

Intravenous Administration of Chicken Immunoglobulin Has a Curative Effect in Experimental Infection of Canine Parvovirus

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Abstract: Chicken immunoglobulin Y (IgY) may provide a new modality in the therapy of various infectious fatal animal and human diseases. This study presents evidence of its efficacy for canine parvovirus (CPV), which is a highly infectious, fatal viral disease in dogs. Hens were injected with formaldehyde-inactivated, tissue culture-derived, field isolates to produce IgY. Additional doses were administered 2 and 4 weeks following the first injection. IgY purified from the collected sera contained two proteins with molecular weights of 68 and 24 kDa. In feline kidney tissue culture, the 50% protective dose (PD₅₀) was found to be 10⁻⁸. The effectiveness of intravenous (IV) IgY immunotherapy was tested in dogs after oral challenge with a highly pathogenic CPV isolate. The recovery rates for the dogs treated with 1,000 and 10,000 PD₅₀ were 25% and 100%, respectively. The higher dose was more effective in generating a protective antibody titer and in suppressing infective virus excretion in stool. We conclude that yolk-derived chicken immunoglobulin can be administered intravenously in dogs and is effective for the treatment of a severe clinical course of experimental CPV-2 infection.

Key words: IgY • Intravenous Immunotherapy • Canine Parvovirus

INTRODUCTION

The economic losses from and health impacts of infectious disease in humans contribute to 43% of the world's total economic burden [1]. Zoonosis is an increasingly important issue. More than 60% of infectious diseases in humans originate from animals [2]. Immunotherapy using chicken immunoglobulin, termed IgY, may provide new modalities for the treatment of infectious diseases in humans and animals. Immunotherapy has been used for the prevention and treatment of human disease caused by cytomegalovirus,

hepatitis A and B, measles, rabies and tetanus [3]. Traditional immunotherapy is carried out by administering mammalian immunoglobulin intramuscularly.

Laying hens have great potential as biological factories to produce specific antibodies [4]. After immunization, IgY is transferred to and accumulates in the egg yolk. This IgY can be easily purified [5]. IgY immunotherapy has been shown to be effective in the prevention and treatment of gastrointestinal diseases in both humans and animals [6]. These antibodies effectively prevent gastrointestinal infections caused by *Escherichia coli* [7], human dental caries caused by *Streptococcus*

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mutans [8], canine distemper infection [9] and canine parvovirus (CPV) [10]. Regarding CPV, oral therapy of IgY has proven to be effective in suppressing the clinical symptoms and viral shedding [10].

The effectiveness of intravenous (IV) application of IgY needs to be explored. Here, we provide evidence that IgY can be administrated intravenously to cure a fatal infection of CPV in its natural host. CPV is a highly infectious fatal disease in dogs characterized by anorexia, fever, vomit and bloody diarrhea [11]. These results will benefit veterinarians by providing a new option for CPV therapy, because CVP is the most frequent fatal infection in dogs. This study also offers new insights into the potency of IV IgY therapy for the treatment of animal and human diseases.

MATERIALS AND METHODS

Ethical Clearance: Ethical clearance for this study was provided by the Ethic Committee of the Faculty of Veterinary Medicine, University of Udayana (No. 102A KE-PH/VIII/2011; dated August 10, 2011).

Experimental Animals

Chicken: Isa Brown laying hens, 20 weeks old, were used for the production of IgY-CPV. The hens were kept in isolated cages at the animal house and provided regular food and water.

Dogs: Sixteen puppies, aged between 2 and 4 months, were divided into four groups each of four. The animals were housed in separated cages in an isolated facility throughout the study.

Virus: Canine parvovirus, derived from field isolates, was inactivated with formaldehyde prior to injection into chickens. Canine parvovirus was obtained from the Depository of Veterinary Research Institute, Bogor, Indonesia. The strain was cultivated in feline kidney cells supplemented with 5% DMEM and incubated at 37°C with 5% CO₂. The 50% tissue culture infectious dose (TCID₅₀) of CPV was determined by culturing a 10-fold dilution of viral suspension in feline kidney cells at a concentration of 2 × 10⁵/mL. The cultures were incubated at 37°C with 5% CO₂ for 5–7 days.

Production, Isolation and Characterization of Anti-Canine Parvovirus IgY: Five chickens were first injected intramuscularly with a suspension containing 2¹³ HA units/mL of CPV and Freund's complete adjuvant. At weeks 2 and 4 after the first injection, additional

injections were administered with the same dose of virus emulsified in Freund's incomplete adjuvant. IgY from egg yolk was purified using an EGG Stract Purification System kit (Promega) according to the manufacturer's instructions. SDS-PAGE electrophoresis was performed to confirm the success of purification. IgY concentration was determined with a Nanodrop ND-1000 spectrophotometer at an absorbance of 280 nm.

In vitro Neutralization Activity of Anti-CPV IgY: A serum neutralization test was performed to evaluate the ability of our anti-CPV IgY suspension to neutralize 100 TCID₅₀ CPV. Calculations were performed using the Reed and Muench methods [12].

Hemagglutination and Hemagglutination Inhibition Assays:

The hemagglutination assay (HA) was performed as previously described [13]. Briefly, the viral suspension was serially diluted two-fold in phosphate-buffered saline (pH 6.8). An equal volume of 0.5% purified pig red blood cells (RBC) was added to each dilution and the mixture was incubated at 4°C. The HA titer was defined as the anti-log of the highest dilution of virus that exhibited complete agglutination of the RBC.

The hemagglutination inhibition (HI) assay was carried out using a previously published protocol [14]. Briefly, the sera were inactivated at 56°C for 30 min, absorbed to RBC and kaolin was added. Then, each serum was serially diluted two-fold in phosphate-buffered saline on a microplate. An equal volume of CPV (8 HA units) was added to each dilution. After incubation at 4°C for 1 h, an equal volume of purified 0.5% pig red blood cells was added and the mixture was incubated overnight at 4°C. The assay was performed in duplicate.

Canine Parvovirus Therapy Using 1,000 or 10,000 PD₅₀

Doses of Anti-CPV IgY: The animals were divided into four groups of each four dogs: 1) the negative control group, which received neither CPV nor anti-CPV IgY, 2) the positive control group, which received 100 TCID₅₀ CPV without IgY, 3) treatment group 1, which received 100 TCID₅₀ CPV followed by intravenous administration of 1,000 PD₅₀ anti-CPV IgY at the onset of clinical symptoms and 4) treatment group 2, which was infected as treatment group 1 and received 10,000 PD₅₀ anti-CPV IgY intravenously, also at the onset of clinical symptoms. Fecal samples were collected daily with sterile cotton swabs and evaluated for virus. Serum samples were also collected daily and these were evaluated for the anti-CPV antibody titer, as measured by an HI assay.

Data Analysis: The data obtained were analyzed using analysis of variance for the effect of therapy on the anti-CPV IgY titer in dog sera, mortality and the titer of excreted virus in dog stools.

RESULTS

Hens were injected with formalin-inactivated CPV at three time points to induce anti-CPV IgY and blood samples were taken. To evaluate the antibody response to these injections, an HI assay was performed on the pooled hen sera. These samples showed anti-CPV IgY titers of 256, 1,024 and 2,048 HI units at 1, 2 and 4 weeks following the first injection, respectively. Next, IgY was purified from egg yolks of the eggs laid by these hens. The mean concentration of this IgY was 5.47 mg/mL. Analysis of the IgY via electrophoresis produced two prominent protein bands with molecular weights of 68 kDa and 24 kDa (Figure 1), which are the expected weights for IgY heavy and light chains. To assess the neutralizing activity of this anti-CPV IgY, a neutralization assay was performed in feline kidney cells. The highest dilution of IgY preparation that completely neutralized field isolates was 10^{-8} (Figure 2).

Next, IgY was tested as a therapeutic against CPV in infected dogs. Blood and stool samples were collected from animals in each of four groups. Our negative control group, which was neither infected with CPV nor treated with anti-CPV IgY, showed antibody titers between 2^1 and 2^2 HI units ($1.39 \pm 0.3 \log_2$) and no virus shedding in the feces (Figure 3; Table 1). No members of this group showed clinical sign of CPV infection during observation. Following oral inoculation of the CPV isolate, all puppies in the positive control group, who were not treated with IgY, were dead at day 7 post-infection (Table 1). The clinical signs exhibited by animals in this group over the course of infection were anorexia, dehydration, vomiting and hemorrhagic diarrhea. Viral titers in the stool of these animals was detectable at day 2 and continued increasing until death. The highest viral titers observed were 2^{10} HA units ($4.05 \pm 3.99 \log_2$), which were detected on day 6 (Figure 3). In the first treatment group, in which infected animals were treated with 1,000 PD₅₀ CPV, one animal survived, while the other three were dead by day 7 (Table 1). Animals in this group showed antibody titers between $2^{3.8}$ and 2^5 HI units ($3.41 \pm 1.57 \log_2$) and fecal virus shedding was detected starting at day 2 post-infection (2^2 HA units) and peaked at 2^8 HA units ($4.07 \pm 3.36 \log_2$) on day 4 post-infection (Figure 3). In the second treatment group, in which infected animals were

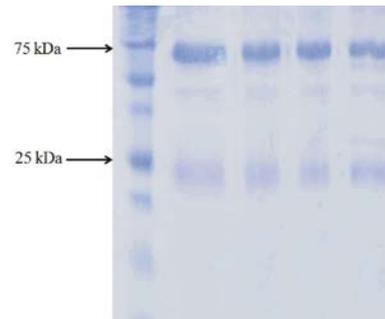


Fig. 1: Band pattern of purified egg yolk IgY resulting from SDS-PAGE. Four preparations of IGY were shown. Left lane was Precision Plus Protein™ Standards (Bio-Rad). The position of molecular weight of 75 and 25 kDa of the marker is indicated. The electrophoreses was conducted in 12.5% SDS-PAGE and stained with coomassie blue



Fig. 2: Neutralizing activity of CPV-specific IgY against CPV infection *in vitro* in feline kidney (FK) cells. The cells show no cytopathic effect (CPE) (left), 50% CPE (middle) and complete CPE (right) after treatment with no IgY, 10^{-8} diluted IgY and 10^{-10} diluted IgY, respectively

treated with the higher dose of 10,000 PD₅₀ anti-CPV IgY, all four animals survived (Table 1). Average anti-CPV titers was $5.60 \pm 2.58 \log_2$ HI unit and the virus titers in their feces were $1.83 \pm 1.58 \log_2$ (Figure 3).

DISCUSSION

Homologous and heterologous specific antibodies are currently used as immunotherapy in humans and animals. Some immunoglobulins are given to patients when they are exposed to infectious agents, long before clinical signs emerge (15, 16). Two common examples for this immunotherapy model are anti-tetanus serum and rabies immunoglobulin (17, 18). Administration of immunoglobulin during the acute phase of infection is not widely utilized and has not yet shown a proven benefit [15]. Moreover, administration of immunoglobulin used to prevent the clinical course of disease are commonly administered via intra-muscular injection or infiltration around bite sites, as in the case of rabies [18].

Table 1: Mean titers of the CPV antibody in the sera and virus excretion in the stool for groups I-IV

Treatment	Mean anti-CPV antibody titer in dog sera (log ₂ HI Unit)	Mean CPV titer in dog stool (log ₂ HA unit)	Survival rate (%)
Group I (Negative Control)	1.39±0.3 ^c	0.00±0.00 ^c	--
Group II (Positive Control)	2.68±1.36 ^{bc}	4.05±3.99 ^a	0
Group III (1,000 PD ₅₀ IgY Therapy)	3.41±1.57 ^b	4.07±3.36 ^a	25
Group IV (10,000 PD ₅₀ IgY Therapy)	5.60±2.58 ^a	1.83±1.58 ^b	100

Different letters in the same column indicate a statistically significant difference between corresponding means (p<0.05).

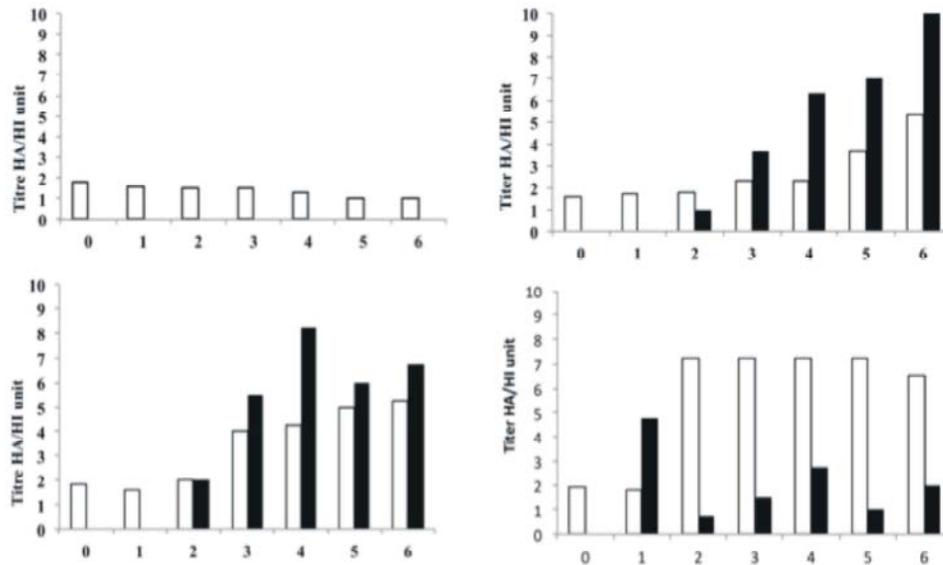


Fig. 3: Daily detection of anti-CPV antibodies in the serum (open bar) and titer CPV virus in the stool (close bar) of experimental animals. Group I: negative control dogs (uninfected and not given IgY therapy). Group II: positive control dogs (challenged with 100 TCID₅₀ CPV, but not treated with IgY). Group III: low-dose treatment group (challenged with 100 TCID₅₀ CPV and treated with 1,000 PD₅₀ IgY anti-CPV therapy). Group IV: high-dose treatment group (challenged with 100 TCID₅₀ CPV and treated with 10,000 PD₅₀ IgY anti-CPV therapy). Oral challenge of pathogenic CPV was conducted at day 0. Anti-CPV antibodies and virus titers were expressed in HI dan HA units, respectively

In this study, we have explored the potential use of chicken immunoglobulin, widely known as IgY, as an intravenous passive immunotherapy for the acute phase of a clinical disease. Intravenous IgG therapy has been published for clinical treatment in human infections, such as West Nile Virus in animal experiments [19] and human cases [20], with variable outcomes. The source of IgG in those studies was human donors, which needed a huge number of donors to provide enough IgG for therapy [21]. We used isolated pathogenic CPV to stimulate antibody production in chickens. The resulting concentration of IgY was very high, up to 5.5 mg/mL and appeared to be enough for immunotherapy. Following purification of IgY from egg yolk, using an appropriate kit, we confirmed that our IgY is the expected size and fully functional. When the samples were used in SDS-PAGE and stained with Coomassie Blue, two dominant bands of approximately

68 kDa and 24 kDa are very clear. These bands likely represent the heavy and light chains of the IgY [22]. IgY differs from mammalian IgG, in that it has four constant regions of heavy chain, instead of three [4]. We demonstrated that the purified IgY showed specific neutralizing activity against CPV (Figure 2). The highest dilution of our IgY that was able to completely neutralize field isolates was 10⁻⁸. This provides clear evidence that the purified IgY was intact.

It is plausible to assert that the prepared IgY in this study must be polyclonal antibodies, which are specific to various viral proteins of CPV. As the laying hens we used were not specific pathogen free, we expect that our purified IgY is a mixture of a specific antibody against a variety of antigens that the birds had encountered over their life spans. We estimate that the percentage of the specific antibody against our immunizing agent is 2–10%,

based on previous reports [23, 24]. Parvovirus is known to be a simple virus, containing three structural proteins (VP1, VP2 and VP3) (reviewed in 25). The capsid is composed of VP1 and VP2. The most abundant protein is VP2 [11]. This protein acts as hemagglutinin in HI assays and receptor binding [25]. In viral replication, VP2 binds to receptors and initiates clathrin-mediated endocytosis [26]. Therefore, the antibody that plays a significant role in *in vitro* and *in vivo* virus replication must be an antibody to VP2. Other antibodies against other structural proteins must have no role, as the virus is released from infected cells via cell lysis [27]. The mechanism of action could be simply that the IgY against VP2 functions to block virus attachment, or, alternately, that through inducing a conformation change in a capsid protein, it indirectly causes VP2 to lose the capacity to bind receptor molecules [28].

Problems in the study of CPV were anticipated, as some reports showed that experimental infection might not produce obvious clinical signs [10,29]. However, we demonstrated that the field isolate used in this study is highly virulent. All animals in the positive control group, who did not receive any therapeutic treatment, developed typical CPV clinical signs and died 7 days post-infection. The observed clinical signs were typical of both natural and experimental infection of CPV [30, 31]. Use of such a highly pathogenic isolate allowed this study to clearly demonstrate the effectiveness of IV anti-CPV IgY administration.

CPV is a highly contagious disease that is transmitted between animals through various routes, including both direct contact and indirect contact [30, 31]. To carefully control for unintended spread, the study was conducted in a tightly controlled situation. No transmission between groups was observed. Animals in the negative control group showed neither protective levels of a serum antibody nor detectable levels of virus in their stool. The negative control animals also failed to exhibit any clinical signs of CPV throughout the study.

To minimize the need for animal subjects, we limited our experiment to two doses of anti-CPV IgY, 1,000 and 10,000 PD₅₀. The 1,000 PD₅₀ dose was found to be insufficient, as only one out of four animals survived. This dose was inadequate to produce a protective level of antibody titer in dog blood and to reduce viral replication, as shown in Figure 3 and Table 1. In contrast, the dose of 10,000 PD₅₀ was protective, with all treated animals in this group surviving to day 7 post-infection. Animals

receiving this dose had a significantly higher antibody titer compared with both the animals receiving the 1,000 PD₅₀ dose and the untreated animals in the positive control group. The 10,000 PD₅₀ dose also significantly reduced the virus titer in the stools.

The incubation period for CPV in this study was 2-3 days (data not shown). As the administration of IgY was carried out at day 2 post-infection, it appears that anti-CPV IgY at the 10,000 PD₅₀ dose was effective for therapy in the early stage of a clinical case. More study is needed to elucidate the window of effectiveness for IV IgY.

Anti-CPV IgY apparently decreases the viremic level. It may neutralize the circulating virus and prevent virus titer burst, which otherwise leads to systemic vascular leakage or vascular permeability, as reported in human B-19 parvovirus [32] and CPV-2 infection [33]. This likely contributes to severe dehydration as the major cause of death for CPV-2 infection, along with the most published pathogenesis of severe damage to the intestines [30]. The blood IgY might prevent infection of the myocardium, which is well known to be the second main tropism of CPV-2 in young puppies [34]. Virus excretion cannot be completely suppressed, as the virus is well known to withstand the acidity of the stomach and the basic environment of bile salt at the intestine. Therefore, we can expect there must have been a direct infection of the intestine following oral inoculation for the infection protocol used in this experimental study. The replication in intestinal cells was most likely at a low level, so it allowed the turnover rate of lymphoid and epithelial cells replenishment, as found previously [35]. This leads to rapid recovery of the animal.

The results of this study clearly indicate that IgY has great potential to cure clinical cases resulting from infectious diseases. More studies are needed to compile its advantages and to understand its possible side effects. As previously stated [36], we can easily produce and purify IgY from egg yolk. The protocol needs little immunizing antigen to generate a high titer antibody, which is deposited in the yolk of a laying hen [36-37]. Producing such an antibody in sheep would need a much higher antigen content [38]. IgY therapy is even safer, less expensive and more efficacious than antibiotics [22]. Because of the heterologous origin for a mammalian host, IgY is thought to be incapable of inducing complement activation and binding to the mammalian Fc receptor [4]. This is an important feature in avoiding an unintended inflammatory reaction.

Another characteristic of IgY is that it has a short half-life, 36 h, which is much shorter than the half-life of mammalian IgG. The half-life of sheep IgG is up to 15 days [39]. The clearance of IgY happens immediately, which avoids possible toxic reactions and immune recognition. Further study is needed to confirm this advantage of IgY in prolonged and repeated administration in mammalian animals. We observed that the half-life of IgY in this experiment was 60 h and no adverse reactions were recorded in the animals after 6 months observation (data not shown).

It could be concluded that yolk-derived IgY can be administered intravenously in dogs and serve as an effective treatment for a severe clinical course of experimental CPV-2 infection. The protocol did not cause any adverse reactions. A high dose seems to perform better than a low dose. More studies are needed to prove the safety of the protocol, especially for repeated treatment of an animal using different IgY to cure various infectious diseases. Pre-clinical studies are likewise required to elucidate the effectiveness of IgY as a cure for various human fatal infectious diseases. This work widens the potential for IV administration of IgY in human and animal medicine.

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