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PII: S0378-1135(18)31496-2
DOI: https://doi.org/10.1016/j.vetmic.2019.03.015
Reference: VETMIC 8255

To appear in: *VETMIC*

Received date: 20 December 2018
Revised date: 11 March 2019
Accepted date: 12 March 2019

Please cite this article as: Armenise A, Trerotoli P, Cirone F, De Nitto A, De Sario C, Bertazzolo W, Pratelli A, Decaro N, Use of recombinant canine granulocyte-colony stimulating factor to increase leukocyte count in dogs naturally infected by canine parvovirus, *Veterinary Microbiology* (2019), https://doi.org/10.1016/j.vetmic.2019.03.015

Use of recombinant canine granulocyte-colony stimulating factor to increase leukocyte count in dogs naturally infected by canine parvovirus

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Running title: cG-CSF increases leukocytes in CPV-2 infected pups

Highlights

- Canine parvovirus is one of the most important cause of morbidity and mortality in young dogs
- G-CSFs is an haemopoietic regulatory glycoprotein that promotes proliferation and activation of leucocytes in bone marrow.
- Treatment with rcG-CSF determines with lymphocytosis and monocytosis, without influencing neutrophils count.

Abstract

Canine parvovirus (CPV) is one of the most important cause of mortality in young dogs and no specific treatment exists. Since prolonged leukopenia greatly increases the risk of death in infected pups, strategies to counteract this decline were investigated. The outcomes of CPV naturally infected pups treated with the recombinant canine granulocyte-colony stimulating factor (rcG-CSF), in combination with the routine therapy, were compared with similarly-managed infected pups not treated with rcG-CSF. A non-randomized prospective clinical trial was performed on 62 CPV infected pups with WBC counts <3000 cells/µL and two different groups were selected based on a non-randomized approach. Group A dogs (31/62) received 5 µg/Kg of rcG-CSF daily from the hospitalization day until WBC reached the reference range (3 to 5 days) and group B (31/62) received 1 ml of placebo injection. All dogs in group A recovered, while five dogs in group B died. The rcG-CSF treatment demonstrated a statistically significant effect on WBC counts (p<0.0001) and, surprisingly, also on lymphocytes and monocytes counts (p<0.0001). There was no significant effect of treatment on neutrophil count (p=0.5502). Although lymphocytes and monocytes are not a specific target for rcG-CSF, our study highlights that rcG-CSF is able to improve haematological parameters compared to untreated dogs and a clear increase in their number was detected, as previously described for humans treated with the homologous molecule.

Keywords: canine parvovirus; recombinant canine granulocyte-colony stimulating factor; therapy; leukocyte counts.

Introduction

Since its emergence in 1978 (Appel et al., 1979), canine parvovirus (CPV) remains one of the most important cause of mortality in young dogs (Goddard and Leisewitz, 2010) determining severe, often fatal, haemorrhagic gastroenteritis (Truyen, 2006). In the 1980s, two antigenic variants, CPV-2a and CPV-2b, emerged and within few years completely replaced the original type 2 (CPV-2). In 2000 a new antigenic type was firstly detected in Italy in the faecal samples of two dogs with haemorrhagic enteritis (Buonavoglia et al., 2001). Currently, these variants are variously distributed in the canine population worldwide (Decaro and Buonavoglia, 2012, 2017).

After CPV penetration through the oronasal route, viral replication begins in the lymphoid tissue of the oropharynx and thymus, and the virus is disseminated to the intestinal crypts of the small intestine, the bone marrow and the lymphoid tissues by haematogenous spread (Macartney et al., 1984; Meunier et al., 1985; Goddard and Leisewitz, 2010). Infection of leukocytes, mainly circulating and tissue-associated lymphocytes, induces acute lymphopenia, often associated with neutropenia (Pollock, 1982). The lymphocytolysis is extensive in the thymus cortex, explaining the severe lymphopenia developed by infected puppies (Goddard and Leisewitz, 2010). Clinical signs consist of anorexia, depression, dehydration, vomiting and mucoid or bloody diarrhoea, frequently associated to fever. Leukopenia is a constant finding, with white blood cell (WBC) count dropping below 2000 cells/mL of blood (Decaro and Buonavoglia, 2012). Due to the destruction of the intestinal villi, puppies with severe CPV enteritis may also develop a severe protein-losing enteropathy.

No specific treatment exists for CPV enteritis and the only management is supportive care with fluid therapy to treat dehydration and re-establish circulating blood volume, as well as to correct electrolyte and acid-base disturbances. In addition, therapy includes correction of hypoglycaemia and

hypokalaemia, combination of antimicrobials and antiemetic, and enteral nutritional support (Prittie, 2004; Goddard and Leisewitz, 2010). In the last decades, attention has been paid to the use of immunomodulatory cytochines in dogs affected with pathologies characterized by marked leukopenia, such as CPV infection (Dhama et al., 2015).

Recently, the major haematopoietic growth factors, also known as colony stimulating-factors (CSFs), from rodents, dogs and humans have been purified, molecularly cloned and distinguished on the basis of the different haematopoietic colony stimulated *in vitro*. Granulocyte-CSFs (G-CSFs) are a class of haematopoietic regulatory glycoproteins that promote proliferation, differentiation, and activation of neutrophils in the bone marrow. Recombinant human G-CSF (rhG-CSF) approved for human beings, has been used in dogs for the treatment of parvovirus-induced neutropenia, but it caused an initial neutrophilia and monocytosis and unfortunately, an abrupt decline to baseline values and below occurred very shortly (Kraft and Kuffer, 1995). The decline in neutrophil and monocyte counts caused by rhG-CSF depends on the development of neutralizing antibodies against the heterologous protein (Lothrop et al., 1988; Hammond et al., 1991; Yamamoto et al., 2009). Consequently, specific, safe and no immunogenic biological growth factors are needed for small animal practice. Recently, the canine G-CSF (cG-CSF) has been isolated and molecularly cloned. Dogs subcutaneously (sc) inoculated with the purified recombinant cytokine (rcG-CSF) increased significantly their leukocyte counts and no severe side effects were observed (Yamamoto et al., 2009).

Since prolonged leukopenia greatly increases the risk of death of CPV infected pups, strategies to counteract this decline were investigated. Primary endpoints of the present study were: i) the evaluation of haematological parameters (total number of WBC and neutrophils, monocytes and lymphocytes count), ii) the treatment outcomes and iii) the duration of hospitalization of naturally infected pups administered with rcG-CSF, in combination with the routine therapy compared with similarly-managed infected pups that were not treated with rcG-CSF. Furthermore, the effect of rcG-CSF treatment on CPV infected pups was also evaluated with regards to the different CPV variants involved in the disease.

Materials and Methods

Study plan

A non-randomized prospective clinical trial was conducted over a period of 18 months. Pups with gastrointestinal clinical signs (lethargy, anorexia, vomiting, diarrhoea) admitted to the Veterinary Hospital Santa Fara of Bari (Italy) were tested for CPV with the commercial rapid antigenic test (SNAP® Canine Parvovirus Antigen Test, IDEXX Laboratories, Westbrook, USA), following the manufacturer's instructions. CPV positive samples were confirmed by real-time PCR with a TaqMan probe (Decaro et al., 2005) and the detected strains were identified by means of minor groove binder (MGB) probe assays able to characterise the CPV variants (CPV-2a, CPV-2b and CPV-2c) (Decaro et al., 2006b) and to eventually discriminate between vaccine and field viruses (Decaro et al., 2006a, 2006c). The inclusion criteria in the study plan were the positivity at the TaqMan PCR assay, the WBC count < 3000 cells/µL, and the onset of the clinical signs (1-2 days before hospitalization). The study was approved by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari, Italy (authorisation no. 08/2016).

A data log was obtained for each dog during every day of hospitalization in order to report breed, age, sex, number and types of vaccines performed, duration of clinical signs before admission, clinical parameters during hospitalization (body temperature, pulse quality, heart rate, respiratory rate, hydration status, body weight), complete blood count, venous blood gas with electrolytes and lactate (RapidPoint 500, Siemens, Munched, Germany), packed cell volume and total plasma protein. All dogs had a peripheral intravenous catheter and received fluid therapy based on their daily hydration status, adding ongoing losses due to vomiting and diarrhea episodes. Crystalloid fluids (Elettrolitica Reidratante III, Novaselect, Potenza, Italy) were used together with dextrose bolus (Glucosio 33%, S.A.L.F. S.p.A., Cenate Sotto, Bergamo, Italy) when hypoglycemia was present, and potassium supplementation based on daily potassium measurements, was administered. Additional treatments included maropitant citrate at 1 mg/kg/day sc (Cerenia, Pfizer Animal Health, NY, USA),

ampicillin/sulbactam at 30 mg/kg by intravenous injection two time daily (IBI, Aprilia, LT, Italy), and cefovecin at 8 mg/kg sc once (Convenia, Zoetis Inc., Kalamazoo, MI, USA). An enteral feeding plan was applied to each dog with a commercial diet (A/D Hill's Pet Nutrition, Inc. Rome, Italy) at 1 ml/kg per os according to the patient's appetite. If pups demonstrated no voluntary appetite they were syringe fed, stopping the feeding if patients showed nausea.

Group A and group B included each 31 CPV positive dogs. Group A consisted of 21 intact males and 10 spayed females, and group B consisted of 19 intact males and 12 spayed females. Recruited animals were purebreds (33) or mixed breed (29) dogs, with an age ranging from 8 weeks to 6 months (median 3 months, Inter Quartile Range, IQR, 3-5 months). Seven out of the 31 dogs of group A and 8 out of the 31 dogs of group B had a complete CPV vaccination plan according to the current guidelines on canine vaccination[1], while remaining pups of both groups had been submitted to an incomplete or incorrect CPV vaccination plan. Dogs of both groups were subjected to conventional therapy as reported, as well as to pain medication when necessary. In addition, dogs of group A received daily 5 µg/Kg of rcG-CSF (Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA, USA) sc, from the day of hospitalization until WBC increased reaching the reference range. The treatment period ranged from 3 to 5 days (except for those patients that died prematurely). The rcG-CSF dose was selected following previously reported data (Rewerts et al., 1998; Mischke et al., 2001; Duffy et al., 2010). Dogs of control group were injected with 1 ml of placebo (sterile saline solution) (Sodio Cloruro 0,9%, S.A.L.F., Cenate Sotto (BG), Italy). Animals of both groups were bled daily (2 ml) and EDTA-blood was used for blood cell counts using the Procyte Dx Haematology Analyser (IDEXX Laboratories, Westbrook, USA). The owners of both dog groups were informed about treatment and informed consent was required before inclusion of their dog in the study.

[1] See: https://www.aaha.org/guidelines/canine_vaccination_guidelines.aspx (accessed online 24 August 2018).

Staff veterinarians were not blinded for patients' allocation in groups. Dogs of both groups were daily monitored starting from the hospitalization day until their discharge. Blood samples were collected once daily immediately before rcG-CSF or placebo injection, at the same time.

Statistical analysis

Data on WBC counts were analysed by a general mixed model for repeated measures; the first level unit was the count in each specific day after the beginning of the treatment, while level two units were dogs. A class variable (the treatment) was used in the model to evaluate differences in WBC profile between treatments; in the model the effect of time and interaction between time and treatment was also evaluated. The different WBC subsets (lymphocytes, monocytes, neutrophils) and total WBC were transformed into natural logarithm before the analysis and then the results of the models were back-transformed for the description. Data were summarised as least square mean of the fitted model. Post-hoc comparisons between groups were adjusted according to Tukey (Kramer, 1956).

Other quantitative variables were shown as mean and standard deviation, if Gaussian distributed, as median and IQR otherwise. The student's t-test or U-Mann-Whitney was used to compare quantitative variables, as appropriate. Qualitative variables were described as counts and percentage. Comparisons between independent groups (i.e. treated vs not treated) were performed by chi-square test or Fisher exact test, as appropriate.

The log-rank test and the Kaplan-Meier curve were applied to compare the survival time from the beginning of the treatment to recovery. The log-rank test was used to compare death rate between treated and not-treated dogs.

A p-value<0.05 was considered for statistical significance. The analysis was done by SAS 9.4 (SAS Institute Inc, Cary, NC, USA).

Results

The study started as a single arm experiment. All CPV infected dogs admitted to the veterinary hospital in the period of the availability of rcG-CSF (approximately 9 months), were treated (group A). In the following period (approximately 9 months), the same number of group A dogs that meets the inclusion criteria, were not treated and represented the control group (B). A total of 62 infected dogs were monitored, 31 dogs per group, each over a period of about 5 days. The population characteristics (sex, age, breed, vaccination plan) and the clinical signs of the pups enrolled into the two study groups were compared and analyzed (Table 1). There was not statistically significant difference in sex between the 2 groups (p=0.5955). The median age was 3 months in both groups (IQR group A: 3-4 months, IQR group B: 2-6 months, p=0.8849). Likewise, no significant difference was observed both in different types of breeds (purebreds and mixed breeds) (p=0.9678) and in CPV vaccination plan (p=0.7668) between the 2 groups. By using the real-time PCR assays, the 31 dogs of group A were found to be infected by CPV-2a (n = 20), CPV-2b (n = 10) or CPV-2c (n = 1). The 31 dogs of group B included CPV-2a (n = 20), CPV-2b (n = 11), while no CPV-2c positive dogs were present. There was no significant difference in the CPV variants involved (p=0.8597). At the time of admission, all dogs were anorexic. No significant differences were observed by Fisher Exact test between groups for anorexia (p=1.0), vomiting (p=1.0) and diarrhea (p=0.0525) (Table 1).

The median number of days of duration of clinical signs before hospitalization was 1 days for group A (IQR, 1-2) and 2 days for group B (IQR 1-2) (p=0.5454), There was no statistically significant difference between groups for days elapsed between the onset of clinical signs and the appearance of marked leukopenia (p=0.6068). All group A dogs recovered, while only 26/31 dogs (83.8%) of group B showed resolution of clinical signs. The median time for the resolution of clinical signs was 3 days for group A and 4 days for group B, and the log-rank test resulted statistically significant (chi-square=27.9351, p<0.0001). Five dogs in group B died and the death rate resulted significantly different between the groups (chi-square =5.3615, p=0.0206).

The difference in cells counts between treated- vs untreated-dogs are shown in Table 2. Treatment demonstrated a statistically significant effect on WBC counts (p<0.0001), with a faster and

more considerable increase in the treated group in comparison with the untreated one (Figure 1). In the treated group, WBC mean increased from 1.71 (95% CI 1.49-1.96) at day 1 to 29.24 x 1000 cells/µL (K/µL) of blood at day 5, while in non-treated group, WBC boost resulted slower, varying from 1.54 at day 1 to 12.17 K/µL of blood at day 5. Except for the first day, the values of the two groups resulted significantly different at each time point, after *p*-value was adjusted according to Tuckey. There was a statistically significant effect of rcG-CSF treatment ($p<0.0001$) on lymphocytes count: the day 1-values were not significantly different between the two groups ($p=0.9139$), but from day 2 to the day 5 there was a stronger increase in dogs of treated group. The two groups were different at day 2 ($p<0.0001$), at day 3 ($p<0.0001$) and day 4 ($p=0.0072$). At day 5 the two groups did not show any significant difference, even if the means had large differences ($p=0.4458$). The treatment gave a statistically significant effect on monocyte counts ($p<0.0001$), but looking at day-by-day comparisons, monocytes resulted significantly different only at day 2 ($p<0.0001$) and day 3 ($p<0.0001$). There was no statistically significant effect of treatment on neutrophil count ($p=0.5502$). Looking at means of the day-by-day comparison, neutrophils had the same trend in dogs of both groups.

Discussion

Development of a safe and effective treatment for parvovirus infection is particularly important, since CPV causes a dramatic depletion of leukocyte count with marked lymphopenia and neutropenia. Therefore, the use of rcG-CSF has been previously evaluated for its immunomodulatory effect, but unfortunately, despite the improvement of WBC counts obtained, some treated patients died after discharge (Duffy et al., 2010). As postulated by those Authors, supporting the idea that rcG-CSF has not a direct role in causing those unexpected deaths, we evaluated the role of rcG-CSF in the treatment of CPV naturally infected pups, looking at WBC, neutrophil, lymphocyte and monocyte counts at time of hospitalization.

Interestingly, the survival rate observed in the present study was different from previously reported (Duffy et al., 2010) and none of the patients treated died after hospital discharge. Owners and/or legal local authorities responsible for the dogs were contacted two weeks later to provide updates on clinical conditions of convalescent dogs and all confirmed that they were alive, growing normally.

The injection of rcG-CSF determined a marked stimulation of lymphocytes and monocytes, but neutrophil counts did not improve as faster as lymphocyte and monocyte counts did, as observed by Duffy et al. (2010). Considering that dogs of both groups showed severe leukopenia, it is plausible that CPV reduced the rcG-CSF neutrophil receptor population or, at least, their ability to bind rcG-CSF, without causing the same effects on lymphocytes and monocytes. Looking at neutrophil counts in recovered dogs of both groups, it seems that their role against CPV infection is marginal, while monitoring of total leukocyte counts yields prognostic information about the likely course of infection, considering that leukopenia is usually proportional to the severity of illness and varies according to the stage of the disease (Green and Decaro, 2012). Despite a significant difference between the baseline neutrophil counts of both groups before the inclusion in the study, it is reasonable to believe that this apparent lack of homogeneity did not affect the obtained results, because the lowest numbers of neutrophils were observed in the treated group and at discharge the counts were in both cases under the reference range. In CPV enteritis, severe neutropenia can not be attributed only to destruction of mitotically active myeloblasts in bone marrow as a direct effect of the virus, but it may be related also to endotoxemia and possible sepsis, as well as a massive loss of neutrophils through the intestinal wall (Goddard et al., 2008). Moreover, neutropenia is strictly affected by two factors, time of infection and infectious dose, variables that could not be exactly determined, being a clinical study.

A direct role of monocyte activity against CPV has not been reported, but monocytes counts should also be monitored during CPV infection, because monocytopenia is always associated with the increase in mortality and an impaired monocyte function could contribute to the development of

secondary infections (Goddard et al., 2008). Although lymphocytes and monocytes are not specific targets for rcG-CSF, our study highlights that the first single injection causes a clear increase in their number as reported by other researchers (Zinkl et al., 1992; Duffy et al., 2010). Current veterinary literature is lacking possible explanations accounting for rcG-CSF stimulation of lymphocytes and monocytes. Few studies reported improved neutrophil counts after rcG-CSF treatment in patients with chemotherapy-induced neutropenia (Ogilvie et al., 1992; Yamamoto et al., 2011), as well as in dogs with normal neutrophil counts or displaying neutropenia associated to other causes (Mishu et al., 1992), but the role of rcG-CSF in stimulating lymphocytes and monocytes has not been yet investigated in these animals.

In humans, the immunomodulatory activity of hG-CSF has been demonstrated and a direct effect on lymphocytes and monocytes has been proved. Sloand et al. (2000) suggested that G-CSF, in addition to its stimulatory effects on hematopoiesis, can both exert immunomodulatory effects on lymphocytes and improve the survival rate and the number of monocytes. Therefore, it can be postulated that a similar scenario could occur in dogs treated with homologous G-CSF. The human cytokine is commercially available and in dogs it induces marked leukocytosis with mature neutrophilia and monocytosis, so that it is also effective for the management of the neutropenia in canine cyclic haematopoiesis. Unfortunately, the long-term use of heterologous G-CSF in dogs causes the development of neutralizing antibodies (Lothrop et al., 1988; Hammond et al., 1991). These antibodies not only neutralized the activity of the heterologous cytokine, but also interfered with and neutralized the effect of the endogenous homologous cytokine (Reagan et al., 1995). In two clinical studies, dogs with CPV-induced leukopenia did not respond to rhG-CSF treatment, and the human recombinant cytokine was not effective either in stimulating neutrophil recovery or in shortening the hospitalization period compared to untreated dogs (Rewerts et al., 1998; Mischke et al., 2001). Several studies demonstrated that in normal patients, homologous CSFs cause an immediate and persistent leukocytosis (Donahue et al., 1986; Metcalf et al., 1986; Welte et al., 1987; Caselli et al., 2016), suggesting that the rcG-CSF in dogs could both prevent the production of neutralizing

antibodies, associated with the administration of heterologous cytokine, and elicit a more effective bone marrow stimulation.

The virus population detected in the present study was homogeneous with both CPV-2a and CPV-2b variants being identified equally in both groups, so there was not any influence of different variants in results observed. No significant differences were observed in the vaccination schedules of the two groups, but considering that CPV2 vaccination does not always induce an immunity response due to the presence of colostrum antibodies, even vaccinated pups get infected with CPV2. So, it appears that vaccination did not affect the results. Finally, rcG-CSF had an impact on the median time for the resolution of clinical signs, which was shorter in the treated group, demonstrating its important role in reducing the time of patients' hospitalization.

This study was a non-randomized clinical trial and staff veterinarians were not blinded for treated or non-treated patients. Considering that the study plan was performed in order to treat indistinctly all dogs recovered, there was not a real choice and any bias should not have applied as a consequence to individual preference. A positive hematological effect of rcG-CSF was observed in the treated group, combined with no death events, while five non-treated patients died. Nevertheless, it is reasonable to ascribe these findings to the high mortality of CPV infection. The small sample size suggests the need to look at these results with caution because the study was not planned to evaluate mortality. Therefore, mortality rate should be further investigated using a greater sample size.

In summary, our results demonstrated that rcG-CSF should be considered an effective drug, so that it could be employed as adjuvant therapy in the treatment protocols of CPV naturally infected dogs. However, additional studies involving a greater sample size are necessary to better investigate the complete role of rcG-CSF, in particular, looking at the patients' survival rates.

Acknowledgments

The Authors would like to thank the Santa Fara Veterinary Hospital team for samples collection and the Appialab s.r.l. laboratorio di analisi veterinarie (Roma, Italy) for their collaboration in hematological samples analysis. The Authors are grateful to Mr Thomas M. Arme, School of Biological Sciences, University of Leicester, UK, for the revision of the English language and to the colleagues of the Department of Veterinary Medicine – Infectious Diseases Section, University of Bari, for their precious and indispensable collaboration.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

None

Declarations of interest

None

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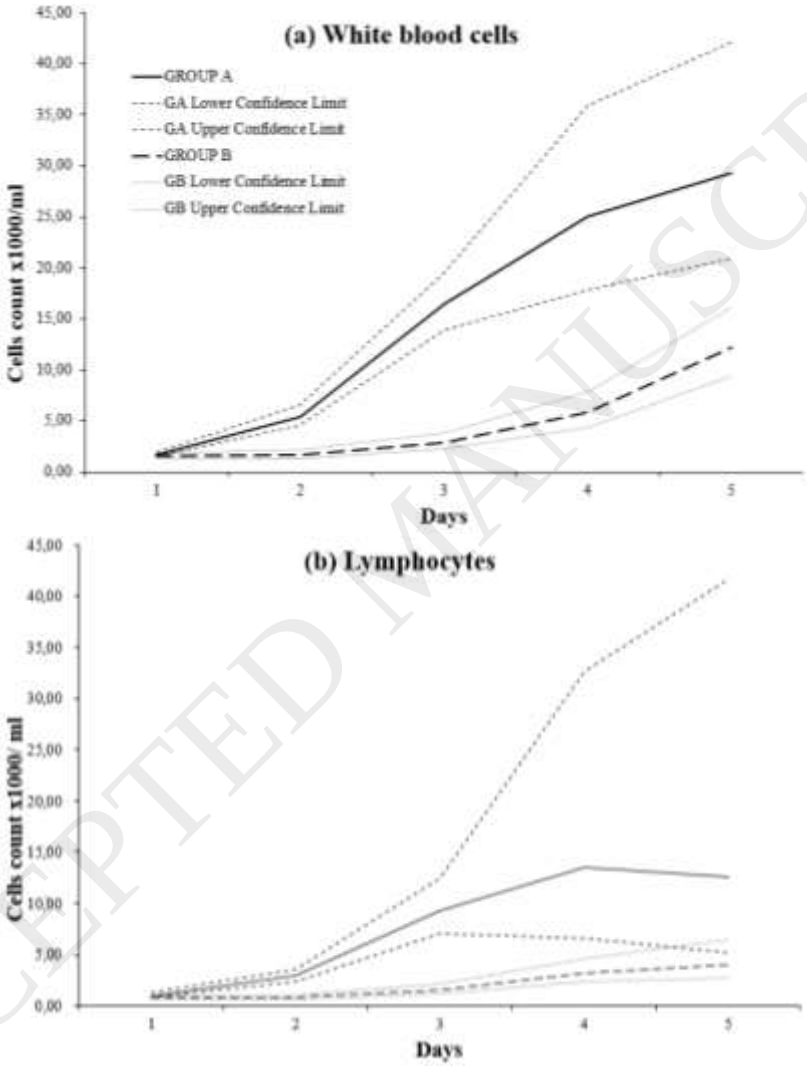
Figure legend

Figure 1. Profile of least-square means and 95% confidence interval by treatment groups (Group A: treated; Group B: not treated) and days after therapy. a) WBC counts. b) Lymphocytes counts. c) Monocytes counts. d) Neutrophils counts.

Figure 1.



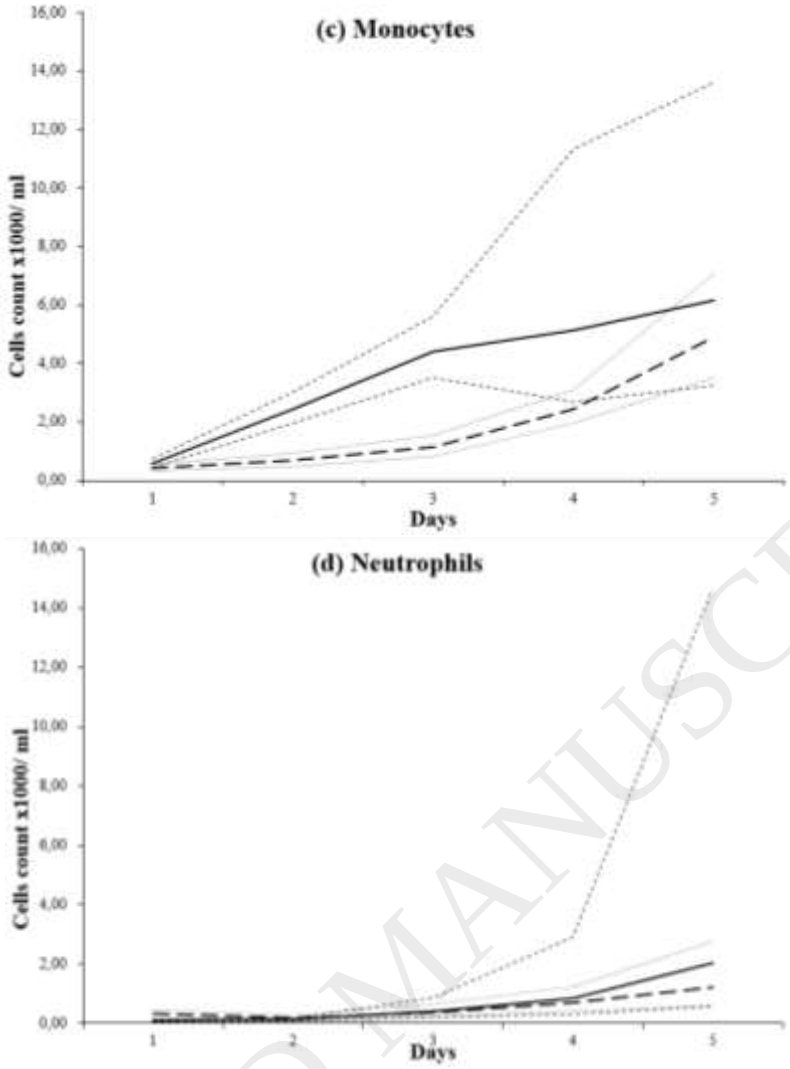


Table 1. Demographic data and CPV variants involved in the study.

Variables	Group A		Group B		P-value
Gender	N	%	N	%	
• **Male**	21	67.7	19	61.3	0.5955
• **Female**	10	32.3	12	38.7	
Median age (IQR)	3 (3-4)		3 (2-6)		0.8849
Breed					
• **Pure breed**	17	54.8	16	51.6	0.9678
• **Mixed breed dogs**	14	45.2	15	48.4	
Vaccination status					
• **CPV2 vaccination plan complete**	7	22.6	8	25.8	0.7668
• **CPV2 vaccination plan incomplete**	24	77.4	23	74.2	
CPV2 variant					
• **CPV2a**	20	64.5	20	64.5	
• **CPV2b**	10	32.3	11	33.5	0.8597^
• **CPV2c**	1	3.2	0	/	
Symptoms					
• **Anorexia**	31	100	31	100	1.000
• **Vomiting**	29	93.5	30	96.77	1.000
• **Diarrhea**	31	100	26	83.9	0.0525

Legend:
N: number of pups
^: without considering 2c variant
Group A: treated
Group B: non-treated
IQR: InterQuartile Range

Table 2. Adjusted means and 95% confidence interval of cells counts. P-values refers to post-hoc adjusted comparison between treated and non-treated groups at each time point.

		Group A			Group B			
	Days^	**Mean (1000cells/µl)**	**LCL* (1000cells/µl)**	**UCL° (1000cells/µl)**	**Mean (1000cells/µl)**	**LCL* (1000cells/µl)**	**UCL° (1000cells/µl)**	**P-value#**
WBC	1	1.71	1.49	1.96	1.54	1.28	1.87	0.9979
	2	5.42	4.54	6.54	1.65	1.27	2.17	<0.0001
	3	16.42	13.95	19.46	2.92	2.31	3.77	<0.0001
	4	24.97	17.89	35.97	5.82	4.40	7.89	<0.0001
	5	29.24	20.94	42.11	12.17	9.40	16.08	0.0028
Lymphocytes	1	0.99	0.79	1.25	0.78	0.61	0.99	0.9139
	2	2.89	2.40	3.53	0.82	0.64	1.08	<0.0001
	3	9.31	7.09	12.51	1.53	1.13	2.10	<0.0001
	4	13.46	6.61	32.63	3.22	2.36	4.53	0.0072
	5	12.62	5.15	41.72	4.05	2.69	6.47	0.4458
Monocytes	1	0.58	0.46	0.74	0.42	0.32	0.56	0.7987
	2	2.42	1.96	3.03	0.68	0.50	0.94	<0.0001
	3	4.40	3.50	5.62	1.13	0.84	1.56	<0.0001
	4	5.12	2.69	11.34	2.44	1.96	3.09	0.5044
	5	6.16	3.24	13.61	4.91	3.53	7.07	0.9999
Neutrophils	1	0.09	0.06	0.14	0.31	0.22	0.46	0.0004
	2	0.13	0.10	0.16	0.16	0.09	0.29	0.9986
	3	0.40	0.20	0.87	0.38	0.22	0.66	1
	4	0.86	0.33	2.91	0.69	0.41	1.24	1
	5	2.05	0.57	14.63	1.22	0.62	2.74	0.9995

Legend:
^ days post treatment
*: Lower Confidence Limit
°: Upper Confidence Limit
adjusted P-value