

Assessment of the CPL-0015 isolate as a vaccine strain for the control of canine parvovirus in Cuba

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ABSTRACT. The safety and protective efficacy of the CPL-0015 Cuban isolate of canine parvovirus type 2 (CPV-2) were evaluated for its possible use as a vaccine strain candidate. The study included a total of 23 healthy Beagle dogs of both sexes, aged 84 days and without specific maternal antibodies against canine parvovirus. Safety was analysed by comparing clinicopathological values, food consumption, body weight, rectal temperature and white blood cell counts for 14 consecutive days between control dogs (n=5) and dogs subcutaneously injected (n=10) with 2 mL (equivalent to two doses) of the CPL-0015 strain with an antigenic titer of $10^{6.0}$ infectious dose₅₀ in cell culture/mL. The protective effectiveness was determined by measuring and comparing anti-CPV-2 IgG levels and clinical signs during 56 experimental days between control dogs (n=2) and dogs inoculated (n=6) with double doses of 1 mL each, separated by a 21-day interval. All animals were challenged orally on day 35 with the virulent strain Cornell-780916 ($10^{5.0}$ infective dose₅₀ in cell culture/mL). The results showed that the CPL-0015 strain did not negatively impact the physiological condition of the exposed animals. The inoculated and challenged animals showed not only significantly increased levels of anti-CPV-2 IgG ($P<0.05$) when compared to days 0, 35, and the control group animals but also had 100% survival without clinical signs of the disease, unlike the control group. It is concluded that CPL-0015 is safe and provides effective protection against homologous virulent strains.

Key words: canine parvovirus, dogs, safety, protection, vaccine.

INTRODUCTION

Canine parvovirus (CPV) is the cause of serious enteric infections in *Canis familiaris*, resulting in acute clinical progression with high morbidity and mortality regardless of age, race, or gender (Aponte *et al* 2020, Dong *et al* 2020). However, several authors consider that certain factors such as younger age, nonconfinement, and lack of vaccination play a key role in its spread, clinical course, and fatal outcome (Zhuang *et al* 2019, Qi *et al* 2020). CPV is characterised by its genetic diversity and alarming ability to cross the interspecies barrier, enabling it to adapt to multiple hosts, both domestic and wild (Miranda and Thompson 2016, Li *et al* 2017, Zhou *et al* 2017, Voorhees *et al* 2019).

The immunisation of domestic dogs with modified live vaccines containing the CPV-2 strain is an effective method of controlling and preventing the clinical development of the disease (Domínguez *et al* 2014, Mukthar *et al* 2021). However, in recent years the use of these formulations has become controversial due to their failure in protecting vaccinated animals (Decaro *et al* 2020, Ying *et al* 2020). Although the cause of these events is multifactorial (Altman *et al* 2017), much attention is being paid to the possible inability of the CPV-2 vaccine strain to induce protection

against the new genetic variants CPV-2a, CPV-2b, and CPV-2c (Ying *et al* 2020). Such suspicions are supported by the antigenic variations detected in emerging lineages associated with several mutations located in the VP2 structural protein, an important antigenic determinant present in the viral capsid (Li *et al* 2017, Sebastian *et al* 2019, Ying *et al* 2020).

As a result, among other aspects, there is an urgent need to evaluate the immunological consequences of the genetic diversity of CPV, the genotypes prevailing in different countries, the antigenic properties of each one, and their possible vaccine potentialities (Zhou *et al* 2017, Sebastian *et al* 2019, Hao *et al* 2020). The antigenic variability observed at the global level does not seem to be present in Cuba. Molecular studies (results not presented) carried out after those published by Fresneda *et al* (2015) from diseased animals, demonstrated the circulation of the CPV-2 strain only. The existence in our archipelago of original pathogenic strains that persist over time is not exclusive to canine parvovirus, it was also reported for *Pasteurella multocida* subsp *multocida* biovar A:1 in rabbits (Lugo *et al* 2019, Domínguez *et al* 2021).

Recent Cuban epidemiological studies revealed that no racial dogs with free access to the street, the consumption of inadequate food including raw meat, and not vaccination are factors that increase the chances of becoming ill from CPV (Pino *et al* 2019, Peña *et al* 2020). In this context, having its own vaccine will make it possible not to depend on international suppliers of vaccines to control the disease. Besides, the possibility of achieving technological sovereignty would reduce the prices of the biological product and promote its commercialisation in the foreign market. This perspective led us to isolate, attenuate in cell culture, and characterise circulating strains native to Cuba (Fresneda *et al* 2015). Accordingly, a vaccine formulation

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was developed with our own technology, adapted to the Cuban epidemiological reality, independently of external strains. Consequently, the present study assessed the safety and protective efficacy of the Cuban isolate, CPL-0015, of canine parvovirus type 2 as a vaccine strain in Beagle dogs without specific maternal antibodies against parvovirus canine.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS

This experiment followed national (GOC-2021-332-EX25) and institutional guidelines for the care and use of animals. All experimental procedures were approved by the Committee of Ethical Review at Grupo Empresarial LABIOFAM (protocol approval number: 06/20; experimental period: October 2020). The selection of the biomodel, age and number of individuals per experimental group took into account several existing international references on clinical evaluations of classic and modern vaccines against canine parvovirus (Langeveld *et al* 2001, Siedek *et al* 2011, Hernández *et al* 2015). The study included a total of 23 healthy Beagle dogs of both sexes, aged 84 days (12 weeks), with no history of vaccination, testing negative for maternal antibodies against canine parvovirus (absorbance <0.18) and free of bacterial (*Salmomella* spp., *Escherichia coli*, *Leptospira* spp., *Borrelia burgdorferi*, *Ehrlichia canis* and *Mycoplasma haemocanis*), viral (Canine parvovirus, Canine hepatitis virus, Canine distemper virus and Canine rabies) and parasite (Nematode spp., Cestode spp., Babessia spp. and Ectoparasite) specific diseases for the species. The animals

were placed in separate cages and were provided with adequate food based on the species and age (three times a day) and free access to water. After the adaptation period (seven days), the animals were randomly distributed (15 for the safety study and 8 for the efficacy study).

IMMUNOGENS

Inoculum. The candidate vaccine strain CPL-0015 (CPV-2) was isolated in 1991, attenuated by 53 passes in Madin-Darby Canine Kidney cells (MDCK) and then stabilised through 27 passes in Crandell-Rees Feline Kidney Cell (CRFK) (Fresneda *et al* 2015). A vial of lyophilised CPL-0015 isolate (Batch 1203010) with a titer of $10^{6.0}$ tissue culture infectious dose (TCID₅₀)/mL was reconstituted with 1 mL of minimum essential medium. This volume was inoculated into the CRFK line supplemented by 2% fetal bovine serum. After incubating for seven days at 37°C and observing the cytopathic effect in more than 80% of the culture, we proceeded to collect, freeze-dry, and preserve it at -70°C. Before use, one of the obtained virals was diluted with water for injection to obtain a titer of $10^{6.0}$ TCID₅₀ in a total volume of 1 mL (figure 1).

Challenge strain. Over the base of previous international experiences (Cunegüendes *et al* 2008, Oliveira *et al* 2010, De Cramer *et al* 2011, Puentes *et al* 2012), the worldwide virulent Cornell-780916 strain (type 2) from the collection ATCC with the number ATCC® VR- 2006™ was selected. Before its use, it was tited in the MDCK cellular line with a value of $10^{6.5}$ and then diluted with minimum essential media to obtain a titer of $10^{5.0}$ TCID₅₀ in a total volume of 1 mL/animal (figure 1).

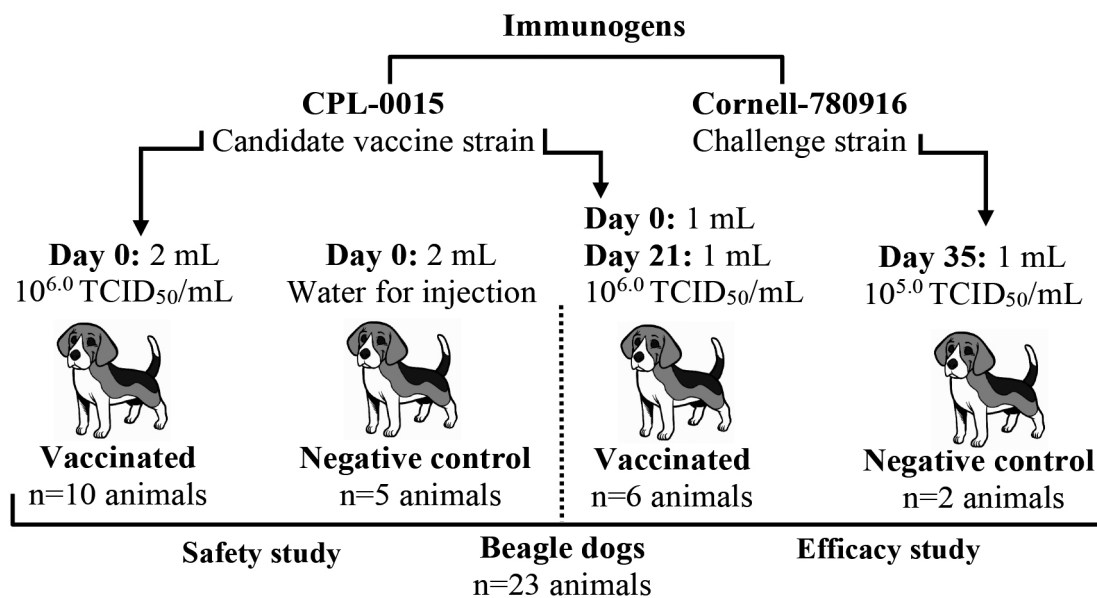


Figure 1. General aspects of the designed studies to evaluate the efficacy and safety of the CPL-0015 strain.

SAFETY STUDY

Two experimental groups were established (figure 1). One group was inoculated with CPL-0015 at a titer of $10^{6.0}$ TCID₅₀/mL (n=10), whereas the negative control group received water for injection (n=5). All animals received double doses (2 mL) subcutaneously in the dorsal area of the neck and were manipulated by the same researcher. The injection site was observed for 14 consecutive days to look for local alterations and the animals were monitored for general clinical manifestations or changes in the rectal temperature. Furthermore, white blood cell counts, food consumption, and body weight were assessed on alternate days.

A clinical assessment system was followed based on possible and typical disease alterations. Scores ranged from 1 to 9 depending on the severity of the clinical alterations (table 1). Euthanasia was performed using a sodium pentobarbital (Pentovet, 150 mg/kg, intraperitoneal) overdose when the score was greater than 7.

EFFICACY STUDY

The animals used in this study were divided into two experimental groups (figure 1). One group (n=6) was inoculated with two subcutaneous doses (1 mL each) of CPL-0015 ($10^{6.0}$ TCID₅₀) with an interval of 21 days between doses (days 0 and 21), whereas the negative control group (n=2) received water for injection in the same volume, frequency, and route of administration as those of the inoculated group. Fourteen days after the second injection (experimental day 35), all animals were challenged orally with the virulent Cornell strain. Clinical follow-up of all animals was performed from day 0 to experimental day 56 using the abovementioned clinical assessment system. The first 35 days, were dedicated to observe the possible adverse reactions of the CPL-0015 initial dose and the recollection, using the same written indicators previously described.

Table 1. Clinical assessment system used in the safety study.

Clinical score	Clinicopathological alterations
1	No symptoms
2	Pain at the injection site
3	Redness at the injection site
4	Unusual skin manifestations at the injection site (erythema, alopecia, etc.)
5	Fever + decreased willingness to eat and drink
6	Mucoid or hemorrhagic diarrhea
7	Vomiting + mucoid or hemorrhagic diarrhea
8	Anorexia + weakness + prostration
9	Death

HAEMATOLOGY AND SEROLOGY

Blood samples for assessing the white blood cell counts of the animals in the safety study were collected on experimental days 0, 2, 4, 6, 8, 10, 12, and 14. In all cases, 1 mL of blood was collected from the lateral saphenous vein into tubes containing an anticoagulant. Additionally, the evaluation of anti-CPV IgG antibody titers was performed on days 0, 35, and 56 using 2 mL of heat-inactivated serum (56°C for 30 minutes). A solid phase, quantitative, indirect enzyme-linked immunosorbent assay system was used (CENPALAB, Cuba). The reading of the samples was taken at 492 nm, and the cut-off value was 0.18 according to the manufacturer's instructions.

STATISTICAL ANALYSIS

For the analysis of immune response variables, data from the inoculated and control groups were compared using the Wilcoxon-Mann-Whitney test. A value of $P < 0.05$ was considered statistically significant. The statistical program used for the analyses was SPSS 12.0 (SPSS Inc, Chicago IL, USA).

RESULTS AND DISCUSSION

Vaccination and use of live attenuated vaccines manufactured using classic production technologies continue to be essential tools for the control of canine parvoviruses worldwide (Puentes 2012, Domínguez *et al* 2014, Mukhtar *et al* 2021). Therefore, controlled clinical studies using autochthonous circulating strains adapted to cell culture are important in order to produce immunoprophylactic formulations adapted to current epidemiological situations.

SAFETY STUDY

Clinical observation for 14 consecutive days after the inoculation of an overdose of the Cuban isolate, CPL-0015, demonstrated the absence of undesirable local or systemic side effects. Analysis of the white blood cell count, rectal temperature, food consumption, and body weight showed that the inoculation of CPL-0015 did not have a negative impact on any animal under these conditions (data not shown). The negative control group, on the other hand, showed similar results and clinical scores (level 1) as those of the inoculated group.

The use of clinical indicators to detect the occurrence of negative events associated with immunoprophylactic formulations (Day 2008) and the evaluation of vaccine antigens (Moore and Hogen 2010) is well-known. In our case, the lack of adverse consequences in animals inoculated with a CPL-0015 overdose supports its suitability for use in puppies. This characteristic of CPL-0015 will be greatly beneficial if used in future formulations because although it is a living virus belonging to the family Parvoviridae, it

shows a high affinity for growing cells having a high rate of mitotic division (Díaz *et al* 2008).

EFFICACY STUDY

Figure 2 summarises the antibody response of the Beagle dogs from both the experimental and control groups at different time intervals. Prior to inoculation (day 0), anti-CPV antibodies were undetectable (values below 0.18) with the technique used, confirming the absence of prior immunological stimulation. On day 35, at the time of the challenge, all animals inoculated with CPL-0015 (days 0 and 21) showed a significant percent increase (490%) in their antibody levels compared to the control group animals who continued to be seronegative. On experimental day 56 (21 days after the challenge), although in general terms significant progressive increases in IgG levels were found

in both groups ($P>0.05$), these were not homogeneous. Animals inoculated with CPL-0015 showed the greatest and most significant percent increases in their antibody levels not only with respect to days 0 and 35 (1,460% and 297%, respectively) but also with respect to the control group (129%) in the same period.

Furthermore, figure 3 shows the results in terms of clinical score for the experimental and control groups before and after the challenge. In animals inoculated with CPL-0015, there was an absence of adverse local or systemic reactions from days 0 to 14 after the second injection (experimental days 35) and signs of disease after the challenge. In contrast, unvaccinated animals challenged with the virulent Cornell-780916 strain began to show progressive clinical signs typical of CPV from experimental day 39 (day 4 after the challenge). Control animals shared the same score until day 13 after the challenge, when

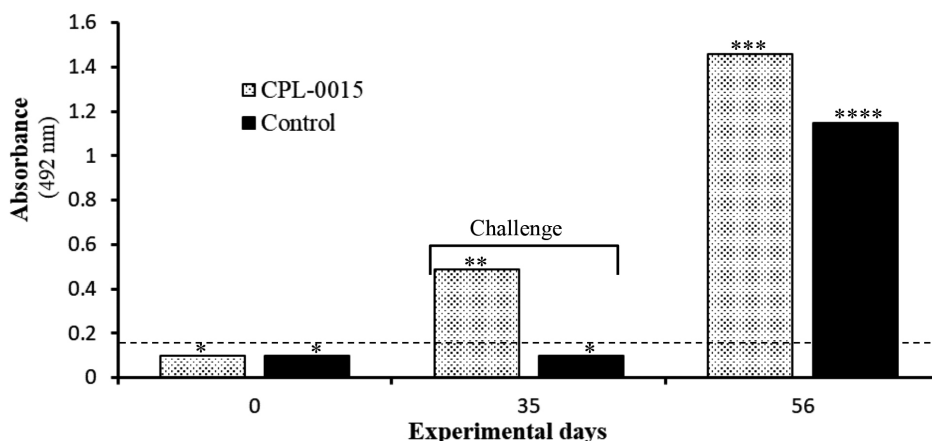


Figure 2. Immune response of Beagle dogs inoculated with strain CPL-0015 and controls.

Note: Different number of symbols indicate significant differences ($P<0.05$) based on the Wilcoxon-Mann-Whitney test.

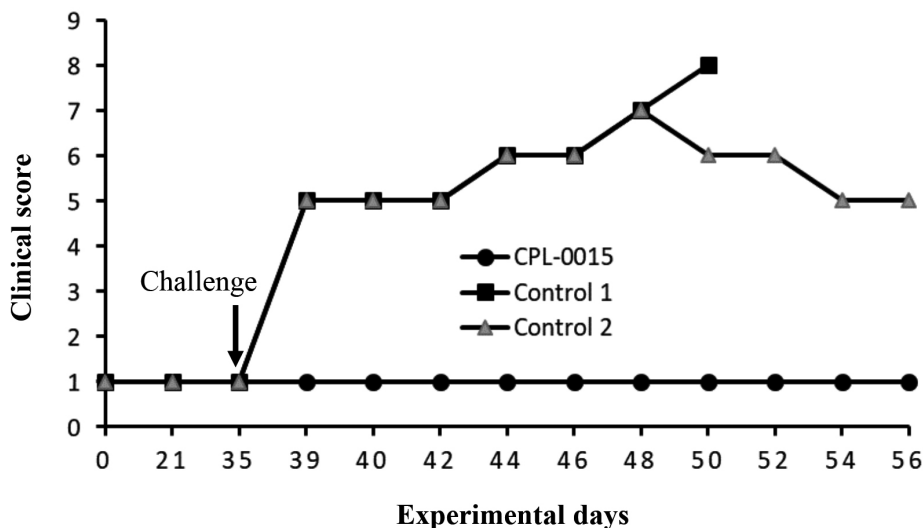


Figure 3. Clinical evaluation of Beagle dogs immunised with strain CPL-0015 (and of negative controls) challenged with the virulent Cornell-780916 strain.

Note: Group vaccinated with CPL 0015 is composed by 6 animals and control group by 2 animals.

one of them progressed towards anorexia, weakness, and prostration, and had to be euthanised.

The remarkable biological differences in terms of seroconversion and survival without clinical signs of disease after the challenge observed between inoculated and control animals demonstrate the ability of the Cuban isolate to induce a powerful protective humoral response. These results are extremely important since they show that the CPL-0015 isolate retained its immunogenic capacity during the process of adaptation to cell culture and was able to induce anti-CPV antibodies in sufficient quantities to neutralise the pathogenic strain (Puentes 2012).

When comparing this result with others reported in the international scientific literature, it can be concluded that the benefits of the CPL-0015 isolate in terms of protection are similar to those obtained with the 17/80 ISS (Pratelli *et al* 2001), Cornell-780916-115 (De Cramer *et al* 2011), 154, and NL-35-D (Larson and Schultz 2008, Siedek *et al* 2011) vaccine strains; all of which are type 2 strains, attenuated in cell culture, and inoculated using a similar route of administration. It is important to highlight that the last two abovementioned vaccine strains, despite being administered with the same biphasic scheme, required a higher viral titer ($10^{7.0}$ TCID₅₀/mL) than the one used in this study ($10^{6.0}$ TCID₅₀/mL) to achieve an effective protective status (Larson *et al* 2008, Siedek *et al* 2011).

The death of 50% of the unvaccinated animals challenged confirm the virulence of the Cornell-780916 strain. This value also corresponded with that reported in the international literature for this type of experimental group (Hernández *et al* 2015, Khatri *et al* 2017). Although there are some variations in the appearance and duration of the clinical signs of CPV, the results of this study were similar to those reported in previously published studies. The onset of disease on day 4 after the challenge is in line with the results described by other authors (Spibey *et al* 2008, Wilson *et al* 2013, Khatri *et al* 2017, Mukthar *et al* 2021) regardless of the challenge strain used, whereas the appearance of fever and mucoid or haemorrhagic diarrhea occurred at similar moments as those described historically by Meunier *et al* 1985, during experimental infections.

These results, together with the absence of the CPL-0015 strain virulence reversal (data not presented), leave open the possibility of continuing with the development of the Cuban vaccine formulation. However, there is still a long way to go, the duration of immunity and stability of the formulation should be explored immediately; as well as the optimization of the vaccination scheme (biphasic and triphasic) against the interference of antiparvovirus antibodies of maternal origin, among other aspects. The perception of the international use of the future Cuban vaccine requires evaluating its behaviour against pathogenic antigenic variants: CPV-2a, CPV-2b, and CPV-2c (non-homologous strains).

Based on the experimental model used and the described experimental conditions, it is concluded that the Cuban

isolate, CPL-0015, proved to be safe and induced effective protection against homologous virulent strains of canine parvovirus.

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