* ***Compend. Cont. Ed. Pract. Vet.****, Yardley, v.6, n.3, p.227-234, 236, 238, 240-241, 1984.*

2 *APPEL, M.J.; PARRISH, C.R. Canine parvovirus type 2. In: APPEL, M.J. (Ed.).* ***Virus infections of carnivores****. Amsterdam: Elsevier, 1987. p.69-92.*

3 *HOSKINS, J.D. Canine viral enteritis. In: GREENE, C.E. (Ed.). Infectious diseases of he dog and cat. 2nd ed. Philadelphia: W.B. Saunders, 1998. p.40-45.*

4 *AFSHAR, A. Canine parvovirus infections: a review.* ***Vet. Bull.****, Weybridge , v.51, p.605-612, 1981.*

5 *SIEGEL, G. Canine parvovirus: origin and significance of a "new" pathogen. In: BERNS, K.I. (Ed.)* ***The parvoviruses****. New York: Plenum, 1984. p.363-383.*

6 *PARRISH, C.R. How canine parvovirus suddenly shifted host range.* ***ASM News****, Washington, DC, v.63, p.307, 1997.*

7 *YOLKEN, R.H. Use of monoclonal antibodies for viral diagnosis.* ***Curr. Top. Microbiol. Immunol.****, Berlin, v.104, p.177–195, 1983.*

8 *CARMICHAEL, L.E.; BINN, L.N. New enteric viruses in the dog.* ***Adv. Vet. Sci. Comp. Med.****, New York, v.25, p.1–37, 1981.*

9 *TERAMOTO, Y.A. et al. Comparison of enzyme-linked immunosorbent assay, DNA hybridization, hemagglutination, and electron microscopy for detection of canine parvovirus infections.* ***J. Clin. Microbiol.****, Washington, DC, v.20, p.373-378, Sept. 1984.*

10 *APPEL, M.J.; SCOTT, F.W.; CARMICHAEL, L.E. Isolation and immunisation studies of a canine parco-like virus from dogs with haemorrhagic enteritis.* ***Vet. Rec.****, London, v.105, n.8, p.156–159, Aug. 1979.*

11 *GORDON, J.C.; ROGERS, W.A. Field evaluation of a canine parvovirus vaccination program, using feline origin modified live virus vaccine.* ***J. Am. Vet. Med. Assoc.****, Schaumburg, v.180, n.12, p.1429–1431, June 1982.*

12 *LUTZ, H. et al. Monoclonal antibodies to three epitopic regions of feline leukemia virus p27 and their use in enzyme-linked immunosorbent assay of p27.* ***J. Immunol. Methods,*** Amsterdam, v.56, n.2, p.209–220, Jan. 1983.

13 *RICE, J.B. et al. Comparison of systemic and local immunity in dogs with canine parvovirus gastroenteritis.* ***Infect. Immun.****, Washington, DC, v.38, n.3, p.1003–1009, Dec. 1982.*

14 SCHADE, R. et al. Chicken egg yolk antibodies (IgY-Technology): a review of progress in production and use in research and human and veterinary medicine. **Altern. Lab. Anim.**, Nottingham, v.33, p.1-26, 2005.

15 LESLIE, G.A.; CLEM, W.L. Phylogeny of immunoglobulin structure and function. III. Immunoglobulin of the chicken. **J. Exp. Med.**, New York, v.130, p.1337-1352, 1969.

16 SHIMIZU, M.; FITZSIMMONS, R.C.; NAKAI, S. Anti-E.coli immunoglobulin Y isolated from egg yolk of immunized chickens as potential food ingredient. **J. Food Sci.**,Champaign, v.53, p.1360-1366, 1988.

17 WIEDEMANN, V. et al. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. **J. Vet. Med. B. Infect. Dis. Vet. Public Health**, Berlin v.38, p.283-291, 1991.

18 YOKOYAMA, H. et al. Detection of passage and absorption of chicken egg yolk immunoglobulin in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. **Am. J. Vet. Res.**, Schaumburg, v.54, p.867-872, 1993.

19 ROSE, M.E.M.; ORLANS, E.; BUTTRESS, N. Immunoglobulin classes on the hen's egg: their segregation in yolk and white. **Eur. J. Immunol.**, Weinheim, v.4, p.521-523, 1974.

20 ROSE, M.E.M.; ORLANS, E. Immunoglobulins in the egg, embryo and young chick. **Dev. Comp. Immunol.**, Tarrytown, v.5, p.15-20, 1981.

21 JENSENIUS, J.C. et al. Eggs conveniently packaged antibodies: methods for purification of yolk IgG. **J. Immunol. Methods**, Amsterdam, v.46, p. 63-68, 1981.

22 KWAN, L. et al. Fractionation of water-soluble and insoluble components from egg yolk with minimum use of organic solvents. **J. Food Sci.**, Champaign, v.56, p.1537-1541, 1991.

23 AKITA, E.M.; NAKAI, S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic E.coli strain. **J. Immunol. Methods**, Amsterdam, v.160, p.207-214, 1993.

24 SHIMIZU, M. et al. Egg yolk antibody (IgY) stability in aqueous solution with high sugar concentration. **J. Food Sci.**, Champaign, v.59, p.763-765, 1994.

25 SHIMIZU, M. et al. Molecular stability of chicken and rabbit immunoglobulin G. **Biosci. Biotechnol. Biochem.**, Tokyo, v.56, p.270-274, 1992.

26 CARMICHAEL, L.E.; JOUBERT, J.C.; POLLOCK, R.V. Hemagglutination by canine parvovirus: serologic studies and diagnostic applications. **Am. J. Vet. Res.**, Schaumburg, n.41, n.5, p.784–791, May 1980.

27 SHIN, N.R. et al. Control of swine respiratory disease using egg yolk antibodies II. Specificity of immunoglobulin Y of hens immunized with bacterial pathogens related with swine respiratory diseases. **Korean J. Vet. Res.**, Seoul, v.41, p.197-202, 2001.

28 SHIN, N.R.; KIM, J.M.; YOO, H.S. Control of swine respiratory disease using egg yolk antibodies III. Immunoprophylactic effect of IgY in mouse model. **Korean J. Vet. Res.**, Seoul, v.41, p.351-356, 2001.

29 SENDA, M. et al. An improved hemagglutination test for study of canine parvovirus. **Vet. Microbiol.**, Amsterdam, v.12, n.1, p.1-6, 1986.

30 BEHN, I.; HOMMEL, U.; OERTE, M.I. Kinetics of IgY formation after immunization of hens with different protein antigens. **ALTEX**, Heidelberg, v.13, p.18-21, 1996. Suppl.

31 VOLLER, A.; BIDWELL, D.E.; BARTLETT, A. **The Enzyme Linked Immunosorbent Assay (ELISA).** Alexandria, Va: Dynatech Laboratories, 1979.

32 MATHYS, A. et al. Comparison of hemagglutination and competitive enzymelinked immunosorbent assay procedures for detecting canine parvovirus in feces. **Am. J. Vet.** Res., **Schaumburg**, v.44, p.152-154, 1983.

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