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Generation and Characterization of Chicken Eggyolk Antibodies against *Canine parvovirus*-2 Infection

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ABSTRACT

Canine parvovirus type 2 (CPV-2) infections causing severe gastroenteritis in puppies and dogs, spreads by direct contact and through feces contamination. Specific antiviral treatments are lacking and veterinarians are left only to treat the symptoms of disease. Vaccines are ineffective in young puppies. Early recognition and aggressive treatment are very important but most of the diagnostic methods are time consuming and require specific equipment. The present study was conducted as a preliminary step to monitor the efficacy of IgY as a passive immunotherapeutic agent to curb the CPV infection in dogs. CPV vaccine containing 10^3 viral particles/ml was used as antigen to immunize 21 weeks old White Leghorn chicken. Eggs were collected and the IgY was purified from the egg yolk using Polson's method. The molecular weight of the antibody was found to be 180kDa using SDS-PAGE. The protein concentration was found to be 4.8 mg/ml of egg yolk. The titre of antibody production in chicken increases after subsequent immunization and remained constant after 60th day. An extraordinary amount of antibodies can be produced from only one hen, approximately 17-35 gram of total IgY/chicken/year, of which 1-10% can be expected to be antigen specific. The purified IgY was found to be specific to the CPV antigen used which was determined using indirect ELISA. The successful completion of this research work will form a platform to use the specific antibodies for therapeutic benefit as an antiviral treatment of CPV2 in dogs and for early diagnosis.

Keywords: Canine parvovirus, gastroenteritis, IgY, ELISA, SDS-PAGE.

INTRODUCTION

Canine parvovirus (CPV) is one of the most common pathogenic viruses. It causes hemorrhagic enteritis in dogs [7]. It is a highly contagious viral infection. Canine parvovirus infection is an emerging disease in India, and the incidence is showing an increasing trend [2, 23]. The virus can survive in harsh environmental condition for a long time and have resistance for many

disinfectants [15]. The disease causes heavy casualty in dogs, particularly in puppies [6, 22, & 26]. Adult dogs host the virus without displaying clinical manifestation [3]. This facilitates the easy spread of infection. The virus is genetically and anti- genetically related to Feline Panleukopenia virus, Mink enteritis virus and Reccoon virus [17].

The genome is a single-standard negative sense DNA that is 5.2 kb in length.CPV-2 emerged in 1978, presumably originating from FPV through a small number of mutations that allowed the cat virus to replicate in dogs [17]. By the mid-1980's, the original CPV-2 strain was replaced by 2 new genetic variants, CPV-2a and CPV-2b. Currently, CPV-2a and CPV-2b along with CPV-2 care prevalent at different proportions in several countries. However, various studies have reported CPV-2b, as the virus responsible for most outbreaks of CPV infection throughout the world. In India, it has been reported that CPV-2b is the major antigenic variant of CPV-2 along with CPV-2a at a smaller proportion, independently without any distinct geographical patterns of evolution [14].

These pathogenic viruses have high morbidity (100%) and frequent mortality upto 10% in adult dogs and 91% in pups [1]. *Canine parvovirus* infection enters through oronasal route. The clinical manifestation of CPV infection depends on the age and immune status of the animal, the virulence of the virus, the dose of infectious virus and the preexisting or concurrent Parasitic, bacterial or virus infections [5, 13]. Parvoviral enteritis can be controlled by vaccination but it is well known that maternally derived antibodies may interfere with the immune response of the puppies. In order to achieve appropriate protection, it is necessary to complete the vaccination schedule [18]. For diagnosis of canine parvovirus infection, faecal and serum sample are taken for detection of viral particle and antibodies by numerous methods such as ELISA, serum neutralization (SN), hemagglutination inhibition (HI), and indirect fluorescent assay (IFA) tests, virus isolation, electron microscopy, haemagglutination, viral neutralization immunofluorescence, latex agglutination, radial haemolysis test, polymerase chain reaction, in-situ hybridization, RFLP, and one-step immunochromatography assays have been used to detect CPV antibodies in puppies [18].

Specific antiviral treatments are lacking and veterinarians are left only to treat the symptoms of disease [11].Treatments are given to prevent secondary bacterial infections by routine administration of antibiotics, while the dog's immune system will actively fight the virus. Vaccines have been used to prevent CPV-2 infection for many years. Adult dogs can be vaccinated and boosted yearly. However, the vaccines are, ineffective in young puppies owing to the presence of maternal antibodies in the puppies' blood. As maternal antibody levels wane, the puppies become susceptible to infection by virus in the contaminated environment [15].

Recently the utilization of Immunoglobulin Y (IgY) from eggs of chickens, which were immunized against certain pathogens, has been the focus of attention in immunotherapy and immunodiagnosis, since the birds can actively transfer immunoglobulin G (IgG) to the egg yolks of their offspring in the same levels of concentration as in mammals [24]. Chicken antibodies can be easily sampled by non-invasive methods, instead of the stressful bleeding of animals. An extraordinary amount of antibodies can be produced from only one hen, approximately 17-35 gram of total IgY per chicken per year, of which 1-10% can be expected to be antigen specific. This huge quantity of available antibodies opens the door for new fields of IgY applications, such

as immunotherapy and immuno prophylaxis applied to several viral and bacterial infections in veterinary and human medicine. In addition, IgY antibodies have no cross reactivity with rheumatoid factors or human anti-mouse antibody. IgY antibodies are unable to activate the mammalian complement system and have no heteroagglutinins [24].

Authors of recent paper suggested that antibody produced from phylogenetically lower animal's species should be preferred in construction of ELISA test for diagnosis of mammalian infectious disease [18]. Thus IgY is valuable for many laboratory diagnosis in which IgG is not suitable [25]. Treatment for young puppies gone vain of vaccination and infected animals can undergo passive immunization by means of oral administration of immune colostrum or immunoglobulins derived from chicken egg yolk (IgY) raised against *canine parvovirus-2*. These IgY reaches gastrointestinal tract and inhibits virus attaching to intestinal epithelial cells. Thus administration of specific IgY may be useful in the treatment of dogs with clinical disease due to CPV-2 [15].

EXPERIMENTAL SECTION

Canine parvovirus vaccine

Canine parovirus antigen was obtained in the form of *Canine parvovirus* Vaccine Inactivated IP (Magavac-P), a commercially available product of Indian Immunological Limited. This vaccine contains tissue adapted strain of Canine parvovirus grown on A-72 cell culture and inactivated with beta-propiolacton containing 10^3viral ppm.

Experimental animal and immunization

Canine parvovirus vaccine dosage (having 10^3 viral particle /ml) was immunized to 21 weeks old White Leghorn hens by injecting into their breast muscles at four different sites [18]. After first immunization, followed by 4 booster dosage were periodically (one week interval) administered (Table 1). After immunization the eggs were collected and stored.

Purification of antibodies from eggs yolk

The egg yolk antibodies were purified according to the method described by Polson *et al*., [20]. The egg yolks were separated and washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The yellow yolk without the adhering membrane was allowed to flow into the graduated measuring cylinder and mixed well with equal amount of buffer-"S" (with NaCl). To the resulting mixture 10.5% PEG 8000 in buffer-"S" was added to a final concentration of 3.5%. The mixture was transferred to a 250 ml Erlenmeyer flask and homogenized in an orbital shaker at 100 strokes min^{-1} for 30 minutes in room temperature. The homogenized mixture was centrifuged at 11,000 rpm for 20 minutes. The supernatant was filtered through double-layered cheesecloth and 42% PEG in buffer – "S" (with NaCl) was added to make final concentration of 12% PEG. Then the mixture was homogenized for 30 minutes and centrifuged at 11,000 rpm for 20 minutes. The pellet was redissolved in buffer – "S" (with NaCl) to the original yolk volume. An equal volume of 4M Ammonium sulphate (pH 7) was added with the redissolved pellet and incubated. The precipitates were centrifuged again and redissolved in buffer- "S" (without NaCl). Purified IgY were desalted by dialysis against buffer "S" (without NaCl) to remove ammonium sulphate. Finally, the contents were removed from the dialysis membrane bag after complete removal of Ammonium sulphate. The dialysed IgY fraction is then subjected to further purification by column chromatography.

Separation of IgY by DEAE Cellulose Column Chromatography

The chicken egg yolk antibodies were purified by DEAE cellulose Ion Exchange Column Chromatography. 25-30grams of DEAE sephadex A-50 cellulose (pre-swollen with distilled water) was added to a beaker and treated with 0.1N NaOH and 0.1N HCl as per procedures until there was a neutral reaction. For purification of immunoglobulin, the column size 2x30 cm was packed first with glass wool to form an even bed and a rubber tube with pinch-cock was attached to the tip of the burette. The column was fixed to stand in vertical position. The DEAE slurry was poured into the column along the sides to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer, pH 8.0. The IgY sample layered on the top of the column and was allowed to run till all the sample had entered the bed. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the un-retained protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0. The protein content of the eluent was estimated by Lowry procedure. The pooled IgY fraction transferred in a dialysis bag was concentrated using polyvinyl pyrolidone [PVP] placed in a Petri-dish at room temperature. The IgY concentration in terms of total protein content was determined and monitored using Lowry's method. The concentrated antibody fraction was then lyophilized and the IgY powder stored under -20°C until further use.

Protein profiling by SDS-PAGE

The protein profile of Anti *Canine parvovirus* IgY antibodies were analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli [9]. The antibody was separated with a 4% stacking and 12% separating gel. IgY were taken (10µl) and treated with an equal amount of sample treatment buffer. The samples were loaded into sample wells along with a known molecular weight markers (Genei Pvt. Ltd, Bangalore) and the electrophoresis was performed at 100V current. When bromophenol blue dye reached at the bottom of the gel, the electrophoresis was stopped. The gel was removed and was stained with coomassie brilliant blue R-250 for 30 minutes. The gel was rinsed with distilled water and destained. The molecular weight of the proteins resolved was estimated in comparison to the molecular weight markers.

Detection of canine parvovirus using ELISA

The immunological specificity of IgY elaborated against *Canine parovirus* antigen was examined by Enzyme-Linked Immuno Sorbent Assay (ELISA) [18]. Polyvinyl micro titration plates were coated with 100µl of the *canine parvovirus* antigen that was diluted with 0.05 M carbonate buffer pH 9.6 and incubated at 4°C overnight. After coating the plates were washed with PBS containing 0.05% tween20 (PBST) and nonspecific binding sites blocked by adding 200µl per well of 1% bovine serum albumin in PBS and incubating the plates at 37°C for 1 hour. Plates were subsequently washed with PBST and incubated with 100µl of either polyclonal chicken antibodies or egg yolk antibodies at appropriate dilutions. Control wells had PBST and pre immune sera served as respective controls. Plates were incubated for one hour at 37°C and subsequently washed with PBST. For the chicken antibodies 100µl of rabbit anti chicken immunoglobulin coupled to horseradish peroxides was added at appropriate dilutions and plates incubated for 1 hour at 4°C. After incubation the plates were washed with PBST and enzyme activity determined by adding 100µl of freshly prepared substrate solution (4mg of O-phenylenediamine dissolved in 10ml of 50mM citrate buffer, pH5.0 containing 10µl hydrogen peroxide). And the plates were allowed to stand at room temperature (dark condition) for 15

minutes. The reaction was stopped by adding 50µl of 4N H_2SO_4 and plates were read at 490nm in an ELISA reader [10].

RESULTS

Detection of IgY antibodies in eggs from inoculated hens

The IgY concentration in terms of total protein content was determined and monitored from the eggs collected before and after each immunization. Protein content of IgY antibodies were estimated using folin-ciocalteau reagent [12]. The optical density of the BSA standard and total protein concentration of the IgY powders was used to plot the graph. The IgY content increased at each booster dose and remained stable after the fourth booster till the 150th day which showed the maximum protein content of 4.8 mg IgY per ml (Table 2).

Purified IgY estimation by DEAE Cellulose Column Chromatography

In DEAE Cellulose Column Chromatography the sample was eluted and fractions were collected. The protein assay was done to all the samples to find the specific IgY concentration. The eluate was collected in aliquots of 5ml and the specific IgY was observed in the 2nd-4th fractions and it was determined by SDS-PAGE.

Characterization of semi-purified antibody

The purity of chicken egg yolk antibodies and their molecular weight were determined by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE),using 12% gel according to the method of Laemmli [9]. IgY was found to be of high molecular weight about 180 KDa. The standard protein marker was also run parallel along with the antibody sample (Fig 1).

Analysis of specificity and dynamics of antibody generation

One of the objective of this study is to find the specificity and dynamics of antibody raised against Canine parvovirus after periodic boosting the immune system of chickens. Partially purified samples are taken for analyzing antibodies, through ELISA method with duplicates. The titrations of specific antibody raised were measured through optical density at 492 nm. Periodic immunization of chickens raises the antibody concentration (anti-CPV IgY) in egg yolk, constantly (Fig 2). The specificity of the antibody to the antigen was also monitored using ELISA which shows the antibody titre to a maximum dilution of 1:10000.

DISCUSSION

Canine parvovirus type-2 (CPV-2) is one of the major diarrhea-causing agents, inducing acute hemorrhagic gastroenteritis in puppies. Canine parvovirus (CPV) infection is one of the most important viral diseases in dogs. CPV-2 is highly contagious and often fatal disease in dogs throughout the world. Vaccine trials demonstrate that current commercial vaccines containing CPV-2 or CPV-2b can provide protective immunity against CPV-2c also [14].

Recently the utilization of Immunoglobulin Y (IgY) from eggs of chickens, which were immunized against certain pathogens, has been the focus of attention in immunotherapy and immunodiagnosis, since the birds can actively transfer immunoglobulin G (IgG) to the egg yolks

of their offspring in the same levels of concentration as in mammals. The ELISA methodology affords the opportunity to provide quick, accurate in-hospital results**.**

Table 1: Immunization schedule

Dosage Schedule	Days	Antigen dose (10^3cells/m)	Site of Injection
1st Dose	0th	1ml	Intramuscular
1st Booster	14th		
2ndBooster	21st		
3rdBooster	28th		
4thBooster	35th		

Table 2: Estimation of protein content of Partially Purified IgY powder

Tube. No	Volume of BSA standard (ml)	BSA concentration (µg)	Optical Density (OD) at 640 nm.	Protein content (mg/ml)
1	Blank	0	0.00	0.00
2	0.2	20	0.26	10.5
3	0.4	40	0.48	20.0
4	0.6	60	0.78	30.5
5	0.8	80	0.92	36.0
6	1.0	100	1.38	60.0
7 (Sample)	0.2	_	1.20	4.8

Table 3: Estimation of Protein concentration in Salted out antibody fractions of Chicken egg yolk

Egg collection (Days)	Total Protein Concentration (mg/ml)
Pre-immune egg yolk	0.3
Day 15	0.56
Day 30	0.72
Day 45	1.31
Day 60	1.80
Day 75	2.36
Day 90	3.87
Day105	3.25
Day 120	4.01
Day 135	4.36
Day 150	4.8

21 weeks old white chickens were immunized by injecting the antigen into its breast muscles at two different sites. The chickens received booster injections with the same antigen at 14 days interval. The eggs were collected and the antibodies were purified from egg yolk using Polyethylene glycol and Ammonium sulphate precipitation method.

The activity and titer of egg yolk antibodies were determined through competitive ELISA at 490 nm [10] showed that the presence of antigen specific antibodies for the *Canine parvovirus*. ELISA is a sensitive technique to find out the antibody titers during different purification processes. The antibody concentration increases as the booster dosage increases. The objective

study of purity, characterization, production rate of specific IgY, against CPV antigen were found effective. Thus, purified anti-CPV IgY can be used for diagnosis of CPV infection. The sensitivity of indirect ELISA systems for the detection of serum antibodies to CPV, CCV and rotavirus was about 2-10 times higher than conventional methods such as HI, VN and CF [4, 21].The egg yolk antibodies were further purified by DEAE cellulose ion exchange column chromatography .The molecular weight of the purified IgY's were confirmed as 180 kDa through SDS PAGE [9].

Fig 1: Characterization of semi-purified antibody

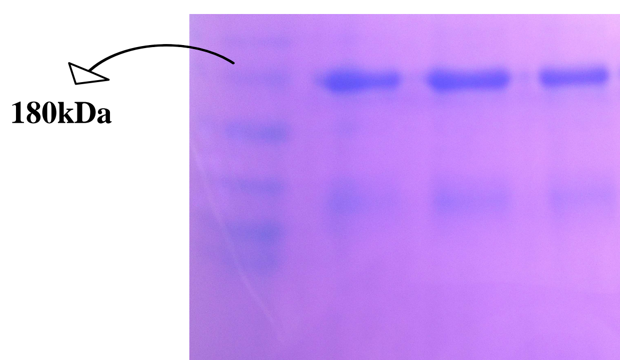
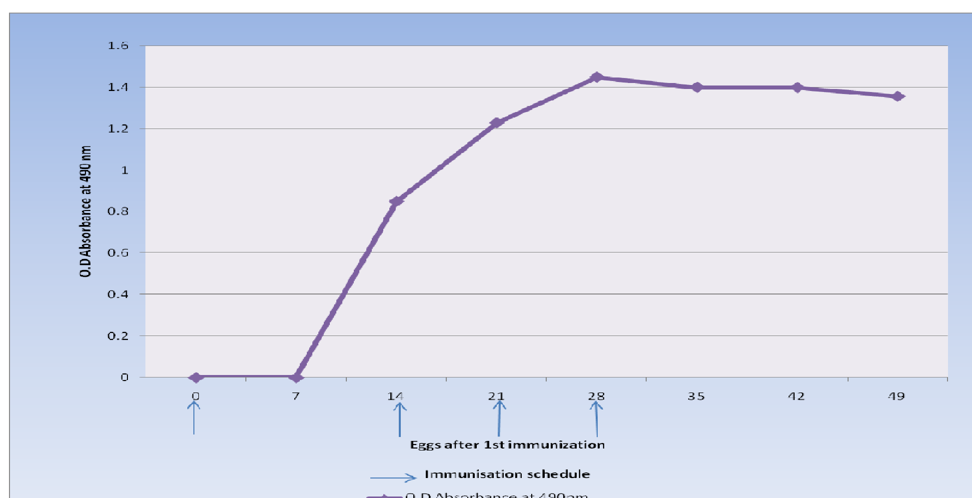


Fig 2: Dynamics of antibody production in egg yolk using indirect ELISA



Oral administration of IgY antibodies specific for CPV-2 do not interfere with the immune response elicited by vaccine and serve to act locally in the intestine similar to lactogenic immunity. Protection conferred did not necessarily prevent infection but, rather, limited the infection to subclinical status. Similar results have been reported with experimental *Rotavirus, Coronavirus* infection [8].

Thus IgY technology in biomedical research has been growing in recent years. It can be concluded that use of IgY in ELISA for diagnosis of *Canine parvovirus-2* infection gives reliable results. This is similar to that concluded by Pokarovo and Nguyen [15, 18]. To undergo trial on

passive immunization in experimental animals, the purity of specific antibody (anti-CPV IgY), antigen-antibody neutralization test are essential.

REFERENCES

[1] MJG Appel; BJ Cooper; H Greisen; LE Carmichael. *J. Am. Vet. Med. Assoc.,* **1978**, 173,1516-18.
[2] SR Chinchkar; B Mohana Subramaniam; N Hanumantha Rao; PN Rangarajan; D Thiagarajan; VA Srinivasan. *Arch. Viral.*, **2006**, 151, 1881-1887.
[3] JS Ettinger; CE Feldman. Text Book of Veterinary Internal Medicine- Diseases of the Dog and Cat. 4th Edition, Eleseiver Inc, Bibliotheque nationale, Paris, **1995**; 646-647, 1354-1355.
[4] LH Ghose; RD Schnagl; IH Holmes. *J. Clin. Microbiol.*, **1978**, 8, 268.
[5] MK Hagiwara; EM Mamizuka; MF Pavan. *Braz. J. Vet. Res. Anim. Sci.*, **1996,** 33, 107-109.
[6] PB Kamalu. *J. Small Anim. Pract.,* **2008**, 26, 663-668.
[7] WR Kelly. *Aust. Vet. J.,* **1978**, 54, 593
[8] M Kuroki; M Ohta; Y Ikemori; RC Peralta; H Yokoyama; Y Kodama. *Arch. Virol.*, **1994,** 138,143–148.
[9] UK Laemmeli. *Nature.,* **1970**, 227**,** 680 - 685.
[10] KA Lee. *J. Biochem. Mol. Biol.*, **2002,** 35, 488-493.
[11] J Lizer. *Vet. Microbiol. Prev. Med.*, **2008,** 587**.**
[12] OH Lowry. *J. Biol. Chem.,* **1951,** 193, 265-275.
[13] P Mcadaragh; SL Eustis; DT Nelson. *Am. J. Vet. Res.*, **1982**, 43, 693-696.
[14] S Nandi; S. Chidri, M Kumar. *Veterinarni Medicina.* **2009,** 54,483–490.
[15] SV Nguyen; K Umeda; H Yokoyama; Y Tohya; Y Kodam. *Can. J.* Vet. *Res.,* **2006,** 70, 62-64.
[16] ADME Osterhaus; GA Drost; RMS Wirahadiredja; TSGM Van den Ingh. *Vet. Quart.,* **1980,** 2,181.
[17] CR Parrish. *Vet. Microbiol.* **1999**, 69, 29-40.
[18] D Pokorova; J France; J Stepanek. *Vet.Med.Czech.,* **2000**, 45(2), 49-54.
[19] RVH Pollock; MJ Coyne. *Vet. Clin. North Am. Small Anim. Pract.*, **1993**, 23, 555-569.
[20] A Polson; MB von Wechmar; G Fazakerley. *Immunol.Commun.,* **1980,** 9, 495.
[21] GF Rimmelzwaan; N Juntti; B Klingeborn; J Groen; FGCM UytdeHaag; ADME Osterhaus. *Vet. Quart*. **1990**, 12, 14-20.
[22] M Sabine; L Herbert; D Love. WHO, AAS and ASM, Canberra, **1982**, 657- 660.
[23] R Sanjukta; L Maheshkumar; B Nagappa; B Joken; I Karuna; S Mandakini. *TANUVAS*, Compendium and Souvenir, **2008**, 65.
[24] R Schade; A Hlinak. *ALTEX*, **1996**, 13(1), 5-9.
[25] R Schade, EG Calzado; R Sarmiento; PA Chacana; J porankiewicz Asplund; H Terzolo, *Altern Lab Anim*, **2005,** 33(2), 129-54.
[26] T Waner; S Mazar; E Keren-Kornblatt. *J. Vet. Diagn. Investig.*, **2004**, 18, 267-270.