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Preparation and in vitro evaluation of canine hyperimmune plasma against *canine parvovirus*: a strategy for passive immunotherapy

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ABSTRACT

The aim of this study was to prepare anti-canine parvovirus hyperimmune plasma through repeated vaccination of dogs and to evaluate its neutralizing capacity and immunoreactivity against viral antigens in comparison with plasma from healthy and infected animals. Four healthy dogs were vaccinated four times at two-week intervals using a live attenuated CPV-2 vaccine and hyperimmune plasma was collected. A CPV-2b strain was isolated from fecal samples of clinically infected dogs and propagated in MDCK cells. Viral identity was confirmed by PCR targeting the VP2 gene. Neutralizing antibody titers were determined using a virus neutralization assay, while CPV-specific antibody titers were measured by indirect ELISA. The immunogenicity against viral structural and non-structural proteins was assessed using Western blot analysis. Hyperimmune plasma exhibited the highest neutralizing titer (1:160±64) compared with healthy (1:26 ± 12) and infected (1:4.5 ± 2.5) groups ($p \leq 0.01$). ELISA optical density values were 0.63 ± 0.11, 0.50 ± 0.12, and 0.28±0.02 for hyperimmune, healthy, and infected plasma, respectively ($p \leq 0.001$). Western blot analysis revealed the presence of reactive antibodies against VP2 (66.0 ± 7.2), VP1 (30.7 ± 14.3), VP3 (45.0 ± 5.3), and NS1 (68.0 ± 7.1) in the hyperimmune group, whereas only low levels of antibodies against VP2 were detected in infected dogs. While antibodies against NS1 were observed exclusively in the hyperimmune plasma. In conclusion, Canine hyperimmune plasma contains high-titer, polyclonal neutralizing antibodies against CPV. These in vitro findings support the probable effectiveness and its potential utility as an emergency passive immunotherapy for CPV infection.

Keywords

Canine parvovirus, Hyperimmune plasma, Passive immunotherapy, Dog

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Abbreviations

CPV: Canine parvovirus
HIP:Hyperimmune plasma
MDCK: Madin-Darby Canine Kidney cell line
PCR: Polymerase Chain Reaction

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Introduction

Canine parvovirus Type 2 (CPV-2) is a highly pathogenic viral agent responsible for hemorrhagic gastroenteritis and acute leukopenia in young dogs, often leading to high mortality rates if untreated [1]. This non-enveloped, single-stranded DNA virus belongs to the Parvoviridae family, subfamily Parvovirinae, and is characterized by rapid genetic evolution [2]. Since its initial identification in the late 1970s, three major antigenic variants, CPV-2a, CPV-2b, and CPV-2c, have been identified and have circulated worldwide, with CPV-2c being the predominant type in many regions [3]. In Iran, recent studies indicate that all three variants are present, with CPV-2a remaining the predominant type [4, 5, 6]. Clinical signs of the disease include severe vomiting, hemorrhagic diarrhea, dehydration, leukopenia, and fever. In untreated cases, mortality rates may reach up to 90% [7]. Currently, no definitive treatment exists; clinical management relies on supportive therapy, including intravenous fluids, electrolyte replacement, broad-spectrum antibiotics, antiemetics, and pain control [8].

Nevertheless, these methods impose high costs on pet owners and often lack sufficient therapeutic efficacy [9]. In recent years, passive immunotherapy has gained attention as a complementary treatment approach. This method involves the administration of pre-formed antibodies derived from external sources, such as hyperimmune sera or concentrated immunoglobulins, into the sick animal to directly neutralize the virus and prevent disease progression [10]. Numerous studies have reported positive effects of passive immunization, including reduced hospitalization duration, decreased viral load, and improved survival rates [11, 12]. However, immunological reactions following serum and antibody injections from different animal species, the unavailability of hyperimmune serum for native strains, the high cost of imported products, and the risk of microbial contamination remain significant challenges in many regions, including Iran [13].

Repeated vaccination of healthy animals with live attenuated virus can lead to the production of hyperimmune sera containing high titers of neutralizing antibodies [14]. These sera can serve as a source for producing therapeutic or diagnostic products. In a study by Kotb et al. (2015), hyperimmune serum produced in horses was able to completely prevent mortality caused by CPV, when administered within the first 72 hours post-infection [10]. Given the conflicting results of previous studies on the efficacy of hyperimmune serum and the need to develop high-quality domestic sources, the present study was designed to prepare and immunologically evaluate canine hyperimmune plasma against CPV-2 using in vitro assays. Active immunity was induced in healthy dogs, and the titers of neutralizing antibodies were assessed through virus neutralization, ELISA, and Western blot assays. Additionally, the antibody response patterns against the viral structural proteins (VP1, VP2, and VP3) and the non-structural protein (NS1) were compared to explore the potential use of this serum in therapeutic and diagnostic applications.

Abbreviations-Cont'd

VP1, VP2, and VP3: viral structural proteins
NS1: Non-structural protein

Results

Assessment of initial health and immune response to vaccination

All four dogs underwent a two weeks quarantine period and screening tests, during which fecal tests for parasites and CPV shedding tests returned negative results. Following completion of the quarantine phase, all animals proceeded to the immunization phase. After administration of four doses of the live attenuated vaccine, no local or systemic adverse effects were observed. The mean serum volume collected from each dog was 260 ± 20 ml. No microbial contamination or hemolysis was detected in any of the samples.

Virus isolation, confirmation and genotyping of the isolated virus

Based on the results, cytopathic effects were observed in the cell culture (Figure 1A). Molecular confirmation demonstrated successful amplification of the expected gene fragments, approximately 583, 420 and 682 bp in length, in positive samples. agarose gel electrophoresis (1.5%) revealed distinct bands at expected position in positive lanes, while no band was observed in the negative control (Figure 1B). These findings confirm that the isolated sample contained the Canine parvovirus genome. Additionally, PCR-based genotyping results showed that the isolated virus yielded positive amplification with the CPV-2b/2a and CPV-2b specific primers. These PCR genotyping sets indicate that the isolated viruses belongs to the CPV-2b strain.

Neutralizing antibody titer in plasma

The mean neutralizing titer in hyperimmune sera (160 ± 64) was significantly higher than that observed in the healthy group (26 ± 12) and the infected group

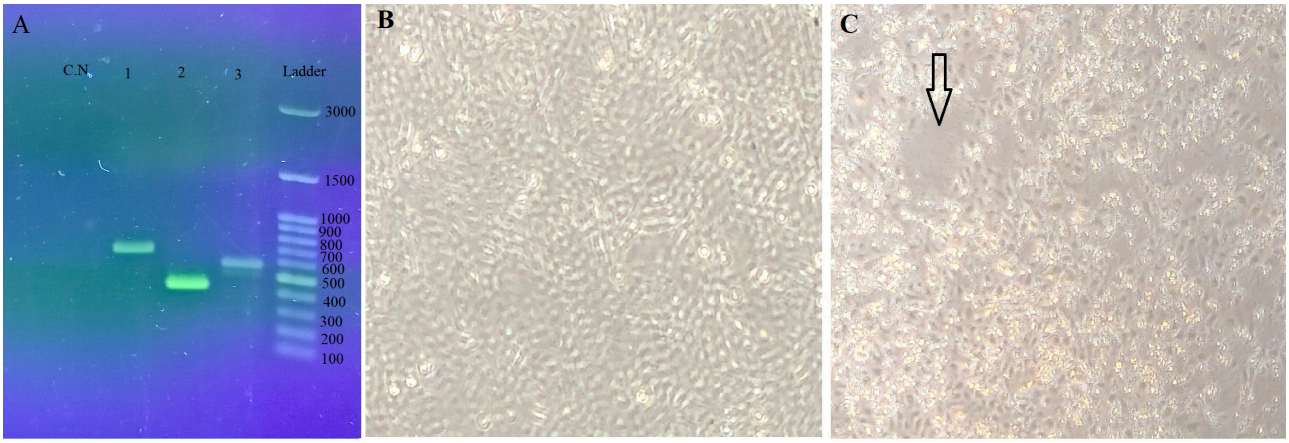

Figure 1.
Confirmation and genotyping of Canine parvovirus extracted from faecal samples of infected dogs. A) Confirmation and genotyping of isolated Canine parvovirus strain. A) Agarose gel electrophoresis of PCR products for the detection of VP2: (1) the PCR product of "Pab" primer pairs, (2) the PCR product of "Pb" primer pairs and (3) the PCR product of "555forc and 555revc" primer pairs. B) Normal culture of Madin-Darby Canine Kidney (MDCK) cell line. C) Occurrence of cytopathic effect (CPE) on the cell culture (indicated by arrow).

(4.5 ± 2.5) ($p \leq 0.001$). The highest individual titer (1:256) was observed in one dog within the hyperimmune group (Table 1). The Spearman correlation between the neutralizing titer and ELISA optical density was r = 0.89 ($p \leq 0.001$).

Specific anti-CPV antibody titer

The mean optical density (OD) value in hyperimmune group was 0.63±0.11, in the healthy group 0.59 ± 0.12, in the infected group 0.28 ± 0.02, and in the negative control sample 0.14 ± 0.004. Pairwise comparisons showed that both hyperimmune and healthy groups had significantly higher antibody levels than the infected group ($p \leq 0.001$) and the control group ($p \leq 0.001$). Additionally, the infected group showed significantly higher values than the control group ($p \leq 0.001$). However, no significant difference was observed between hyperimmune and the healthy group ($p > 0.05$)

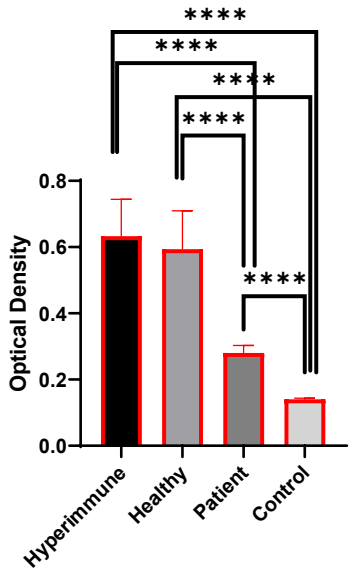

Figure 2.
The specific anti-Canine parvovirus titer in healthy, infected, and hyperimmune dogs evaluated by ELISA. The significant difference greater than 0.001 showed by four stars

Table 1.
Neutralizing titer of parvovirus by plasma from healthy, infected, and hyperimmune dogs.

Dog IDs	SN titer for Hyperimmune Dogs	SN titer for Healthy Dogs	SN titer for infected Dogs
1	1:128	1:32	1:8
2	1:256	1:32	1:2
3	1:128	1:8	1:4
4	1:128	1:32	1:4
Overall Mean	1:160 ± 64	1:26 ± 12	1:4.5 ± 2.5

Western blot results

The intensity of the VP2 band in hyperimmune group was 66.0 ± 7.2 luminous units, which was 1.6 times higher than the healthy group and 50 times higher the infected group (Table 2 and Figure 3A and 3B). VP1 and VP3 proteins were clearly detected in hyperimmune and healthy sera, with intensities of 30.7 ± 14.3 and 45.0 ± 5.3 units, respectively. The NS1 was specifically present in hyperimmune sera (68.0 ± 7.1 units), healthy (12.9±1.5 and remained below the detection threshold in infected group (≤ 2 units). Dot blot analysis of whole virus particles yielded results

Table 2.
Average intensity of Western blot bands in plasma analysis of healthy, infected, and hyperimmune dogs with Canine parvovirus (optical units ± standard deviation). ND: below the detection threshold. Statistically significant differences are indicated by different lowercase letters.

Antigen	Hyperimmune	Healthy	Infected	Statics
Whole-Virus	170.2 ± 9.5 [a]	91.2 ± 15.0 [b]	2.7 ± 2.0 c	F=312, $P \leq 0.001$
Vp1	30.7 ± 14.3 [a]	6.1 ± 2.9 [b]	ND	t= 4.8, $P = 0.039$
Vp2	66.0 ± 7.2 [a]	41.6 ± 3.4 [b]	1.3 ± 0.9 c	F=189, $P \leq 0.001$
Vp3	45.0 ± 5.3 [a]	11.9 ± 0.3 [b]	ND	t=18.7, $P = 0.003$
NS1 (N)	68.0 ± 7.1 [a]	12.9 ± 1.5 [b]	2.1 ± 1.8 c	F=215, $P \leq 0.001$

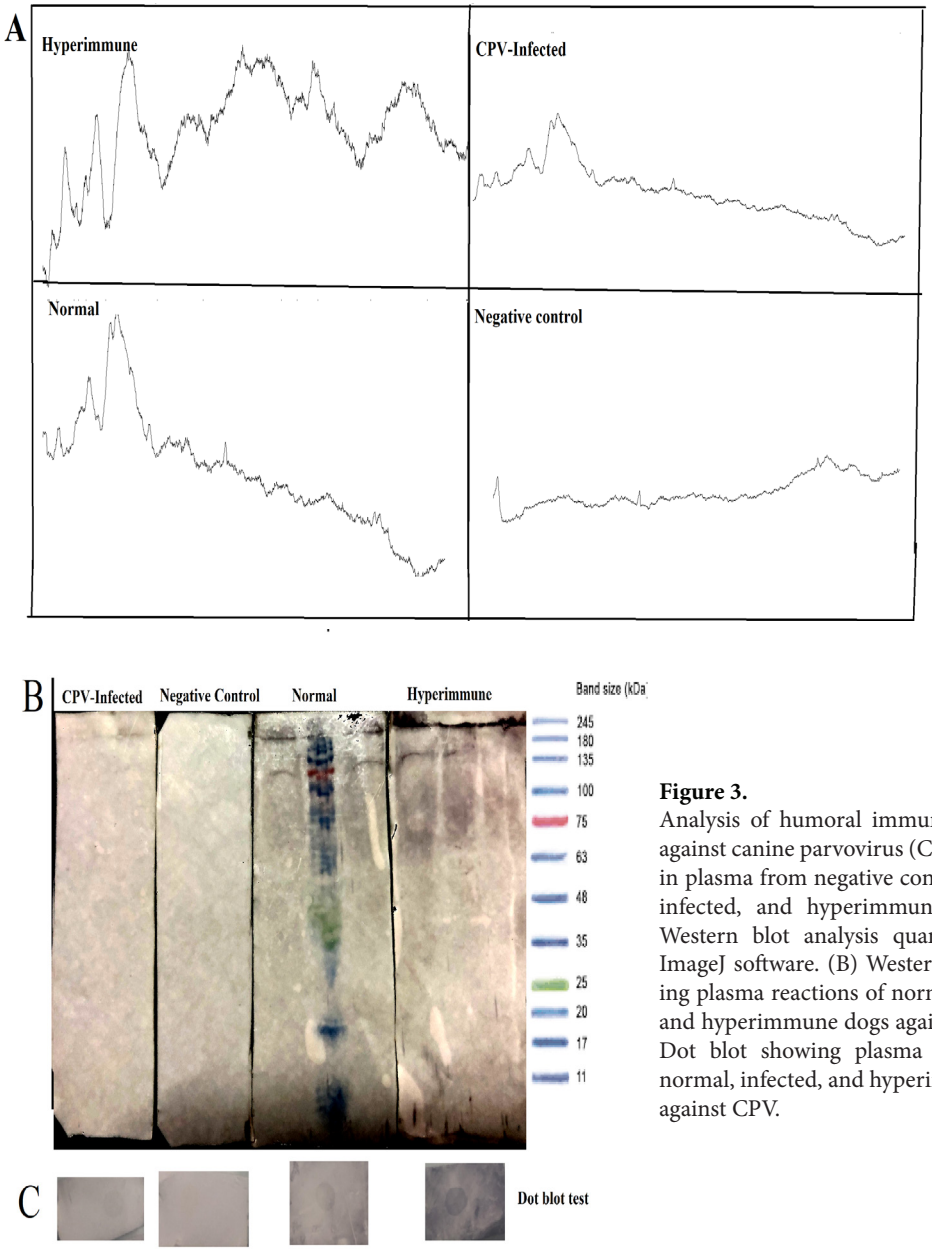

Figure 3.
Analysis of humoral immune responses against canine parvovirus (CPV) antigens in plasma from negative control, normal, infected, and hyperimmune dogs. (A) Western blot analysis quantified using ImageJ software. (B) Western blot showing plasma reactions of normal, infected, and hyperimmune dogs against CPV. (C) Dot blot showing plasma reactions of normal, infected, and hyperimmune dogs against CPV.

consistent with western blot assay, showing that the dot corresponding to hyperimmune plasma exhibited the highest reaction intensity, while the infected plasma showed the lowest intensity (Figure 3C).

Discussion

The aim of this study was to prepare and conduct in vitro evaluation of canine hyperimmune plasma against canine parvovirus. In the present study, canine

parvovirus was propagated in the MDCK cell line, with cytopathic effects (CPE) first observed on day three post infection. Although A-72 and CRFK/F81 cell lines are frequently employed for Canine parvovirus propagation, previous reports have demonstrated that MDCK cells are also permissive to CPV infection and capable of supporting productive viral replication [15]. Nevertheless, replication efficiency and absolute infectivity titers may vary among different cell lines. In the present study, all virus titration and neutralization assays were performed using the same cell line and virus stock, ensuring valid relative comparisons of neutralizing antibody responses between experimental groups. Additionally, PCR-based genotyping demonstrated that the isolated CPV strain belong to the CPV-2b variant. The virus neutralization assay provides a functional assessment of antibody-mediated inhibition of viral infectivity, supporting the relevance of these findings for passive immunotherapy. While $TCID_{50}$-based virus neutralization assay in current study provides a functional evaluation of antibody-mediated inhibition of viral infectivity; the inclusion of molecular quantification and standardized neutralization metrics could further enhance analytical resolution.

The appearance of cytopathic effects (CPE) at day three suggests a high replication capacity of the local CPV strain in MDCK cells. This is consistent with Kaur et al [15], who reported CPE appearance on the fifth day. The shorter time to CPE appearance in the present study may be attributed to two factors: the use of freshly passaged cells (less than 24 hours old), which are more sensitive to the virus, as well as the presence of mutations in the GH loop (positions 297 and 426) that enhance the virus's binding to the TLR receptor [16]. These findings indicate that the optimal culture conditions (37 °C, 5% CO_2, and DMEM supplemented with 2% FBS) are sufficient for the rapid replication of the studied CPV strains.

The mean titer of 1:160 in hyperimmune sera was significantly higher than that of normal serum (1:26) and infected sera (1:4.5) ($p \leq 0.001$). This 50-fold difference is consistent with two key studies; Kotb et al. (2015) reported a titer of 1:024 in horses (10), and Larson et al. (2024) recorded a titer of 1,320 in dogs receiving monoclonal antibodies, resulting in a 100% reduction in mortality [12]. Similarly, Dash et al. (2020), reported 25% reduction in mortality rate following administration of hyperimmune serum [11]. The neutralization assay using CPV-2b strain and antibody titer showed significant difference among evaluated sera. The result could be interoperated as necessity of enhancement of humoral immunity of the infected animals in acute phase of the disease using hyperimmune sera or polyclonal IgG. The hyperimmune serum used in this study was prepared using CPV-2 vaccine, which is designed to provide protection against all CPV-2 strains. Therefore, the resulting hyperimmune serum may be effective in infected animals against various variants of virus.

The detection of VP2, VP1, VP3, and NS1 exclusively in hyperimmune sera, along with the absence of VP1 and VP3 in the patient's serum, aligns with the findings of Nelson et al. (2007) [14]. They reported that these proteins are expressed at low levels (<5%) in parvoviruses and elicit a weak immune response during natural infection. Furthermore, NS1, detected clearly only in hyperimmune sera. This observation is consistent with the study by Miao et al. (2022), who demonstrated that antibodies against NS1 appear only after repeated vaccination [17]. Based on current result, the infected animals in acute phase of the disease, have significant reduced titer against CPV-2 in all in-vitro tests. So, the lower immunogenic protein like as NS1 was appeared only in hyperimmune animals or healthy animals. These proteins may therefore have potential to be used for serologically diagnosis of the animals in acute phase of the disease.

Spearman's correlation coefficient was 0.89 between neutralization titer and ELISA titer, and 0.85 between ELISA optical density and the sum of Western blot band intensities, indicating that these three methods are highly interchangeable. This finding is consistent with Decaro et al. (2006), who reported a coefficient of 0.82 between the neutralization and ELISA tests [18]. Therefore, in clinical settings where neutralization tests are time consuming, ELISA with a cutoff OD ≥ 0.45 (sensitivity 92% and specificity 88%) may serve as a rapid screening tool.

Asil et al. (2023), reported that CPV-2 disrupts B cell differentiation and suppresses both humoral and cellular immune responses by significantly reducing IL-6, CD4+, and CD8+ levels in peripheral leukocytes. This condition is associated with a severe form of the disease in dogs (CPVD-Severe), characterized by leukopenia, thymic atrophy, and high mortality. The reduction in antibody titers in affected dogs compared to healthy dogs can be attributed to this immune suppression, as well as to the neutralization of antibodies by the virus during the acute phase of the disease [19]. Similarly, Ulas et al. (2024), indicated that using standard supportive treatment, the survival of the infected animals was only 57.1%, whereas the addition of recombinant interferon-ω improved the neutrophil/lymphocyte ratio and increased circulating lymphocytes, raising survival to 85.7% [20]. These finding indicates that cellular immune recovery during the acute phase of the disease is critical, as

supportive therapy alone may be insufficient in severe symptomatic cases .

Given the suppression of active immunity during the acute phase of the disease, passive immunotherapy represents as the most rational therapeutic strategy. In the current study, the hyperimmune serum exhibited a neutralization titer of 1:160 and an OD_{450} of 0.63, demonstrating 50 times higher titer than the neutralizing capacity of infected serum. In addition to targeting VP2, the hyperimmune serum also produced antibodies against VP1, VP3, and NS1, providing multi-epitope coverage and reducing the risk of antigenic escape. According to a simplified pharmacokinetic model, canine IgG has a half-life of 8–10 days. Following intravenous injection of 5–10 mg/kg of hyperimmune serum with a titer of 1:160, serum levels of ≥ 1:80, which considered as a protective titer [21, 22], may be maintained for more than 72 hours. This period coincides with peak viremia (days 3 to 5 post infection), providing sufficient opportunity for initial control of infection before severe occurrence of intestinal damage. In the present study, the neutralizing titer of the prepared hyperimmune serum was 1:160, which is lower than that of commonly imported products (typically 1:512); while simultaneously decreasing dependence on imports conditions. However, considering the lower cost of domestic production, it can reduce treatment expenses by up to 50%. Therefore, despite lower absolute titers, the locally produced hyperimmune serum is not only pharmacokinetically effective but also may represent as a more economically viable and accessible therapeutic alternatives.

Conclusion

For the first time in southwestern Iran, a CPV-2b variant was isolated and characterized, demonstrating high replication capacity in MDCK cells. Multi-steps vaccination of healthy dogs with a live attenuated vaccine induced the production of indigenous hyperimmune sera with a neutralization titer of 1:160, significantly higher than that observed in both healthy and naturally infected dogs. Western blot analysis identified VP2 as the dominant immunogenetic antigen, while VP1, VP3, and especially NS1 were detected in hyperimmune sera and healthy dogs. The method described in current study for preparation of canine hyperimmune serum using CPV-2 vaccination yielded promising in vitro results. The prepared hyperimmune serum or its purified polyclonal IgG can be manipulated for preparation of a safe and endotoxin free product that could be receive license for in vivo evaluation of its ppharmacokinetics and efficacy in infected dogs with parvovirus.

Materials and Methods

Vaccination and preparation of hyperimmune sera

All experimental procedures, including animal care, treatment, and sample collection, were conducted in accordance with the ethical guidelines approved by the Ethics Committee and the regulations for working with animals at Shahid Chamran University of Ahvaz, under approval number 1404.088. In this study, four young adult female indigenous dogs, aged between 1.5 and 2.5 years, were obtained and individually housed in separate cages for two weeks. During this period, clinical examinations were performed, and a complete anthelmintic drug (Caniverm) was administered. After confirming the animals' health and the absence of prior parvovirus infection using an immunochromatography test kit (Hangzhou Evegen Biotech, China), a specific monovalent parvovirus vaccine (BioVeta brand) was administered subcutaneously in four doses at two-week intervals. Ten days after the final injection, a total of 500 mL of blood was collected from each dog in two occasions at two weeks intervals using special blood transfusion bags. Plasma was separated using standard centrifugation methods and stored at –20°C until efficacy testing was performed.

Collection of fecal and blood samples

Four dogs presenting at the Veterinary Faculty Clinic of Shahid Chamran University of Ahvaz tested positive for parvovirus antigen via immunochromatography kit. From each case, 5 g of fecal sample and 2 mL of blood were collected and transported to the Virology laboratory. Fecal samples were homogenized in sterile PBS and centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and filtered through 0.22 µm syringe filters for decontamination. The filtered liquid was then treated with antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL) prior to virus isolation. Plasma was separated from the collected blood samples using standard methods. Additionally, plasma samples from four healthy dogs, which tested negative for the antigen and showed no clinical signs, were also prepared.

Virus isolation

Virus isolation was performed using the MDCK (Madin-Darby Canine Kidney) cell line cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% fetal bovine serum (FBS). MDCK cells were used for virus isolation as a canine origin epithelial cell line that has previously been reported to support the replication of canine parvovirus with typical cytopathic effects [15]. When cell monolayer reached approximately 40–50% confluence, the cells were infected with 0.5 mL of virus-containing fluid and incubated at 37°C for 1 hour to allow virus adsorption. Following adsorption, inocula were removed, cells were washed with sterile PBS, an appropriate volume of DMEM containing 2% FBS was added to the flasks, which were then incubated at 37°C in a 5% CO2 atmosphere for 3-4 days until cytopathic effects (CPE) were observed. Following the appearance of CPE, the flasks were stored at -20°C, then thawed. The contents were transferred to sterile Falcon tubes and cell debris was removed by centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was used as the virus stock [13]. The virus infectivity (TCID50) of the stock was determined using the Reed-Muench method to ensure the appropriate virus concentration for the virus neutralization assay [16].

PCR test for confirmation and genotyping of the isolated virus

To confirm that observed CPE resulted from Canine parvovirus infection and not nonspecific cytotoxicity, the identity of the propagated virus was molecularly confirmed by PCR amplification of a VP2 gene fragment (583 bp) from infected cell cultures [23]. Furthermore, to determine the CPV genotype of the isolated virus, two separate PCR assays were performed using genotype-specific primer sets targeting CPV-2a/2b, and CPV-2b, respectively [24], as detailed in Table 3. Nucleic acids were first extracted from the viral source using the Sinapure DNA extraction kit (Sinaclon Company, Iran) following the manufacturer's instructions. PCR was then performed in a 25 µL reaction volume, comprising 12.5 µL of Amplicon 2x master mix, 1 µL each of forward and reverse primers at 10 µM concentration, 7.5 µL of nuclease-free water (Sinaclon Company, Iran), and 3 µL of template DNA. The cycling profile for all primer sets and rounds included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes. PCR products were electrophoresed alongside a 100 bp DNA ladder (Sinaclon, Iran) on a 1.5% agarose gel containing safe dye (Sinagen, Iran) at 100 volts for 60 minutes and visualized under UV light using a transilluminator (Uvtec, England).

Table 3.
Sequence of the used primers for identification and genotyping of *Canine parvovirus*.

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Target Genotype	Reference
555forc	CAGGAAGATATCCAGAAGGA	583	All	[23]
555revc	GGTGCTAGTTGATATGTAATAAACA			[24]
Pbs	CTTTAACCTTCCTGTAACAG	428	B	[24]
Pbas	CATAGTTAAATTGGTTATCTAC			
Pabs	GAAGAGTGGTTGTAAATAATT	682	A / B	
Pabas	CCTATATAACCAAAGTTAGTAC			

Virus neutralization test

The infectivity of the prepared virus stock was calculated as the 50 % tissue culture infectious dose (TCID50) using the Reed and Muench method [16]. Subsequently, a dose of 100 TCID50 was used in the virus neutralization assay. The neutralizing capacity of hyperimmune plasma was compared with plasma of healthy and infected dogs with Canine parvovirus. After collecting plasma samples from each group, the samples were filtered through 0.22 µm syringe filters for potential decontamination. To remove non-specific and inhibitory factors present, the plasma samples were first incubated at 55°C for 30 minutes. Each sample was then diluted at a ratio of 1:2. Next, 100 TCID50 of the active virus was mixed with an equal volume of the diluted plasma. To facilitate the binding reaction between the antibodies (present in the plasma) and the antigen (virus), the mixture was incubated at room temperature for 1 hour. Subsequently, MDCK cells were co-cultured in 96-well plates with each dog plasma dilution mixed with the virus, in triplicate. The microplates were incubated at 37°C in 2% CO2 and monitored daily under a microscope. At the end of the incubation period, the neutralizing antibody titer against parvovirus in the tested plasma samples was determined based on the presence or absence of cytopathic effects.

Assessment of parvovirus-specific antibody titer

Inactivated and freeze-thawed antigens of isolated parvovirus strain from infected dogs were diluted in carbonate-bicarbonate buffer (pH 9.6) and coated onto ELISA plates (Jet Biofil, China) at a concentration of 1 µg/well. The plates were incubated at 4 °C overnight. After that they been washed with PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked by addition 250 µL of 4% skimmed milk to each well and incubation for 2 hours at room temperature. After additional three washing step same as before, plasma samples from 4 healthy dogs, 4 infected dogs, and 4 hyperimmune dogs, diluted according to optimized recipe, were added to each well and incubated for 1 hour at room temperature. Following another washing step, rabbit anti-dog IgG at 10 µg/mL was added, followed by another one-hour incubation at room temperature. Addition 100 µL of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Immuno Chemistry Technologies Company, USA) at a 1:20,000 dilution and incubation at room temperature for one hours was conducted in followed step. In the final step, 60 µL of tetramethylbenzidine (TMB) substrate was added to each well, and after 10 minutes, 60 µL of stop solution was added. The optical density of each sample was measured at 450 nm using a spectrophotometer.

Antibody reaction with parvovirus immunogenic proteins

A Western blot test was conducted to compare the pattern of immunogenic proteins of parvovirus in infected, hyperimmune, and healthy dogs. Freeze-thawed viral antigens at equal concentrations equal to 200 µg/mL were used for this assay. Initially, antigens were mixed with sample buffer containing 0.1 M beta-mercaptoethanol and boiled for 10 minutes. A total of 25 µL was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 120 minutes. The antigens were then transferred to nitrocellulose membranes using the wet transfer method. Active sites on the membranes were blocked with 4% skimmed milk overnight at 4°C. Optimized 5% serum dilutions from infected, hyperimmune, and healthy dogs were separately applied to each sample lane. Subsequently, a secondary Anti-dog antibody of rabbit origin was added at a concentration of 10 µg/mL, followed by an HRP-conjugated antibody diluted 1:7.500. In the final step, alpha-chloronaphthol substrate was added, and after 15 minutes

of incubation in the dark, the membranes were washed with distilled water to stop the reaction. Photographs of the test results were taken, and the molecular weight and intensity of the viral immunogenic protein bands in the test groups was defined using ImageJ software. The molecular weights of the Western blot bands were used to identify the corresponding CPV antigens based on previous studies. The visible bands with molecular weights of 82.3, 64, 65.7, and 76.6 kDa corresponded to VP1, VP2, VP3, and NS1, respectively [25-27]. Dot blot assay was performed by coating 1 µL of CPV antigens (200 µg/mL) onto PVDF membrane. The membrane was incubated at room temperature until dry. Afterward, the membranes were washed three times with PBS-T buffer and blocked with a 4% skim milk solution for 2 hours. The subsequent steps were carried out as described in the Western blot section.

Statistical analysis

The study results were analyzed using SPSS software with a one-way analysis of variance (ANOVA) method. Statistical significance was set at $p \leq 0.05$.

Authors' Contributions

All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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