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Antibody Response of Mice to the Bali Isolate of Canine Parvovirus Propagated in Madin-Darby Canine Kidney Cell Culture

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ABSTRACT

Canine parvovirus (CPV) infection is still common among dogs, leading to severe disease with high mortality. The potential of a local isolate of CPV as an effective vaccine to prevent the disease warrants investigation. This study aimed to determine the antibody response in mice against a Bali isolate of CPV propagated in the Madin-Darby Canine Kidney (MDCK) cell culture. The virus was purified using polyethylene glycol (PEG)-6000 and mixed with an Aluminum hydroxide adjuvant. Fifteen 7-week female mice were divided into three treatment groups: treatment group 1 (PEG-purified virus and Adjuvant), treatment group 2 (crude unpurified virus and adjuvant), and treatment group 3 (adjuvant without virus), with five replicates per group. The Bali isolate of CPV was successfully replicated in MDCK cells, achieving a titer of 2^{10}-2^{11} hemagglutination (HA) units after eight serial passages through the cell culture. The virus was confirmed as CPV by immunocytochemistry test using a monoclonal antibody and hemagglutination inhibition (HI) test using chicken anti-CPV polyclonal antibody. Following the first immunization, the antibody endpoint titer in mice immunized with PEG-purified CPV (5.6) was significantly higher than those immunized with crude unpurified CPV (4.2) and adjuvant without CPV (1.4). Similarly, after the second immunization, the antibody endpoint titer in mice immunized with PEG-purified CPV (7.6) also remained significantly higher than those immunized with crude unpurified CPV (6.4) and adjuvant without CPV (0.8). Significant increases in antibody endpoint titer were observed after the second immunization in mice immunized with PEG-purified CPV and crude unpurified CPV, but not in those given adjuvant without CPV. The Bali isolate of CPV propagated in MDCK cell culture induced a robust antibody response in mice, suggesting it's a potential as an alternative vaccine candidate for preventing CPV infection in dogs.

Keywords: Bali, Canine parvovirus, Madin-Darby Canine Kidney, Mice, Vaccine

INTRODUCTION

Canine parvovirus (CPV) infection in dogs is characterized by severe gastroenteritis with a high mortality rate (Voorhees et al., 2019). Persistent vomiting and diarrhea, which can lead to dehydration and damage to intestinal mucosa, are the main causes of death in infected dogs (Nandi and Kumar, 2010). Although dogs of all ages can be affected, the disease tends to be more serious in puppies compared to adult dogs. In addition, myocarditis, commonly found in young dogs, contributes to the fatality of CPV infection (Decaro et al., 2020). The case fatality rate of CPV infection can reach 91% in untreated dogs. However, if the disease is treated properly and in the early stage of the disease, its survival rates can reach 80-95% (Prittie, 2004: Ling et al., 2012). Effective biosecurity measures, such as disinfection of kennel equipment and environments, along with proper vaccination, are essential to prevent CPV infection in dogs (Mazzaferro, 2020). Despite the availability of various vaccines, cases of CPV infection remain prevalent among dogs indicating that current vaccines do not provide complete prevention (Harelas et al., 2022).

Vaccination failure appears to be the main factor contributing to the prevalence of CPV infection among dogs in Indonesia (Harelas et al., 2022). Such failure can be due to different variants of CPV field isolate with virus strain used for preparation of vaccine, high titer of maternal antibody at the time of vaccination, and the low potency of available vaccines, which fail to induce protective immunity in dogs. The presence of antigenic variants among CPV isolates has been widely reported (Alexis et al., 2021; Maganga et al., 2023). The antigenic disparity between field isolates and CPV vaccine has long been under investigation, with combo vaccines consisting of both local isolates and classical CPV strains showing improved protection rates against field CPV isolates (Mittal et al., 2014; Woolford et al., 2017). Previous studies have also demonstrated that anti-CPV antibodies neutralize the homologous type of CPV in a much higher titer than the heterologous type of the virus (Cavalli et al., 2008; Kour et al., 2023), indicating that vaccines prepared using local isolates are likely to induce better protection against local wild-type CPV.

Two main CPV types (CPV-1 and CPV-2) have been identified, each comprising several subtypes based on their genetic, antigenic, and pathogenic characteristics in dogs (Decaro and Buonavoglia, 2012). However, CPV-2 is more

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heterogenous and includes many variants, such as CPV2a, CPV2b, and CPV2c (Decaro et al., 2020; Maganga et al., 2023). Mutation in the gene encoding for its major viral protein 2 (VP2) has contributed to the presence of new CPV variants in the fields (Alexis et al., 2021). The CPV vaccines available for vaccination of dogs are generally prepared using original CPV-2. It is possible then that the vaccine is not capable of inducing a fully protective immune response against CPV infection when new CPV variants are present in the field (Decaro et al., 2020).

Exploration of novel CPV strains in Indonesia has not yet been conducted. However, several CPV variants have been identified in Asia, such as CPV-2a in China (Zhao et al., 2013) and CPV-2b in India (Nandi et al., 2010). An isolate of CPV from an infected dog in Bali was confirmed as CPV by polymerase chain reaction (PCR). This Bali isolate of CPV replicates effectively in Madin-Darby Canine Kidney (MDCK) cell culture and yields a high titer after eight serial passages. The present study was therefore conducted to evaluate this virus isolate for its potential use as a vaccine candidate to control CPV infection in dogs.

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Animal Ethics Committees of the Faculty of Veterinary Medicine, Udayana University, Indonesia with the Animal Ethics Approval Certificate number: B/282/UN14.2.9/PT.01.04/20023.

Virus and cells

The virus used in the present study was isolated from a deceased Bali dog exhibiting signs of gastroenteritis. The dog was submitted for necropsy to the Veterinary Pathology Laboratory by a co-assistant student during his co-assistantship in the Veterinary Pathobiology Department (registration number: Koas/409/2021). Following necropsy, specimens, such as feces, organs, and blood were collected and sent to the Veterinary Virology Laboratory, Faculty of Veterinary Medicine, Udayana University, Indonesia for confirmatory PCR testing. The dog was recorded in the student report as a positive case of CPV infection. Organ samples, including the intestine, liver, and spleen were processed and used as inoculum for MDCK cells. The MDCK cells were obtained from Veterinary Investigation Center Regional II, Bukittinggi, West Sumatera, Indonesia.

Adaptation and propagation of canine parvovirus in Madin-Darby canine kidney cell culture

The propagation of the Bali isolate of CPV in MDCK cell culture was carried out as explained in the following steps. Organs such as the intestines, spleen, and liver were collected from the infected dog and homogenized in a mortar to prepare 20% organ suspension in serum-free cell culture media. After centrifugation at 3000 x g for 10 minutes, the supernatant fluid was collected, filtered through 0.2 µm syringe filter, and used as inoculum for MDCK cells. The MDCK cell cultures, grown in minimum essential medium (MEM) with 5% fetal bovine serum (FBS) (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) at approximately 70% confluency, were prepared in 25 cm^2 tissue culture flasks. The medium was discarded and the cells were inoculated with 1 ml of the prepared inoculum. After incubation for 90 minutes at 37^oC, the inoculum was discarded and replaced with MEM containing 3% FBS. The cells were incubated at 37^oC for 7-8 days until clear cytopathic effects were observed. The infected cells underwent three freezing and thawing steps (Parthiban et al., 2011). The cells and the medium were collected in a 15 ml centrifuge tube and centrifuged at 5000 x for 10 minutes.

Titration and identification of canine parvovirus by hemagglutination and hemagglutination inhibition test

The titer of CPV in the infected MDCK cells was determined by hemagglutination (HA) test. Following the processing of CPV-infected MDCK cells, the CPV titer was assessed using the HA test, and the virus was subsequently identified using hemagglutination inhibition (HI) test (Cavalli et al., 2008). For the titration of CPV, 50 µl supernatant fluid was subjected to serial two-fold dilution with phosphate buffered saline (PBS: pH 6.8) in 96-well V-bottomed microtitration plates. Fifty µl of 0.5% porcine red blood cells in PBS was added to each well and incubated overnight at 4^oC. Normal uninfected MDCK cell culture medium was used as a negative control. A well-characterized CPV isolate published by Suartini et al. (2014) was used as a positive control. The HA titer of CPV was expressed as the antilog of the highest virus dilution capable of completely hemagglutinating 0.5% porcine red blood cells. To identify CPV, the HI test was performed. A serial two-fold dilution of chicken anti-CPV antibody in PBS (pH 6.8) was performed in 96-well V-bottomed microtitration plates (Suartini et al., 2014). Twenty-five µl of each antibody dilution was mixed with 25 µl CPV containing 8 HA units in each well and incubated for one hour at room temperature. Fifty µl of 0.5% porcine red blood cells in PBS was then added to each well and incubated at 4^oC overnight. The virus isolate was confirmed as CPV if the antibody inhibited the hemagglutination of porcine red blood cells by the virus.

Detection of canine parvovirus replication in Madin-Darby canine kidney cells

Replication of CPV in MDCK cell culture was detected by immunocytochemistry (ICC) following a similar but modified procedure based on Astawa et al. (2018). The MDCK cells grown in 24-well plates at approximately 70% confluency were inoculated with CPV at 1 MOI (multiplicity of infection). Normal uninfected MDCK cells were used as a negative control. After three days of incubation at 37°C, the supernatant medium was removed and the cells were fixed with cold 80% acetone in PBS for 30 minutes at -20°C. The acetone was then removed and the cells were treated with 3% H_2O_2 for 20 minutes. Subsequently, 200 µl of anti-CPV monoclonal antibody (Santa Cruz, sc-57961, Texas, USA) diluted 1:100 in Da Vinci Green antibody diluent (Biocare Medical, Pacheco, USA) was added to each well. Following incubation for 1 hour at room temperature, the cells were washed twice with Phosphate-buffered Saline-Tween (PBS-T: PBS with 0.5% tween 20. pH 7.4) and 200 µl of Histofine Simple Max PO (multi) (Nichirei Biosciences INC, Tokyo, Japan) was added. Following another hour of incubation at room temperature, the cells were washed five times as described above and 200 µl of 4-chloro-1-naphthol substrate solution (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was added. CPV-infected cells were identified by the presence of dark blue-purple precipitate, mainly in the nuclei of the infected cells (Ulyashova et al., 2011).

Preparation of virus for immunization of mice

Several flasks of MDCK cells at approximately 80% confluency were infected with CPV. Seven days after infection, the infected cells underwent four freeze-thaw cycles. The infected cell culture was then centrifuged at 5000 x g for 5 minutes. The supernatant was collected and used as a virus stock for the immunization of mice. The virus titer was then determined by the HA test and confirmed again by the HI test. For the preparation of PEG-purified CPV, the same method described below for ELISA antigens was used. The virus was inactivated with binary ethylenimine (BEI) and mixed with Alum Alhydrogel adjuvant (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) in a 50:50 ratio of virus to adjuvant.

Inactivation of virus

The virus was inactivated using binary ethylenimine (BEI) according to the methods described by Mondal et al. (2005) and Elbagory et al. (2021). The BEI solution was prepared by dissolving 0.6 M of 2-bromoethylamine hydrobromide (BEA) in 0.5 M of sodium hydroxide (NaOH) and incubation at 37°C for 1 hour to convert BEA into BEI. The resulting BEI was mixed with the virus stock at a final BEI concentration of 0.03 M. After incubation for 30 hours at room temperature, 2% sodium thiosulfate ($Na_2S_2O_3$) was added to neutralize the BEI in the virus stock.

Immunization of mice

A total of 15 female mice, aged seven weeks with an average body weight of 20 grams, were used in the present study. They were divided into three treatment groups included treatment group 1 (PEG-purified CPV + adjuvant), treatment group 2 (crude CPV + adjuvant), and treatment group 3 (adjuvant without virus), five 5 mice in each group. Each mouse was immunized twice with 0.2 ml of vaccine at two-week intervals. Approximately 200 µl blood was collected from the orbital vein 10 days after each vaccination and incubated for 2 hours at room temperature. After centrifugation at 1000 x g for 5 minutes, serum was collected in 1.5 ml Eppendorf tubes and stored at -20°C until use. The titer of anti-CPV antibody in mouse serum was determined by enzyme-linked immunosorbent assay (ELISA) test.

Titration of antibody by enzyme-linked immunosorbent assay

The antigen for the ELISA test was prepared using CPV grown in MDCK cell culture as described above. The virus was purified by using polyethylene glycol (PEG) and sodium chloride (NaCl) precipitation method (Boisvert et al., 2010). On day 8 post-infection, the cells were freeze-thawed 4 times, after which chloroform was added at a ratio of 1 ml chloroform to 9 ml infected cell stock. The mixture was vortexed and centrifuged at 10,000 x for 30 minutes. The supernatant was collected and the virus in the supernatant was precipitated by adding 7.5% PEG 6000 and 1.5 M NaCl (final concentration) for 16 hours at 4°C with shaking. The mixture was then centrifuged at 15,000 x for 45 minutes at 4°C. The pellet was diluted with 5 ml of 10 mM Tris-HCl (pH 7.5) and dialyzed overnight against the same buffer. Checkerboard titration was then conducted to determine the optimal dilution of the PEG-purified CPV for use in the ELISA test. The purified virus was then used as an antigen for the ELISA test at the dilution of 1:100 in the coating buffer.

Each well of 96-well ELISA microplates was coated with 100 ul purified parvovirus antigen diluted 1:100 in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) at 4°C for 18 hours. After washing twice with PBS, each well was blocked with 200 µl of 5% skim milk diluted in PBS and left at 37°C for 1 hour. Serial two-fold dilutions of mice sera, starting at 1:100, were prepared in PBS-T containing 2% skim milk (Oxoid, Hampshire, UK). Additionally, each well received 100 ul of sera samples from each dilution, followed by a 1-hour incubation at 37°C. Three normal mouse

sera samples were also prepared to calculate the cutoff value. The microplate wells were then washed three times and 100 ul of anti-mouse IgG-HRP (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) diluted 1:2000 in PBS-T containing 3% skim milk was added into each well. The microplate was incubated for one more hour at 37°C. Following five washes with PBS-T, 100 µl of TMB (3,3', 5,5'-Tetramethylbenzidine) substrate (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was added to each well. After incubation for 15 minutes at 37°C, 50 µl of stop solution (2 N H_2SO_4) was added to each well.

The absorbance values of the substrate in microplate wells were then determined using an ELISA reader with a 450 nm filter (BioTek ELX800 Microplate reader, Cole-Palmer, Illinois City, USA). The cutoff point value was calculated by adding three standard deviations (SDs) to the average optical density (OD) value of 3 negative samples run on the ELISA (Larsen et al., 2021). This cutoff value was then used to determine the dilution endpoint antibody in each serum sample. Given that each serum sample was diluted in a serial two-fold dilution starting at 1:100, the dilution endpoint of each serum sample was determined as $1/100 \times 2^x$, where x is $\log_2$ of serum dilution. The antibody endpoint titer is defined as the reciprocal of the highest dilution of a serum that gives a reading above the cutoff value (Makarova et al., 2011). The antibody endpoint titer was determined by utilizing the dilution endpoint as 100×2^x, where x is $\log_2$ of antibody dilution. In the current study, the antibody endpoint titer was expressed as the antilog_2 of the dilution endpoint using 1:100 dilution as the starting point.

Statistical analysis

Antibody titers were analyzed using one-way ANOVA and Tukey's multiple comparisons tests to determine the significant differences among treatment groups, utilizing SPSS v.23 (Armonk, NY, USA: IBM Corp). A p-value less than 0.05 was considered statistically significant.

RESULTS

Growth characteristics of canine parvovirus in Madin-Darby canine kidney cells

The replication of CPV in MDCK cells was characterized by cytopathic effects (CPEs), observable at day 4 post-infection and becoming more evident after day 6 post-infection. Optimum CPEs were observed at day 7 post-infection, although not all MDCK cells died due to Canine parvovirus infection. The CPEs in MDCK cells induced by Canine parvovirus infection were characterized by the detachment of cells from the walls of the flasks (Figure 1). Other forms of CPE such as syncytium were not observed in MDCK cells infected with the Bali isolate of CPV. The initial titer of CPV grown in MDCK cells was 2^8 and increased gradually following several passages in MDCK cells. After passage 8, the HA titer of the virus reached 2^{11} HA units (Table 1).

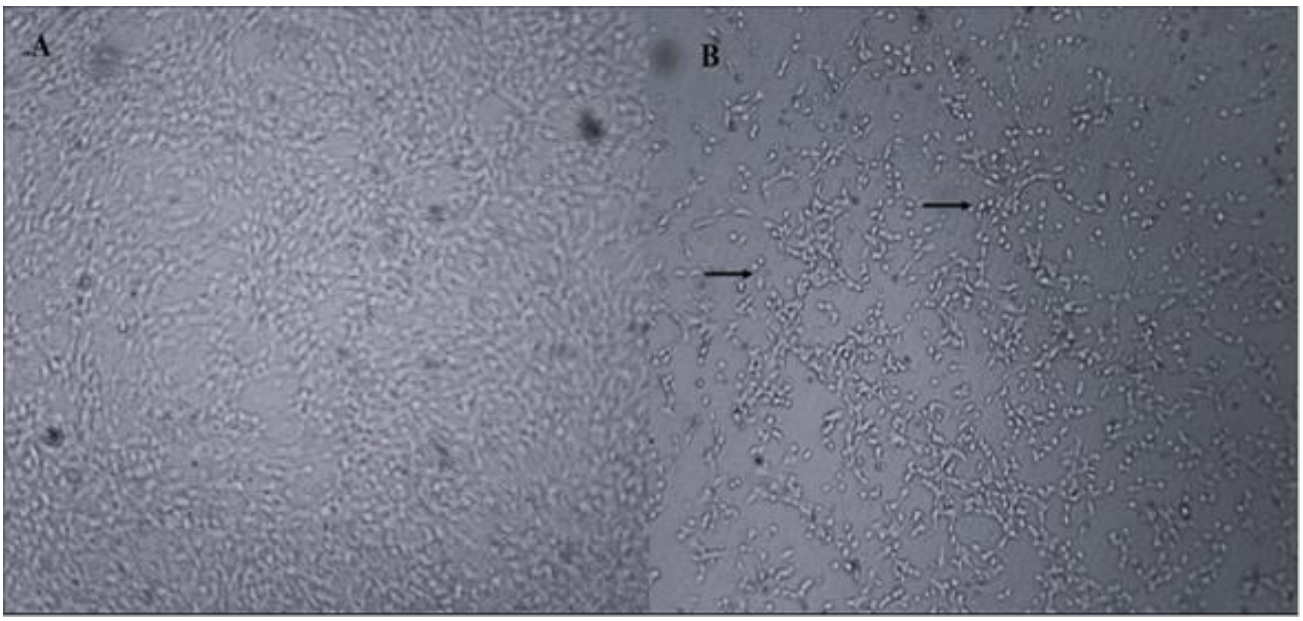

Figure 1. Cytopathic effects induced by canine parvovirus infection in Madin-Darby Canine Kidney cell culture. **A**: Normal MDCK cells, **B**: CPV-infected MDCK cells. Cytopathic effects are characterized by detaching cells from the surface flask where the cells were grown (arrow).

Table 1. Titers of the Bali isolate of canine parvovirus following passages in Madin-Darby Canine Kidney cell culture

Passage 1	Passage 2	Passage 4	Passage 6	Passage 8
2^8 HA units	2^9 HA units	2^9 HA units	$2^{9.5}$ HA units	2^{11} HA units

HA: Hemagglutination

Evidence for the replication of canine parvovirus in Madin-Darby Canine kidney cells

The replication of the CPV Bali isolate in MDCK cell culture was further confirmed by an immunocytochemistry test using an anti-CPV monoclonal antibody as a detection antibody. CPV-infected MDCK cells were visible as dark blue-purple stains, predominantly in the nuclei and, to a lesser extent, in the cytoplasm of the infected cells. In contrast, uninfected cells appeared as unstained cells in the nuclei and cytoplasm of the cells (Figure 2). Approximately 40% of cells were infected by day 3 post-infection. Further evidence for CPV replication in MDCK cells was obtained using an HI test by chicken anti-CPV antibody. As it is shown in Figure 3, the anti-CPV antibody was capable of inhibiting CPV to hemagglutinate porcine red blood cells at the antibody titer of titer 2^2 HI units (Figure 3).

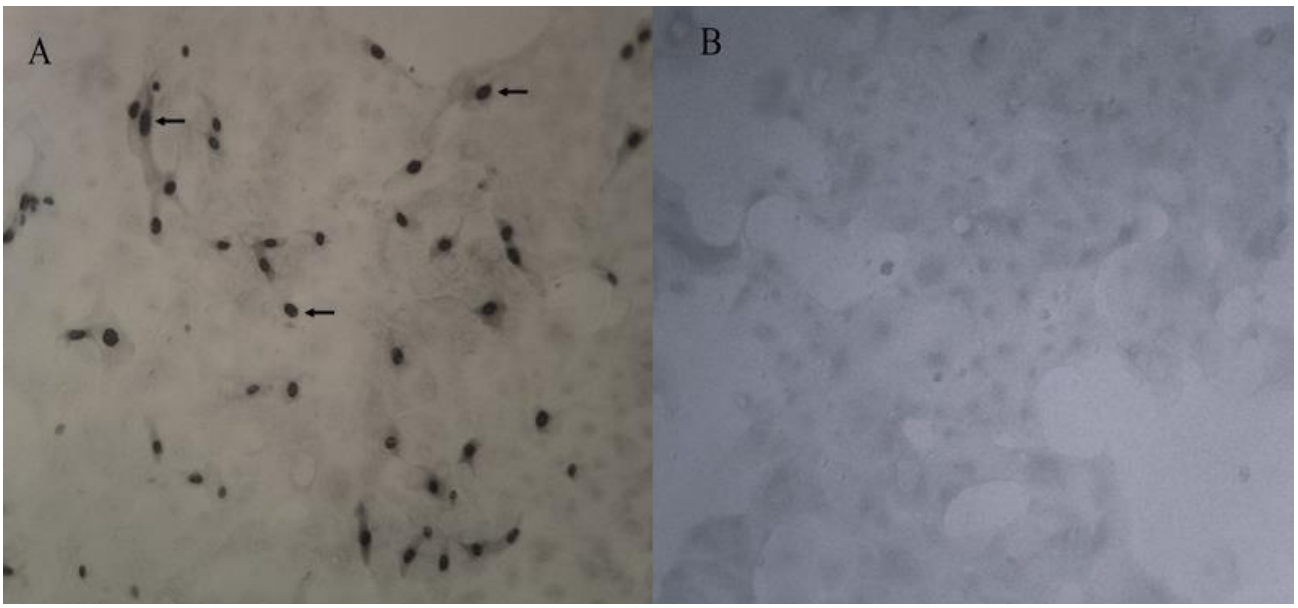

Figure 2. Madin-Darby Canine Kidney cells Infected with CPV at day 3, detected by immunocytochemistry using a monoclonal antibody against CPV. **A**: Infected cells appear bluish purple mainly in the nuclei and, to a lesser extent, in the cytoplasm (arrow), **B**: Uninfected cells.

Figure 3. Inhibition of porcine red blood cell hemagglutination by chicken anti-CPV antibody. Inhibition of hemagglutination is indicated by settling porcine red blood cells at the bottom of a V-shaped microtitration plate (arrow).

Antibody response of mice against inactivated Canine parvovirus antigen

Sera from mice were analyzed for anti-CPV antibodies following the first (primary) and the second (booster) immunizations. The average dilution endpoints of 5 serum samples after the first immunization were $1/100 \times 2^{5.6}$, $1/100 \times 2^{4.2}$, and $1/100 \times 2^{1.4}$ for PEG-purified CPV, crude unpurified CPV, and adjuvant without CPV, respectively (Figure 4).

Following the second immunization, dilution endpoints of those samples were $1/100 \times 2^{7.6}$, $1/100 \times 2^{6.2}$, and $1/100 \times 2^{0.8}$ for PEG-purified CPV, crude unpurified CPV, and adjuvant without CPV, respectively (Figure 5). The endpoint titers of anti-CPV antibody were expressed as the antilog_2 of the dilution endpoints (antilog $1/100 \times 2^{x}$). Following the first immunization, the antibody endpoint titer of the mice immunized with PEG-purified CPV (5.6) was significantly higher than those of the mice immunized with crude-unpurified CPV (4.2) and adjuvant without CPV (1.4) ($p < 0.05$). After the second immunization, the antibody endpoint titer of the mice immunized with PEG-purified CPV (7.6) remained significantly higher than those of the mice immunized with crude unpurified CPV (6.4) and adjuvant without CPV (0.8) ($p < 0.05$). A significant increase in antibody endpoint titers was observed after the second immunization compared to the first ($p < 0.05$), with notable enhancement in the mice immunized with PEG-purified CPV and crude unpurified CPV, but not in those immunized with adjuvant without CPV (Figure 6).

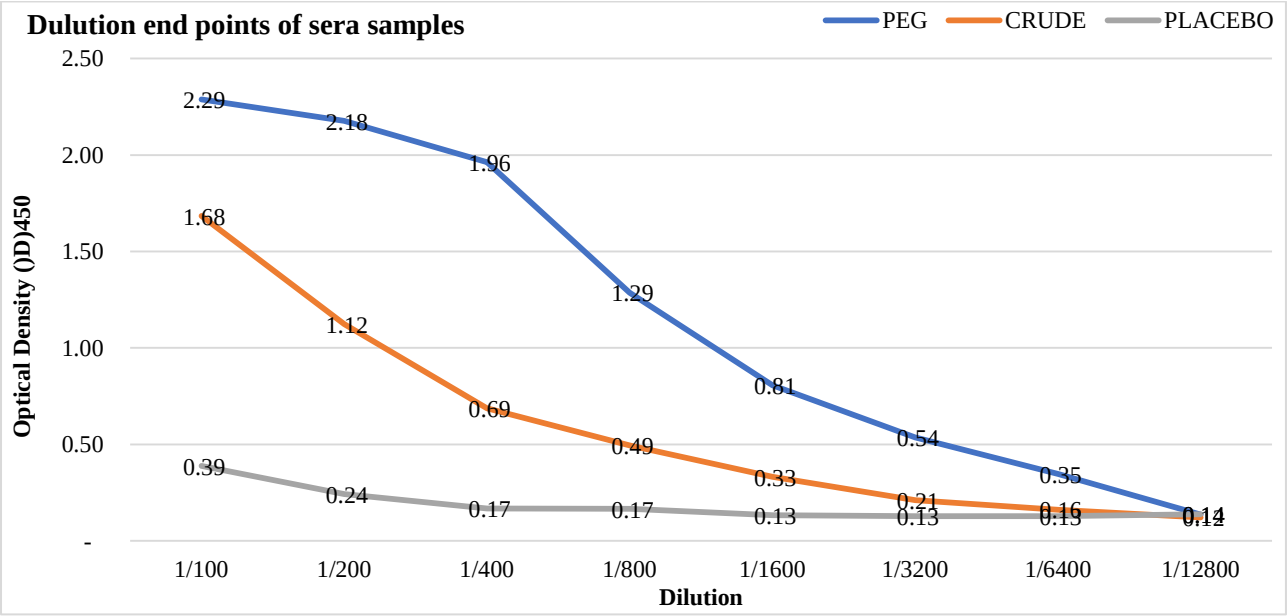

Figure 4. Dilution endpoints of mouse serum samples following the first immunization with CPV antigen determined by the ELISA test. PEG: Immunized with PEG-purified CPV antigen, CRUDE: Immunized with crude unpurified CPV-antigen, and PLACEBO: Immunized with PBS without CPV antigen

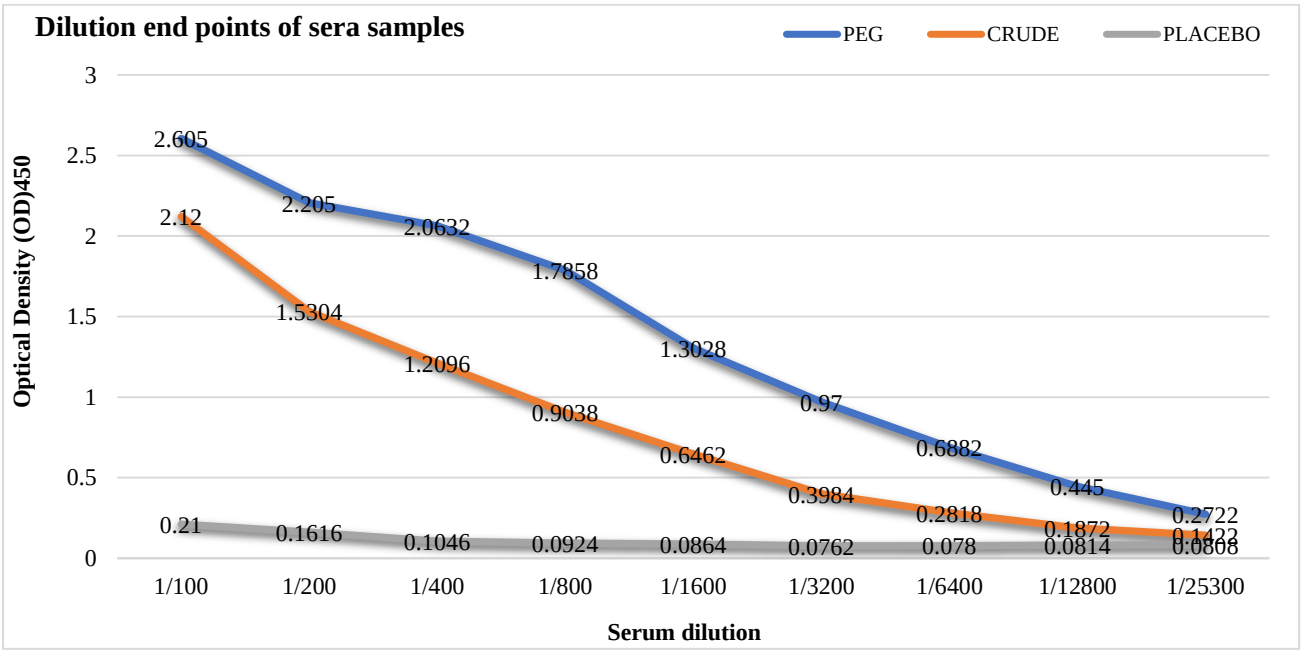

Figure 5. Dilution endpoint of mice sera samples following second immunization with CPV antigen examined by ELISA test. PEG: Immunized with PEG-purified CPV antigen, CRUDE: Immunized with crude unpurified CPV-antigen, PLACEBO: Immunized with PBS without CPV antigen.

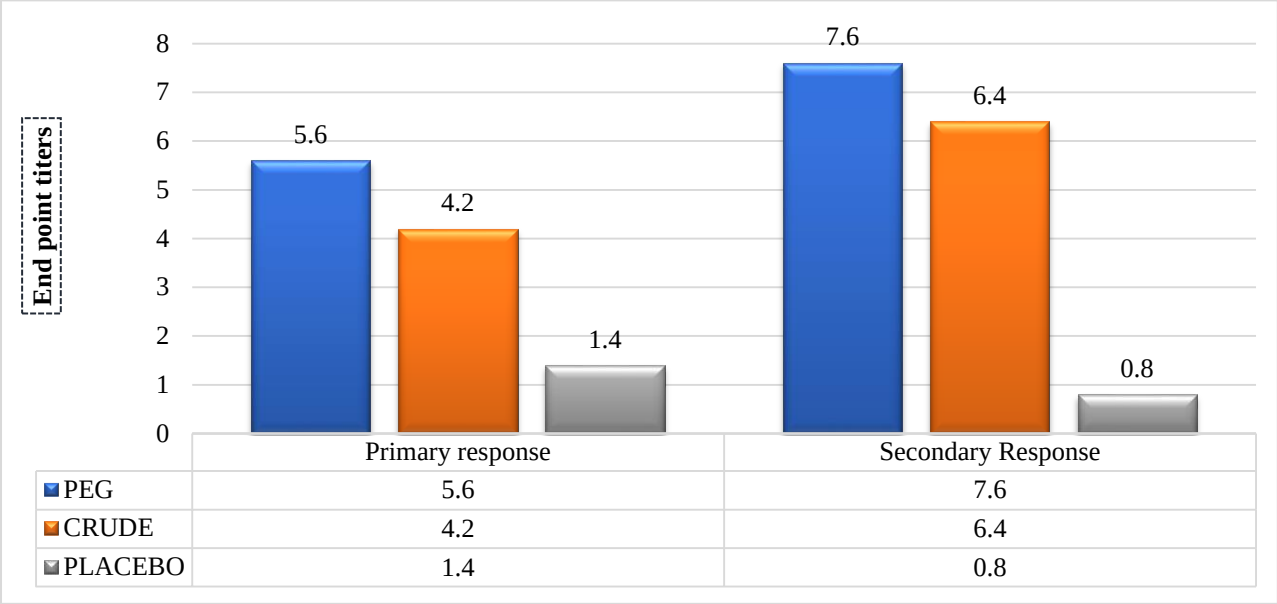

Figure 6. Antibody endpoint titers of mouse serum samples following the first and second immunizations with CPV antigens. PEG: Immunized with PEG-purified CPV antigen, CRUDE: Immunized with crude unpurified CPV-antigen, and PLACEBO: Immunized with PBS without CPV antigen

DISCUSSION

Madin-Darby Canine Kidney (MDCK) cells are epithelial mammalian cell lines derived from dog kidneys. They have been widely used in various studies including viral infections, cell stress responses, and vaccine production. These cells have undergone an immortalization process, allowing them to grow unlimitedly in culture (Capellini et al., 2020). MDCK cells were initially designed for the production of influenza virus vaccine (WHO, 1995). They have been widely used to propagate many viruses such as influenza virus (Kim et al., 2018; Wu et al., 2020; 2021) and Canine parvovirus (Kaur et al., 2015; Sharma et al., 2016).

In the present study, the Bali isolate of CPV efficiently replicated in MDCK cells, inducing clear cytopathic effects characterized by cell rounding and detachment. However, many cells did not detach from the surface of the flasks at day 7 post-infection, indicating that some cells may have developed insensitivity to CPV infection. This phenomenon is also observed in other viruses grown in MDCK cell culture. In the influenza virus, for instance, re-cloning MDCK cells from a single cell has shown to enhance their sensitivity to influenza virus infection (Zinnecker et al., 2024). Similarly, obtaining cells with heightened sensitivity to CPV infection may necessitate the re-cloning of MDCK cells from a single cell. Previous studies have demonstrated that CPV replicates in MDCK cell cultures (Kaur et al., 2015; Sharma et al., 2016). However, virus replication in MDCK cell cultures is generally less efficient compared to Crandell-Rees Feline Kidney (CrFK) cell culture (Jaune et al., 2019). Therefore, the use of MDCK cells to produce CPV vaccine has not been reported. Researches by Hyeon et al. (2023) and Dai et al. (2020) indicated that CPV is more adaptable to replicate in CrFK cells as compared to the same in MDCK cells. Consequently, when CPV is replicated in MDCK cells, the resulting titer is typically lower than that achieved in CrFK cells.

The reason is not known why CrFK cells are more sensitive to CPV infection compared to MDCK cells. Whereas MDCK cells were initially designed for the propagation and production of the influenza virus vaccine (WHO, 1995), CrFK cells were initially used for the isolation and production of the feline virus vaccine (Lappin et al., 2005). CrFK cells have been shown to be highly sensitive to CPV infection (Parthiban et al., 2011: Hyeon et al., 2023). This suggests that while MDCK cell clones are more suitable for influenza viruses as compared with CPV, CrFK clones are more so for both feline and Canine viruses.

The titer of CPV propagated in MDCK cells was assessed using the HA test. The test is a simple and widely-used method to determine the titer of CPV in fecal and tissue samples of infected dogs. However, the test is less reliable when there are high levels of non-specific hemagglutinating factors in the samples (Nandi et al., 2019). The results of present study showed that the HA titer of the Bali isolate of CPV propagated in MDCK cells was comparable to the titer of CPV propagated in CrFK cells. In the previous studies, the HA titers of CPV obtained from infected CrFK cells at passage three varied from 2^6 to 2^{11} HA units (Parthiban et al., 2011: Hyeon et al., 2023). Additionally, the results of the present study indicated that serial passages in MDCK cells increased the titer of the virus in MDCK cells. A report detailing the methods by which passages can enhance CPV titer in MDCK cells is currently unavailable. However, in the influenza

virus, serial passages can increase the titers of the influenza virus adapted to grow in MDCK cells. Generally, after a series of passages, a stable titer of the influenza virus was obtained after three passages in MDCK cells, and a high titer of the virus was obtained after passage 20 (Genzel et al., 2010). A similar phenomenon appeared in CPV adapted to grow in MDCK cells and a higher titer of the Bali isolate of CPV might be obtained after further passages.

Evidence supporting the replication of the Bali CPV isolates in MDCK cells was shown by the immunocytochemistry test. This test, which detects the viral antigen in cells, has widely been used for the detection of virus replication in infected cell cultures. The test is simple and very useful to detect the virus in cell cultures when CPEs are not present, as seen with rabies virus replication in BHK-21 cells (Astawa et al., 2018). The results of the present study showed that CPV replication was characterized by the presence of viral antigens predominantly in the nuclei and, to a lesser extent, in the cytoplasm of infected cells (Figure 3). It was clear that by day three, some 40% of cells seemed to have been infected. Parvovirus is a small DNA virus that replicates in nuclei of infected cells and the presence of CPV antigen in the nuclei of MDCK cells provides further evidence for viral replication in the cells (Mattola et al., 2022). However, the process of virus replication and the synthesis of viral proteins takes place in the cytoplasm of cells (Mäntylä et al., 2020), matching with the ICC test that the CPV protein was also detected in the cytoplasm of the infected cells.

The replication of the Bali isolate of CPV in MDCK cells was also confirmed by the HI test in which chicken anti-CPV IgY could inhibit the virus to hemagglutinate porcine red blood cells (Figure 5). The HI test is commonly used to detect anti-CPV antibodies in the serum of vaccinated dogs (Cavalli et al., 2020). and can also be employed to detect the presence of CPV in the suspected samples, such as feces and tissues from infected dogs (Nandi and Kumar, 2010). It is important to note that the HA test may be affected by non-specific hemagglutinating factors, particularly when using serum as the antibody source, which can interfere with test results (Cavalli et al., 2021).

Active attenuated CPV vaccines are generally considered more effective and efficient in inducing a protective immune response in dogs. However, inactivated CPV vaccines still remain in use and BEI is a viral inactivating agent commonly used for the preparation of inactive vaccines (Hasan et al., 2017; Elbagory et al., 2021). The use of BEI-inactivated CPV for preparation of vaccine has been reported in previous studies. Previous studies have reported that BEI-inactivated CPV can induce antibody response in puppies (Elbagory et al., 2021). Similarly, it was found in this study that the BEI-inactivated Bali isolate of CPV induces antibody response in mice. Nevertheless, there is a need to further boost the virus concentration to improve the immune response. The current study clearly indicated that the virus concentration was elevated by PEG-precipitation of CPV, leading to a higher antibody titer in mice. Instances of using PEG as a precipitating agent for concentrating viruses such as infectious bursal disease virus (Leong et al., 2023), and SARS-CoV-2 have been widely reported (Lucansky et al., 2023).

Vaccines, whether active attenuated or inactivated, are available to prevent CPV infection in dogs (Hedgespeth and Fogle, 2021). The present study evaluated a local CPV isolate inactivated with BEI as a potential vaccine candidate. Since a local and virulent virus provides a vaccine with better antigenic homology to the field virus, utilizing the inactivated virus as a vaccine is preferable. For example, during the foot-and-mouth disease outbreaks in Pakistan, the vaccine to control the disease was prepared using inactivated local isolate propagated in adherent BHK-21 cell culture (Razak et al., 2023). The ability of local CPV isolate to induce immune response in mice indicates that this isolate holds promise as a candidate for developing a vaccine to control CPV infection in dogs.

CONCLUSION

The Bali isolate of CPV was able to grow in MDCK cell culture reaching the HA titer of 2^{11} HA units after 8 passages. The virus was confirmed as Canine parvovirus both by immunocytochemistry test using monoclonal antibody against CPV and by hemagglutination inhibition test using chicken anti-CPV antibody. The Bali isolate of CPV induced antibody response in mice with the highest response observed using PEG-purified CPV antigen compared to crude-unpurified CPV antigen. However, as this is an initial stage of vaccine development, further research is necessary to explore its potential for use as a vaccine, possibly using dogs as experimental animals instead of mice. In addition, it appeared that further passages of the virus in MDCK cells are still required to increase the titer of the virus obtained from infected MDCK cell cultures.

DECLARATIONS

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Authors' contributions

I Nyoman Mantik Astawa and Gusti Ayu Yuniati Kencana conceived and designed the experiment, collection, and data analysis. Both authors read and approved the final draft of the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by the authors.

Availability of data and materials

All data of this study are available upon reasonable requests from authors

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