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Generation, Purification and Characterization of Avian Egg yolk antibodies against Canine parvovirus – 2

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Abstract:

Canine Parvovirus -2 (CPV – 2) is one of the most commonly occurring viral disease of young dogs less than 2 months old causing high morbidity and mortality rate. Only supportive care is given to the affected animals due to the non-availability of specific antiviral treatment for CPV -2 infections. Hence, there is an urgent need to develop novel approaches for treating the animals. An alternate method of treatment which is specific to any disease is passive immunization with polyclonal antibodies. Considering the advantages of avian egg yolk antibodies (IgY) when compared with the mammalian antibodies, several studies are focused on the use of specific IgY antibodies for passive therapy. The present study has been undertaken with the aim of generation of specific avian egg yolk antibodies (IgY) against CPV – 2 vaccine strain using BOVANS strain of White Leghorn chicken. The kinetics of the purified IgY against CPV -2 by Sodium chloride is determined based on the total protein content and specific CPV -2 antibody HI titer. The purity and the virus neutralization capability of the purified specific IgY was determined by SDS PAGE and by *in-vitro* assay using A-72 cell line respectively. The HI titer of specific CPV -2 antibody titer in the chicken serum reached maximum (Chicken 1 - 512 & Chicken 2 - 256 HI units) in 30th week of their age and started declining gradually after 32 weeks of age. The HI titer of the purified IgY against CPV – 2 reached maximum at 27th week of age. The titer of specific CPV – 2 antibodies maintained up to 32 weeks and declined gradually from 33 week onwards. The birds were given booster doses intermittently based on the antibody titer to increase its levels in the egg yolk. In-order to maintain a high titer of specific antibodies in the egg yolk against any antigen, the birds shall be administered booster doses of antigen based on the antibody titer. The neutralizing ability of the generated and purified IgY antibodies against the field isolate of CPV -2 strain demonstrated that the generated antibodies can be used for passive therapy by a suitable parenteral route of injection based on the clinical trials.

Keywords: CPV – 2 Avian IgY antibodies, Canine Parvo Avian Egg yolk (IgY) antibodies, Passive therapy, CPV – 2 HI titer, CPV -2 Neutralization assay

1. Introduction

Canine parvovirus (CPV-2) is one of the most important pathogen of canines emerged in the year 1978. The occurrence of this virus has been reported from America, Asia, Australia, Europe and New Zealand. It was reported that the two types of canine parvo viruses, CPV- 2 and CPV – 1 are

infecting canines (Nandi and Kumar, 2010**).** This virus is highly contagious in nature, spreads through fecal - oral route or oro - nasal route. They are extremely stable in the environment and probably readily transmitted across long distances by fomites.

The rapid replication of CPV – 2 in the host is totally relies on the presence of actively proliferating cells like intestinal crypts and lymphoid organs (Bloom & Kerr, 2006). Due to this tropism, the most common clinical signs are vomitting and diarrhea in association with anorexia, depression and fever. The loss of fluid and protein through the gastrointestinal tract leads to severe dehydration and hypovolemic shock. The other gastrointestinal signs are lymphopenia and eventually panleukopenia. The breakdown of the intestinal barrier facilitates the passage of bacteria and or endotoxins in the blood stream of the dog results in septicemia (Goddard and Leisewitz, 2010). Coagulation disorders and septic shock are therefore commonly present in CPV – 2 infected animals and contribute significantly to the disease severity and lethality (Ramsey, 2008 and Schoeman, *et al*., 2013).

This virus causes immune suppression due to lymphocytolysis and depletion of lymphocyte precursors in the bone marrow (Hoskins, 1998). It also causes acute hemorrhagic enteritis and myocarditis. The morbidity of the virus is 100% and the mortality is up to 10% in adult dogs and 91% in pups (Nandi and Kumar, 2010).

The clinical infection of CPV-2 is predominantly prevented by humoral immune response. The antibodies produced by this immune response are capable of neutralizing most of the viruses. Maternal antibodies play a vital role in preventing the pups from parvoviral infection. Infections occur predominantly in young animals after maternal antibodies declined to low levels (Hoskins, 1998 and Larson and Schultz, 1997).

The high cost treatment of CPV - 2 infection places the practitioner and the owner in a difficult situation. Since there is no antiviral treatment which is specific for CPV, only the supportive care is provided to the suffering pups. Therefore, an urgent need to develop novel approaches to treat and manage CPV -2 infections. An alternate method of treatment is passive immunization with polyclonal antibodies and it is possible to avoid any side effects that might result from vaccination. Due to various advantages of IgY antibodies when compared with mammalian antibodies, much research works are being carried out with the objective of effective usage of IgY antibodies for passive immunization applications (Jennifer *et al.,* 2012).

Use of IgY antibodies has been an accepted practice since 1996. The Veterinary Office of the Swiss Government (Office Vétérinaire Fédéral) approved this practice in 1999. Oral administration of specific egg yolk antibody is an attractive approach against gastrointestinal infection in humans and animals (Barati *et al*., 2016).

Prior to their usage the egg yolk antibodies have to be purified. The commonly used purifying agents are Ammonium Sulphate (Akita and Nakai, 1992, Tong *et al*., 2015 and Ko and Ahn, 2007), Poly Ethylene Glycol (Pauly *et al*, 2011), Sodium Chloride (Petr *et al.,* 2013) and Capralyic acid (Yan Brodsky *et al*., 2012). The specific antibody titer in the purified IgY shall be determined either by HI (Carmichael *et al.,* 1980.), ELISA (Ko and Ahn. 2007) or by Virus neutralization (Briggs *et al.,* 1998) tests.

The protective effect of immunoglobulins derived from chicken egg yolk (IgY) against the infection by CPV-2 isolate was evaluated in beagle dog model by challenging the animals orally with a pathogenic strain of CPV -2. The animals that received IgY powder with CPV – 2 antibodies did not exhibit any symptoms or exhibited very mild symptoms based on the dose given, whereas the control group animals exhibited classical sign of CPV – 2 infection. These results indicate that the IgY antibodies are useful in protecting the dogs from CPV-2-induced clinical disease (Nguyen *et al*., 2006). The similar type of observation was reported by Naveenkumar *et al*., 2019.

The effectiveness of intravenous (IV) IgY immunotherapy was tested in dogs after oral challenge with a highly pathogenic CPV – 2 isolate by Gusti *et al.,* (2014). The recovery rate for the dogs treated with 1,000 and 10,000 PD were 25% and 100%, respectively. The higher dose was more effective in suppressing the infective virus excretion in stool.

In the present study the kinetics of the specific IgY antibodies raised against CPV -2 by Sodium chloride method was assessed in terms of total protein content, HI titer and Virus neutralization tests.

2. Materials and Methods

2.1 Chemicals and reagents

In the present work the chemicals used were of analytical grade and procured from reputed manufactures Water for injection (WFI) was used for the preparation of reagents and buffers.

2.2 Glassware and consumables

All Glassware were washed well and rinsed with Water for Injection (WFI). They were sterilized in hot air oven and autoclave before starting the experiment.

2.3 Birds

To generate specific avian egg yolk antibodies against CPV - 2, three numbers of thirteen weeks old BOVANS strain of White leghorn (Mediterrarian) breed were procured from a local commercial poultry farm. Two birds were used for immunization and one bird was used as control.

2.4 Immunization

Immunization procedures were followed as described by Sankareswaran *et.al*, (2011) with slight modification in the age group of birds. Instead of 21 weeks old birds, 18 weeks old birds were used for immunization as the egg laying period of the birds starts from 19 to 21 weeks of their age (Silveira *et.al.,* 2014). Each bird was immunized with 1.0mL of commercially available inactivated, Aluminium Hydroxide adjuvanted CPV- 2 vaccine containing 10^6 viral particles (based on the label claim). This vaccine was administered at 4 different sites of their breast muscles as this route is the most preferable route of immunization which can elicit higher antibody response when compared with antigens immunized through subcutaneous route (Chang *et.al.,* 1999).

2.5 Collection and storage of eggs

Eggs were collected from each bird separately from 21 weeks onwards. The eggs were labeled with the details of identification number and date of collection. The collected eggs were stored at 4°C till their processing as the IgY antibodies are stable in the egg yolk under refrigerated conditions for at least 12 months (Larson *et.al*. 1991).

2.6 Maintenance, Preservation and Storage of A - 72 cell line

A-72 cell line was obtained from “Centre for Animal Health Studies, Madhavaram Milk colony, TANUVAS” in a 25 cm^2 flask. For this work DMEM (Lonza, CAT. No: BE12-604F), Trypsin EDTA (Hi-Media, CAT. No. TCL048) and Bovine serum (Cell clone, CAT. No. CCS – 500-S-A-U) were used. The propagation, maintenance, preservation and storage of cell line was carried out as per the standard protocols (McAteer, J.A and Davis, J. 1994).

2.7 Propagation and Preservation of CPV – 2 field isolates

Two field isolates of CPV -2 were obtained from “Centre for Animal Health Studies (CAHS), Madhavaram Milk colony, TANUVAS”. They were propagated in A 72 cell line. When 90% CPE was observed, the flasks were kept at -80º C for 1 to 2 hrs for freezing. After freezing the flasks, the fluid was thawed at 4º C. Like this freeze thaw was carried out for 5 times to extract the viruses effectively from the infected cells. After freeze thawing the infected fluid was collected in the sterile centrifuge tubes and centrifuged at a RPM of 1200 RPM for 10minutes to remove the cell debris. The clear fluid was aliquoted in 0.5mL quantity in sterile microfuge tubes and stored at -80º C till further usage.

2.8 Haemagglutination (HA) Test

The chicken and porcine RBCs were prepared as per the procedures described by Praveenkumar et al., and the HA test was carried out as per Carmichael *et al*., (1980). Two field isolates of CPV -2 obtained from CAHS were tested for their HA activities. The positive HA result was indicated by the absence of button formation. The HA titer was expressed as the reciprocal of highest dilution of virus showing agglutination.

2.9 Haemagglutination inhibition (HI) Test

The haemagglutination inhibition (HI) assay for chicken sera samples and purified IgY was carried out as per the procedures described by Carmichael *et al.,* 1980 with small modification using

CPV -2 field isolate number 2 as this virus strain was sensitive to chicken RBC. The HI titer was expressed as the reciprocal of highest serum dilution inhibiting the haemagglutination reaction.

2.10 Egg Processing

Based on the sera HI titer for CPV – 2, eggs of bird number 1 were processed. Eggs laid for a week were pooled together for processing. The eggs were cleaned by wiping its outer surface with sterile 70% IPA. Egg shell was carefully removed. Albumin was discarded and the remaining albumin was removed by rolling the yolk on a sterile non-shedding wipes. The egg yolk was carefully separated in a beaker and washed with water for injection for 3-5 times and placed on sterile non-shedding wipes to remove the excess water. Yolk membrane was punctured and the yolk was collected in a measuring beaker to measure its volume.

2.11 Purification of IgY by Sodium Chloride Method

WFI is added seven fold to the measured egg yolk volume and thoroughly mixed until the egg yolk is completely dissolved. pH of the mixture was checked and adjusted to 5 by using 0.5 M HCL. The mixture was left overnight at -20°C for freezing for proper delipidation. The frozen sample was kept for thawing at 4°C. Thawed sample was centrifuged at 11000 rpm for 20 minutes at 4°C. Pellet was discarded and supernatant was taken for filtration. Three layered cotton was placed over the funnel and the supernatant was filtered. This portion is called as water soluble fraction. Volume of the filtered sample was measured and 8.8% Nacl (Spectrum, CAT. No. S0108) was added to this. pH of the sample was adjusted to 4.0 by using 0.5M Hcl. The sample was incubated for 2 hrs at 4°C in shaking incubator. The sample was centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was dissolved in PBS of $1/10^{th}$ of yolk volume. The sample was kept for dialysis at 4°C until the salt is completely removed. After dialysis the required quantity of purified IgY was filtered by using 0.2µ syringe filter for neutralization assay and stored at -80°C. The remaining quantity was stored at 4°C for further analysis.

2.12 DOT Blot

In-order to ensure that the generated IgY antibodies are against CPV – 2, a dot blot assay was performed. For this adequate size of Nitrocellulose membrane (Amersham Life Science – Hi Bond – C Super; Pure NC, 0.45µ) was wetted in Tris Buffer Saline (TBS) and placed over the TBS wetted Whatmann filter paper**.** The wells in the lower part of the apparatus other than the sample injection wells were covered using the hydrophobic sticker. Thus the membrane over the filter paper is sandwiched between the upper and lower part of dot blot apparatus. The apparatus was connected to vacuum pump for suction. Once the set up was done, 50 µL of CPV - 2 antigen was loaded. After loading, the vacuum pump was switched on to absorb the sample and the controls on the membrane. Then the whole apparatus was dismantled and the membrane is separated out. The membrane was dried by incubating it at 37˚C. The dried membrane was blocked by incubating the membrane in blocking solution (2% BSA in TBS) at 37˚C for 45 minutes. Then the membrane was washed 2 to 3 times in TBS. The washed membrane was once again mounted in the DOT blot apparatus as described above and 50µLof positive serum, purified IgY contains specific CPV – 2 antibodies and negative control IgY was loaded. Then the membrane was washed with washing buffer. After washing the membrane was incubated in TBS (with 2% BSA) containing Rabbit Anti chicken IgY (Sigma, CAT. No. 12341), the secondary antibody at 37˚C for 1 hour. After the completion of the incubation time the membrane was washed using the washing buffer for 2 to 3 times. After washing the washed membrane was incubated in TMB substrate (Sigma, CAT. No. T8665) until the formation of dark color.

2.13. Determination of $CCID_{50}$

The Cell culture infective dose ($CCID_{50}$) for CPV -2 field isolate 2 was carried by titration of the virus in the 96 well cell culture plates. A series of 10 fold dilution of virus was carried out up to $10^{-6.0}$ and from that dilution fold two fold serial dilutions were carried out up to $10^{-7.5}$. 50µL of DMEM with 10% foetal calf serum was added to all the wells. 100µL of virus from the dilution series $10^{-6.0}$ to $10^{-7.5}$ was added to each of 10 wells. After the addition of virus, 100µL of A-72 cells were added at the concentration of 6000 cells / well. The plates were incubated at 37°C for 5 days in a CO_2 incubator

maintained at 5% CO_2 and RH of 99%. The plates were observed every day for cytopathic effect (CPE). Fifty percent end points were calculated using Reed and Munch formula.

2.14 Total Protein Estimation

The total protein content (mg/mL) of the purified IgY was measured photometrically at 280nm and calculated according to Lambert – Beer law with an extinction coefficient of 1.33 for IgY (Pauly *et.al*., 2011).

2.15 Purity by SDS PAGE

The purity of the purified IgY antibodies were tested by Sodium dodecyl sulfate-PAGE method. The test was carried out under reducing conditions using Mini-PROTEAN II Cell (BioRad) as per the procedures described by Ko and Ahn 2007. The purity of various IgY preparations was estimated using 10% SDSPAGE, and Coomassie brilliant blue R-250 (BioRad) was used to visualize the protein bands. Medium - range SDS PAGE molecular weight marker of 16 to 98 kDa (Geni, Bangalore, CAT No.3 PPMWM 112375001730) was used.

2.16 Virus Neutralization Assay

The neutralization ability of the purified IgY antibodies raised with vaccine strain against field isolate of CPV – 2 was determined by virus neutralization assay using 100 $CCID_{50}$ of CPV -2 field isolate as described by Kouji *et al.,* 2006. In this assay 10 fold serial dilutions of purified IgY was carried out up to $10^{-3}.^{0}$ Thereafter 5 fold dilution was carried out till $10^{-8.0}$. 50µL of DMEM with 10% foetal calf serum was added in all the wells. Then 50 µL of serially diluted antibodies were added into 10 wells for each dilution. After this 50µL of 100 $CCID_{50}$ virus was added in all the wells. The plate was incubated for 1 hr at 37°C in a CO_2 incubator maintained at 5% CO_2 and RH of 99%. After 1 hr of incubation 100µL of A-72 cells were added at the concentration of 6000 cells / well. The plates were incubated at 37°C for 5 days in a CO_2 incubator maintained at 5% CO_2 and RH of 99%. The plates were observed every day for cytopathic effect (CPE). Fifty percent end points (PD_{50}) was calculated using Reed and Munch formula.

3. Results

3.1 HA titer of the field isolates of CPV -2

The HA titer of different viral harvest of CPV -2 field isolates were presented in table -1. The results indicate that, the isolate number 1 can react only with Swine RBC, whereas, the isolate number 2 can react with both Swine and Chicken RBCs. Hence isolate number 2 was used to estimate the HI titer of both serum and purified IgY antibodies.

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Table 1: HA Results of CPV -2 field isolates with Swine RBC and Chicken RBC

Isolate No.	Passage level	HA Titer (Log 2)	
		Swine RBC	Chicken RBC
1	VP0	2^9	Nil
	VP1	2^8	Nil
	VP2	2^8	Nil
	VP3	2^8	Nil
2	VP0	2^7	2^7
	VP1*	2^6	2^7
	VP1*	2^8	2^8
	VP1*	2^7	2^7

VP0 – Virus fluid obtained from CAHS, VP1, 2 & 3 – Virus Passages, * Viral fluid harvested on different days.

3.2 Immunization Schedule

The immunization schedule followed for the entire study was presented in the table – 2.

Table 2: Immunization Schedule

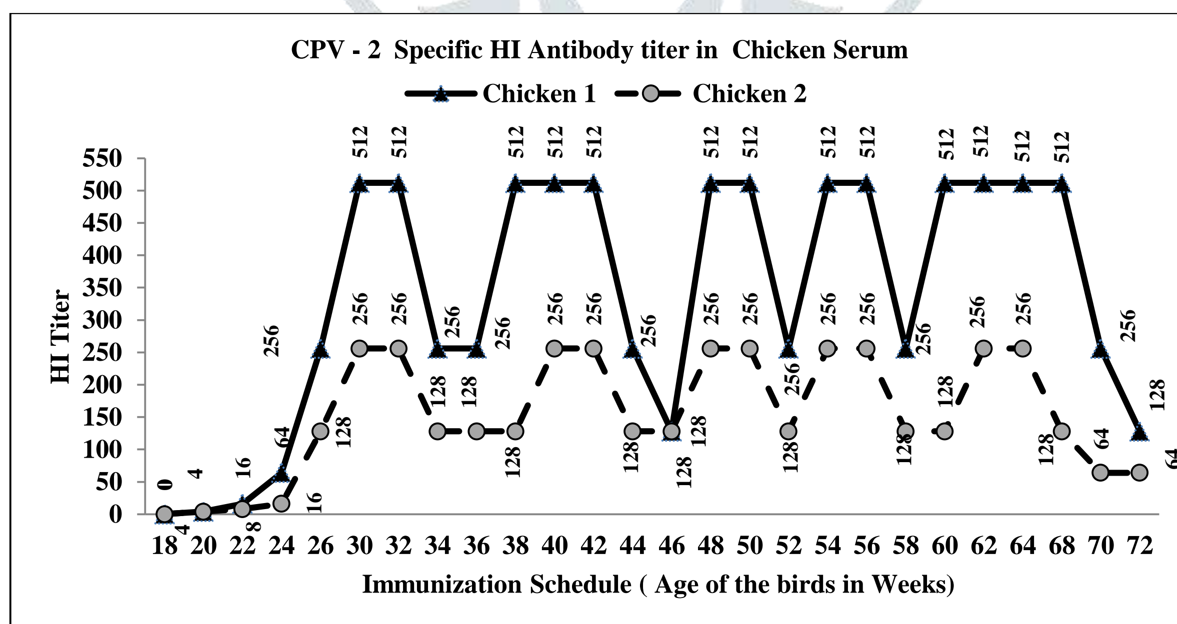
Age of Bird (Weeks)	Remarks
18	Primary
20	Booster - 1
22	Booster - 2
24	Booster - 3
26	Booster - 4
36	Booster - 5
46	Booster doses were administered after observing the declining of Specific CPV – 2 antibody HI titer
52	
58	
64	

Five booster doses were given initially and further booster doses were given to maintain the specific CPV -2 specific antibodies in the chicken serum and in the egg yolk.

3.3 HI titer of chicken serum

The HI titer of specific antibodies against vaccine strain of CPV -2 in chicken sera samples collected from chicken 1 and 2 at different weeks is presented in figure – 1. It was observed that in the immunized chickens the specific anti CPV antibodies reached maximum titer in 30th week *i.e.* after 4th booster dose was administered. After 2 weeks i.e. at 32nd week the antibody titer was started declining gradually at 26th week of age. Once the booster dose was administered at the age of 36th week, the titer reaches maximum after 2 weeks. The similar phenomenon was observed throughout the study period. Based on the HI results the eggs from chicken 1 were processed for the purification of IgY antibodies.

Fig 1: CPV - 2 Specific HI Antibody titer in Chicken Serum



3.4 Egg processing

Eggs collected in a particular week were pooled together for processing. The number of processed eggs varied from 5 to 7 nos. The volume of egg yolk ranges from 9 to 11 mL. Only 7 mL of egg yolk was collected from the eggs laid in between 21 to 23 weeks.

3.5 Total protein content of IgY

The total protein content (mg/mL) in purified IgY of eggs collected in different weeks is presented in table 3. Initially the protein content is less and gradually increased and constant after certain weeks. The observed mean protein concentration was 7.16 ± 0.9 mg /mL. There was a slight decrease observed in the concentration of protein from 68th week onwards.

3.6 DOT Blot

The result of DOT blot is presented in the plate – 1. The dark color indicates that the developed IgY antibodies are specific to CPV – 2.

Plate No. 1. DOT Blot

Legends: 1- Negative control (Control IgY) 2- Immunized chicken serum 3- Purified IgY

3.7 Purity of IgY

SDS PAGE results indicating that the purified antibodies are having more than 90% purity. The result of purity is presented in the plate no.2.

Plate: 2 Purity of IgY by Sod. Chloride method (Reduced condition)

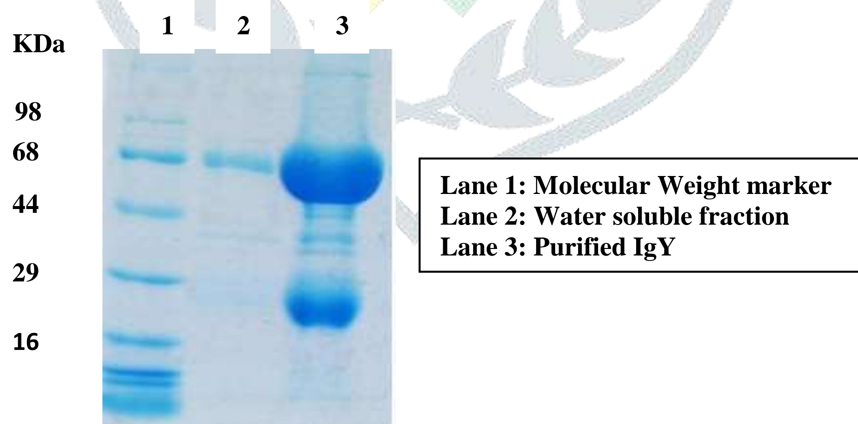


Table 3: Total Protein content in Purified IgY of eggs collected in different weeks

Age of Birds (Weeks)	Protein Conc. (mg/mL)	Age of Birds (Weeks)	Protein Conc. (mg/mL)	Age of Birds (Weeks)	Protein Conc. (mg/mL)
21	6.23	39	7.01	57	7.35
22	6.42	40	6.28	58	7.01
23	6.15	41	7.23	59	6.54
24	6.92	42	7.40	60	6.82
25	7.02	43	9.09	61	7.82
26	6.75	44	7.84	62	7.36

27	7.25	45	8.57	63	6.94
28	6.95	46	6.95	64	6.52
29	7.02	47	9.94	65	8.54
30	7.36	48	8.20	66	7.21
31	6.73	49	8.97	67	8.02
32	6.53	50	8.34	68	6.10
33	6.95	51	8.01	69	5.64
34	7.34	52	7.47	70	5.03
35	7.10	53	7.76	71	6.20
36	6.82	54	7.21	72	5.70
37	6.54	55	7.54	-	-
38	6.76	56	6.97	-	-
Mean					**7.16**
SD					**0.90**

3.6 Kinetics of Specific IgY antibody titer

The kinetics of specific IgY antibody titer (HI) is presented in the figure – 2. From this figure it was observed that the specific antibody titer reached maximum in 27th week and maintained up to 31st week. A declining in antibody titer was observed from 33rd week onwards and reached the lowest level at the age of 36th week. Once the booster dose was given a gradual increase in the antibody titer was observed and maintained at peak for a couple of weeks and started declining. Similar observations were made during the entire study period.

The HI titer and protein concentration was plotted in a graph (Fig: 3) to find out any correlation between protein concentration and titer. But the study results indicated that there was no correlation observed between both.

3.7 Neutralization assay

Samples of purified IgY antibodies showing high HI titer was subjected for neutralization test using A-72 cell line. The sample of high HI titer of 512 was able to neutralize 100 $CCID_{50}$ virus at a log dilution of $10^{-4.5}$ *i.e.* the PD_{50} of the purified antibody was log 4.5. During this test it was observed that the CPE was started on 2nd day and complete CPE was observed on 5th day. No CPE was observed in the wells where the purified antibody was able to neutralize the virus and as well as in the control wells. The various stages of CPE observed in the neutralization test were presented in plates 3 to 8.

Fig 2: Kinetics of Specific Ig Y antibodies titer

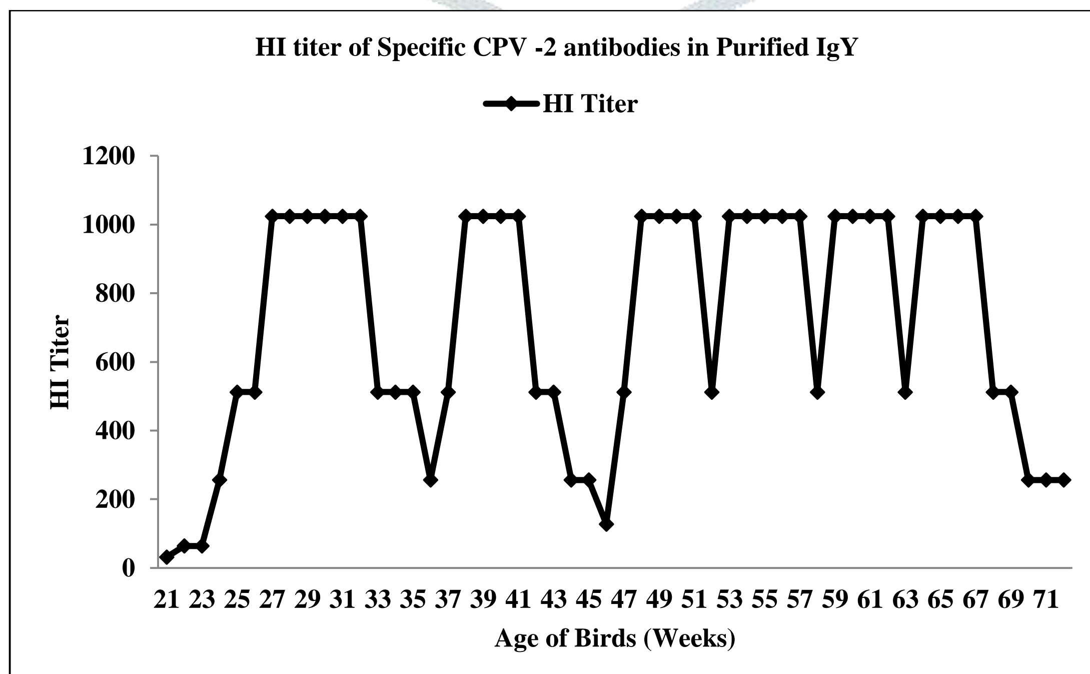


Fig 3: Total Protein concentration of Purified IgY vs. HI titer of Specific antibodies of CPV – 2

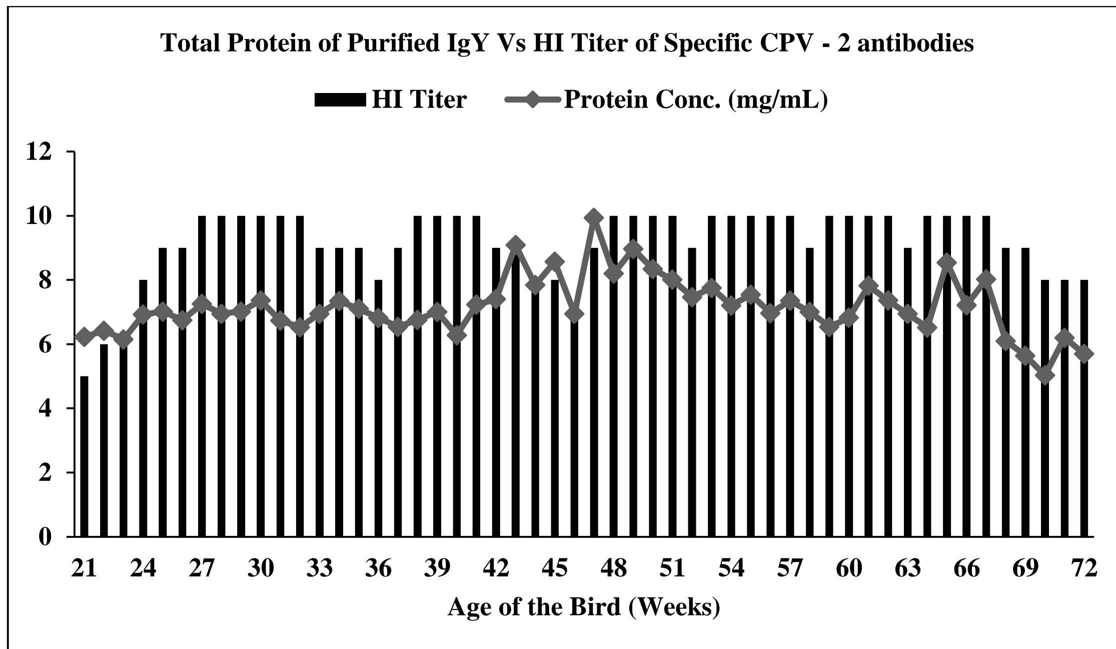


Plate 3 -8 : Neutralization test in A- 72 cell line

Plate: 3 – Day 1 prior infection

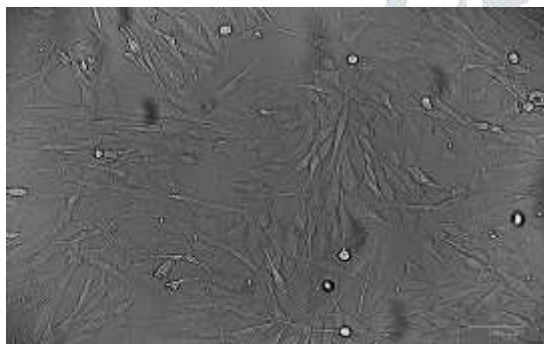


Plate: 4 – Day 2 of post infection

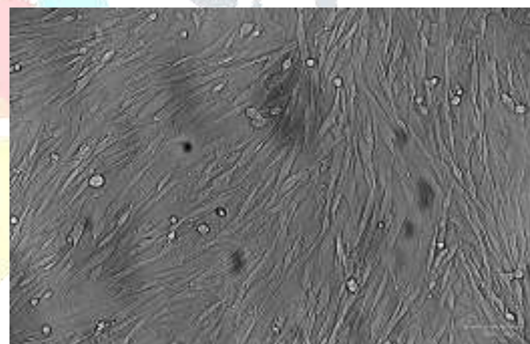


Plate: 5 – Day 3 post infection

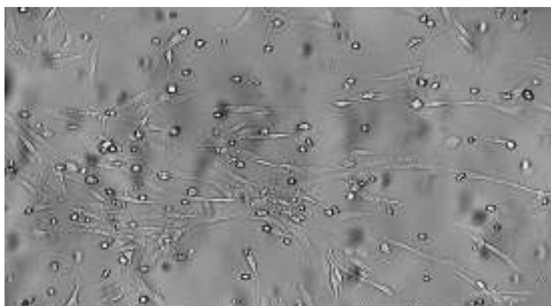


Plate: 6 – Day 4 post infection

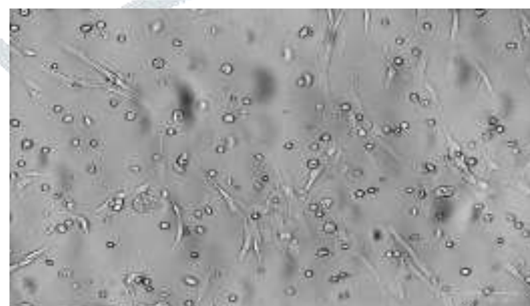


Plate: 7 – Day 5 post infection

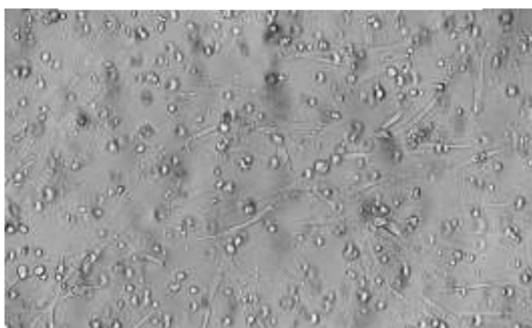


Plate: 8 – No CPE in neutralized well with purified IgY

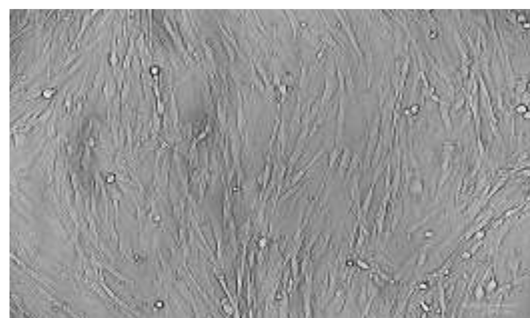
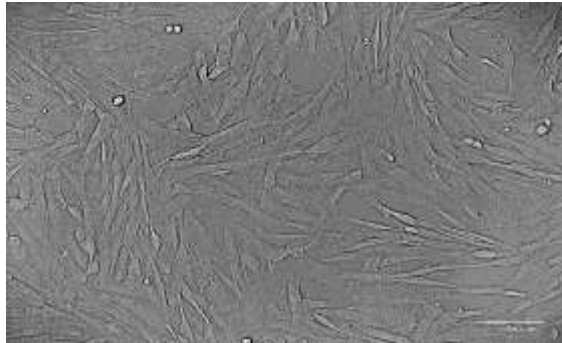


Plate: 9 – Control cells



4. Discussions

CPV - 2 is one of the most commonly occurring viral enteritis of young dogs less than 2 months old. This disease causes high morbidity and mortality rate in unvaccinated canines. Since there is no specific antiviral treatment available, only the supportive care is provided for the suffering dogs. Therefore, there is an urgent need to develop novel approaches to treat and manage CPV -2 infections. An alternate method of treatment is passive immunization with polyclonal antibodies especially in case of immuno compromised canines. Avian egg yolk antibodies (IgY) are recognized as an alternate sources of antibodies in place of mammalian antibodies since Immunoglobulin Y (IgY) is functionally equivalent to immunoglobulin G (IgG). Hence, several studies are focused on the use of specific IgY antibodies for passive immunization applications (Jennifer *et al.,* 2012).

CPV -2 field isolate number 2 gave positive HA test results with chicken and swine erythrocytes similar like earlier reports (Bayati *et al.,* 2010). There was no difference in the HA titer of the isolates either with Chicken RBC or with Swine RBC. Hence based on the sensitivity of the isolates, researchers can use either chicken RBC or Swine RBC. The HA with chicken RBC of isolate 2 could be due to the presence of haemmagglutinins receptors on virus surface which may be of glycoprotein in nature and have the ability to bind on the surface of the avian and swine erythrocyte. It was observed that the time taken for HA reaction is rapid when compared with swine RBC, may be due to chicken RBCs are bigger, nucleated, denser and heavier (Khan *et al.,* 2006).

Initially the chickens were immunized with 5 booster doses with a commercial vaccine preparation to elicit specific CPV – 2 egg yolk antibodies. Chicken No.1 showed higher immune response when compared with bird No. 2. Hence eggs from the bird number 1 were taken for the purification of IgY antibodies. The immunized chickens attained peak HI titer on 30th week *i.e.* after the 4th booster dose administered at the age of 26th week. After couple of weeks the HI titer was declined gradually. After administering booster doses of vaccine once again the sera HI titer was reached maximum and declined gradually which was similar to the observations reported earlier (Kovacs – Nolan *et al*., 2001). Similar type of phenomenon was also observed in the specific antibodies HI titer in the purified IgY (Fig. 1). This observation indicate that, to maintain high titer of specific antibodies either in the serum or egg yolk, the chickens have to be immunized at least once in 4 to 5 weeks interval till the completion of their maintenance period.

SDS-PAGE, stained with coomassive blue, showed that the purity of the IgY prepared by NaCl method was greater than 95%. Two predominant bands with approximate molecular weight of 68 kDa (heavy chain) & 24 kDa (light chain) were observed.

The specific neutralizing activity of the purified IgY antibodies against CPV - 2 (Plate 8) was demonstrated using A – 72 cell line. The highest dilution of the IgY that was able to completely neutralize the field isolate was log $^{4.5}$ indicating that the purified IgY was intact. It is a known fact that the purified IgY antibodies are polyclonal, which are specific to various viral proteins of CPV. It was reported earlier that the percentage of the specific antibody against the specific immunizing agent is 2–10% (Gusti *et al*, 2014). As the laying hens used were not specific pathogen free, it was expected that the IgY generated and purified was a mixture of antibodies specific against a variety of antigens that the birds had encountered over their life spans. Parvovirus is a simple virus containing three structural proteins namely, VP1, VP2 and VP3 (Hoelzer, and C.R. Parrish 2010). The capsid is composed of VP1 and VP2. The most abundant protein is VP2 (Decaro *et al*., 2006). This protein acts as

hemagglutinin in HI assays and receptor binding (Hoelzer, and C.R. Parrish 2010). Therefore, the antibodies generated against VP2 are protective in nature and preventing the animals from CPV -2 infection.

The results of this study clearly indicate that IgY has great potential as a remedy for the clinical cases resulting from infectious diseases. More studies are needed to compile its advantages and to understand its possible side effects. As previously stated (Camenisch *et al*., 1999), it is easy to generate and purify IgY from egg yolk. For this, little amount of immunizing antigen are sufficient to generate a high titer antibody, when compared to sheep which need a much higher antigen content (Woolley and Landon, 1995). IgY therapy is even safer, less expensive because of the heterologous origin for a mammalian host. Hence IgY is thought to be incapable of inducing complement activation and binding to the mammalian Fc receptor (Narat, M., 2003). This is an important feature in avoiding an unintended inflammatory reaction. Another advantage of IgY is that it has a short half-life, 36 h, which is much shorter than the half-life sheep IgG (15 days). Hence the clearance of IgY happens rapidly from the blood stream, which avoids possible toxic reactions and immune recognition.

5. Conclusions

From this study it could be concluded that in-order to maintain desired level of specific antibodies against CPV – 2 the birds have to be immunized with frequent booster doses based on the kinetics of the antibody titers observed in the egg yolk. The data on neutralizing capability of the generated and purified antibodies further strengthened the concept of passive therapy for suffering canines after establishing safety and efficacy of the product in preclinical and clinical trials.

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