

Short communication

Oral administration of modified live canine parvovirus type 2b induces systemic immune response

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25 **Highlights:**

- 26 • Interfering levels of maternally derived antibodies (MDA) can hamper the immune response
- 27 against canine parvovirus type 2 (CPV-2) vaccination.
- 28 • Fourteen pups with MDA titres ranging from 1:20 to 1:160 were orally administered a
- 29 commercial modified live CPV-2b vaccine.
- 30 • All pups but one seroconverted after a single vaccine administration.
- 31 • The remaining pup raised an immune response after administration of a second vaccine dose
- 32 • Oral administration of CPV-2 vaccines can help overcome MDA interference
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51 **Abstract**

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53 Different strategies have been proposed to overcome maternally derived antibodies (MDA)
54 interference with canine parvovirus type 2 (CPV-2) immunisation, including intranasal vaccination,
55 which presents some practical limitations. In the present study, the results of the oral administration
56 of a commercial CPV-2b modified live virus (MLV) vaccine in pups with MDA are reported. The
57 CPV-2b vaccine was orally administered to 14 6-week-old pups with a bait. Blood samples and
58 rectal swabs were collected at different days post-vaccination (dpv) to determine CPV-2 antibody
59 titres and DNA loads. Thirteen pups were positive to serological and virological tests after the first
60 vaccination and one pup became positive after the second vaccine administration. The findings of
61 this study suggest that systemic immunity against CPV-2 may be achieved by the use of an MLV
62 CPV-2b vaccine administered orally even in the presence of MDA titres that usually interfere with
63 vaccination.

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65 **Key words:** pups; canine parvovirus; maternally derived antibodies; oral vaccination.

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70 **1. Introduction**

71 Canine parvovirus type 2 (CPV-2) is the causative agent of a severe, highly contagious
72 gastroenteric disease in pups [1]. Since its first emergence in the mid-1970s, the original type CPV-
73 2 has evolved giving rise to new antigenic variants designated CPV-2a, CPV-2b and CPV-2c,
74 which have completely replaced the original virus and are now distributed worldwide [2], [3].

75 Typically, CPV-2 infects 4-to-12 week-old pups especially during the decline of maternally derived
76 antibodies (MDA) [2]. Modified live virus (MLV) vaccines are prepared by using either the original
77 type CPV-2 or its variant CPV-2b. Despite the high efficacy of vaccines, outbreaks of infection
78 continue to arise in vaccinated dogs, likely as a consequence of the MDA interference, which
79 prevents an active immune response by vaccinated pups. Thus, in order to avoid or reduce this
80 interference, vaccines should be administered to pups only after waning of MDA [2]. Different
81 strategies have been proposed to overcome the MDA interference, including the administration of
82 high-titre vaccines [4] or intranasal vaccine administration [5].

83 In a previous study [5], CPV-2b MLV vaccine administered intranasally induced high antibody
84 titres even in the presence of high MDA titres. The intranasal vaccination can significantly reduce
85 the MDA interference, resulting particularly effective in infected kennels. However, this procedure
86 is not easy to perform, requires an adequate containment of the animal and, consequently, it can
87 cause stress and/or the accidental loss of part of the vaccine dose during the administration.

88 In order to overcome the limitation of intranasal vaccination, we selected an alternative approach
89 based on the oral administration of a MLV vaccine. Therefore, in the present study, the results of
90 the oral administration of a commercial modified live CPV-2b vaccine in pups with MDA are
91 reported.

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93 **2. Materials and Methods**

94 **2.1 Dogs**

95 The study was approved by the Ethics Committee of the Department of Veterinary Medicine of
96 University of Bari (Protocol number 756 – III/13).

97 Fourteen privately owned healthy pups presented to the Department Veterinary Teaching Hospital
98 for preventive medicine examination and veterinary counseling were included in the study. All
99 owners were informed of the nature of the study and gave their written consent (Client Informed
100 Consent Form for clinical trials). The study was conducted on one litter comprising 11 pups (A1 to

101 A11) and on 3 other pups (C₁, C₂ C₃) belonging to three different owners. Litter A consisted of 11
102 English short haired pointers (6 males and 5 females), whose mother had received last vaccination
103 against CPV-2 18 months before parturition, while puppies C₁ (male), C₂ and C₃ (females) were
104 mongrel dogs. All pups at the start of the study were 40 days old.

105 **2.2 Vaccine**

106 Canigen® Puppy 2b vaccine (Virbac srl, Milan, Italy), containing a MLV CPV-2b strain
107 (CPV 39) and having a titre of $10^{5.6} - 10^{7.5}$ Tissue Culture Infectious Doses (TCID₅₀)/dose, was
108 used during the study (Lot n. 72ZT; expiry date: May 2020).

109 **2.3 Experimental procedure**

110 The pups were orally vaccinated using a bait containing the vaccine dose. Each bait consisted of a
111 friable biscuit filled, immediately prior to use, with one dose of vaccine (1 ml) and freely taken by
112 pups. The chewing of the bait resulted in a persistence of the vaccine in the oral cavity for 1-2 min.

113 Clinical examinations were performed daily to evaluate the general conditions of the pups.

114 Blood samples were collected at 0 (T0), 15 (T15) and 30 (T30) dpv to determine individual CPV-2
115 antibody titres. Rectal swabs were collected at 0 (T0), 4 (T4), 8 (T8) and 15 (T15) dpv for
116 virological testing (vaccinal virus shedding).

117 At T15, in the absence of seroconversion (increase of antibody titres), pups were vaccinated again,
118 using the same protocol (route of vaccination, sampling, serological and virological investigations).

119 In particular, the following sampling was scheduled: T19 and T23 (for virological testing), T30 (for
120 serological and virological testing).

121 **2.4 Serological testing**

122 Antibody titers against CPV-2 were evaluated by haemagglutination inhibition (HI) test, which was
123 performed in V-shaped 96-well microtitre plates, at +4°C, using 0.1% pig erythrocytes and 10
124 haemagglutinating (HA) units of CPV-2b strain 29/97 [6].

125 Serial twofold dilutions of each serum, starting from 1:10 to 1:10240, were prepared in phosphate
126 buffered saline (PBS, pH 7.2). An equal volume (25 µl) of virus (10 HA units) was added to the

127 wells containing the dilutions of the sera. Thereafter, the plates were incubated at room temperature
128 for 60 min, then refrigerated at +4°C for 30 min before adding to each well 50 µl of the cold
129 erythrocyte suspension. HI titres were read after 12 hours at +4°C and expressed as the reciprocal of
130 the highest serum dilution that completely inhibit the HA activity.

131 **2.5 Virological testing**

132 Faecal samples were homogenised in PBS (10% w/v) and subsequently clarified by centrifugation
133 at 8,000 rpm for 5 min. Viral DNA was extracted from the supernatants of faecal homogenates by
134 boiling for 10 min and chilling on ice. To reduce residual inhibitors of DNA polymerase activity to
135 ineffective concentrations, the DNA extract was diluted 1:10 in distilled water [7].

136 CPV-2 DNA was detected by real-time polymerase chain reaction (PCR) using a conventional
137 TaqMan probe [7], whereas virus characterisation was obtained by a panel of minor groove binder
138 (MGB) probe assays able to predict the viral type [8] and to discriminate between vaccine and field
139 strains of CPV-2 [9], [10].

140 **2.6 Statistical analysis**

141 Normality of distribution of continuous variables was assessed by Shapiro-Wilk test. Antibody
142 titers at T0 and T15 were summarized as median and interquartile range and compared to each other
143 by the non-parametrical Wilcoxon signed rank test with continuity correction. Viral DNA copies at
144 T0, T4, T8, T15, T19, T23 and T30 were summarized as median and interquartile range and
145 subjected to the non parametrical Friedman test. Pairwise comparisons using Wilcoxon signed rank
146 test with Bonferroni post-hoc test were performed to evaluate significant differences in viral DNA
147 copies between T0, T4, T8, T15, T19, T23 and T30. Statistical analyses were performed by using
148 the freely available online tool EZR [11]. A p-value<0.05 was considered for statistical
149 significance.

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151 **3. Results**

152 **3.1 Vaccine administration**

153 Thirteen puppies chewed and then ingested the bait containing the vaccine dose, freely and without
154 any difficulty. Only pup C2 showed an initial distrust for the bait and expelled it twice from the
155 oral cavity, before ingestion. However, the bait was better tolerated by this pup at the second
156 vaccine administration. None of the pups showed local or general reactions following oral
157 vaccination.

158 **3.2 Serological testing**

159 The results of serological tests are shown in Table 1. The sera taken at T0 showed HI MDA titres
160 ranging from 1:20 to 1:160. All pups orally vaccinated, except pup C2, seroconverted after the first
161 vaccination, displaying at T15 HI titres ranging from 1:1280 to 1:10240. In the comparison between
162 antibody titers detected at T0 and T15 a statistically significant difference (p-value = 0.001293) was
163 observed.

164 The first oral vaccination of pup C2 did not result in seroconversion. This pup showed at T0 and
165 T15 HI MDA titres of 1:160 and 1:40, respectively. According to the protocol described above, pup
166 C2 was vaccinated a second time at T15, using the same procedure as for the first vaccination. At
167 T30 (15 days after the second vaccination), the pup seroconverted, showing an HI titre of 1:1280.
168 Therefore, the vaccine was able to induce active seroconversion in all pups having HI MDA titers
169 ranging from 1:20 to 1:80, as well as in 1 (A9) out of 2 pups displaying an HI titre of 1:160 at the
170 time of the vaccination.

171 **3.3 Virological testing**

172 The results of virological tests are shown in Table 2. All rectal swabs taken at T0 were negative for
173 CPV-2 by the TaqMan real-time PCR assay [7].

174 At T4, 6 puppies (A3, A4, A9, A10, A11 and C3) were positive for CPV-2. By using MGB probes
175 able to discriminate between the three CPV-2 variants [10] and between vaccine and field CPV-2b
176 [9], the virus in the swabs was characterized as CPV-2b vaccine strain CPV 39, with viral loads
177 ranging from 3.1×10^2 to 4.75×10^3 DNA copy numbers 10^{-1} μ l of template.

At T8, additional 7 pups (A1, A2, A5, A6, A7, A8 and C1) tested positive for the vaccine strain. At T15, the rectal swabs of all pups but C2 tested positive for CPV-2b strain CPV-39. The copy numbers ranged from 3.89×10^3 to 1.57×10^7 DNA copies 10^{-1} μ l of template at T8 and from 6.13×10^3 to 9.06×10^4 DNA copies 10^{-1} μ l of template at T15.

At T19, the vaccine strain was detected in the swabs of all dogs, including the C2 pup and the copy numbers 10^{-1} μ l of template ranged from 3.8×10^2 to 5.29×10^3 . The viral shedding was detected in rectal swabs of pup C2 up to T30 while other pups tested negative at T23 and T30.

Therefore, the vaccine virus was detected at T4 (6 pups), T8 (7 pups) and T19 (pup C2, four days after the second vaccination) and lasted for 11 days (A1, A2, A5, A6, A7, A8, C1, C2) or 15 days (A3, A4, A9, A10, A11, C3).

Overall, the comparison between viral DNA copies at T0, T4, T8, T15, T19, T23 and T30 showed statistically significant differences (p-value = 0.000000000677). The pairwise comparisons revealed significant differences in viral DNA copies between T4 and T19 (p-value = 0.0051), T8 and T19 (p-value = 0.0051), T8 and T23 (p-value = 0.0487) T8 and T30 (p-value = 0.0077), T15 and T19 (p-value = 0.0051) and T15 and T30 (p-value = 0.0051).

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194 **4. Discussion**

One of the primary obstacles in immunisation of dogs against CPV-2 is the persistence of high levels of MDA in pups which may interfere with the development of the vaccine-induced immunity. The administration of high-titre vaccines [4] and the intranasal vaccination [5] are currently the only strategies proposed to overcome the MDA interference. Compared to the parenteral inoculation, the intranasal administration of the vaccine has the advantage of stimulating a better local immunity together with a faster humoral response [2], [5]. However, this route of administration has many practical limitations with regards to the forced containment of the animals and the possible loss of part of the vaccine dose during the inoculation into the nostrils. For these reasons, the intranasal

203 vaccination may not be easy and may cause stress, suffering and/or alteration of the animal welfare.
204 Based on the above, the need for alternative vaccination routes against CPV-2 is evident.
205 In the present study, oral administration of a commercial MLV CPV-2b in pups induced a systemic
206 immune response (in terms of HI antibodies) already at the first vaccine administration. All pups
207 with HI MDA titres up to 1:80 and 1 out of 2 pups with MDA titres of 1:160 seroconverted after the
208 first vaccination, showing that oral administration of the vaccine is able to overcome MDA titres
209 that interfere with vaccination using the parental route [2]. Only pup C2, which displayed a MDA
210 titre of 1:160 and had some problems in ingesting the bait, failed to seroconvert after the first
211 vaccination but responded to the second vaccination. In 6-week-old pups, such MDA levels were
212 not so common in the past, but nowadays repeated vaccination of dams and or continuous virus
213 circulation are leading to a longer-term persistence of MDA especially in breeding kennels [2,
214 personal observation].
215 The successful oral vaccination was also confirmed by the results obtained from the virological
216 tests. In fact, all the pups shed the virus for 11-15 days after the vaccination, albeit with different
217 trends and peaks of viral titres. This is in agreement with a previous study showing that dogs
218 parenterally administered a MLV CPV-2b strain shed the vaccine virus in their faeces for 12 mean
219 days [12]. In contrast, Freisl and colleagues [13] observed an MLV CPV-2 shedding up to 28 days
220 in 20% of the vaccinated dogs, but they used a CPV-2 (original type) vaccine, which was found to
221 be shed for a longer period with respect to MLV CPV-2b [12]. In addition, in the Freisl's study
222 dogs shed the virus discontinuously and at very low titres [13].
223 The main drawback of the present study is that only the immunogenicity of the vaccine
224 administration (in terms of antibody response) was evaluated, whereas the efficacy evaluation
225 would have required the challenge of the vaccinated dogs 22-24 days after vaccination, along with
226 the recruitment of a control group consisting of unvaccinated dogs subjected to the challenge with
227 the pathogenic virus. Albeit strictly required by the European Pharmacopeia, in the present study
228 the challenge test was not performed to avoid suffering of the infected pups (control group) and

229 because this was conceived as clinical study not subjected to authorization of the Ministry of Health
230 but only to the advise of the Ethics Committee. Indeed, it is recognized that seroconversion for
231 CPV-2 not only represents a marker of vaccine immunogenicity but also it can be correlated with
232 clinical protection [12], [3], [14]. This approach is consistent with the principles of the 3Rs
233 (Refinement, Reduction and Replacement) and avoids efficacy studies based on infectious
234 challenges *in vivo* that result in the death or euthanasia of the infected animals.

235 In conclusion, the present study demonstrates that the oral administration of a MLV CPV-2 vaccine
236 is not only immunogenic, but also easy and it do not cause any stress to the animals that ingested
237 the bait spontaneously and without containment. However, the failure of the first vaccination of pup
238 C2 suggests that the palatability of the bait could be improved, especially when it is intended for
239 pups accustomed to a milk diet.

240 Moreover, further studies are required to establish the ability of oral administration of MLV CPV-2
241 to induce immune response in pups, even in the presence of higher ($\geq 1:160$) interfering MDA titres.
242 Although the application of the 3R principles is acceptable under a scientific point of view, the
243 eventual registration of a commercial oral vaccine will inevitably require oral challenge studies
244 according to the European Pharmacopeia.

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246 5. References

247 [1] Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hubinger AM, Hughes J,
248 Mietzsch M, Modha S, Ogliastro M, Péntzes JJ, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall
249 P, Tijssen P, Ictv Report Consortium. ICTV Virus Taxonomy Profile: Parvoviridae. J Gen Virol.
250 2019; 100(3):367-368.

251 [2] Greene CE, Decaro N. Canine viral enteritis. In: Greene CE, ed. *Infectious Diseases of the Dog*
252 *and Cat*. 4th ed. St Louis, MO: Elsevier Saunders. 2012; 67-80.

253 [3] Decaro N, Buonavoglia C. Canine parvovirus post-vaccination shedding: Interference with
254 diagnostic assays and correlation with host immune status. *Veterinary Journal*. 2017; 221: 23-24.

255 [4] Burtonboy S, Charlier P, Hertoghs S, Lobman M, Wiseman A, Woods S. Performance of high
256 titre attenuated canine parvovirus vaccine in pups with maternally derived antibody. *Vet. Rec.* 1991;
257 124: 377-381.

258 [5] Martella V, Cavalli A, Decaro N, Elia G, Desario C, Campolo M, Bozzo G, Tarsitano
259 E, Buonavoglia C. Immunogenicity of an intranasally administered modified live canine parvovirus
260 type 2b vaccine in pups with maternally derived antibodies. *Clin Diagn Lab Immunol*. 2005;
261 12(10):1243-5.

262 [6] Cavalli A, Martella V, Desario C, Camero M, Bellacicco AL, De Palo P, Decaro N, Elia
263 G, Buonavoglia C. Evaluation of the antigenic relationships among canine parvovirus type 2
264 variants. *Clin Vaccine Immunol*. 2008; 15: 534-539.

265 [7] Decaro N, Elia G, Martella V, Desario C, Campolo M, Di Trani L, Tarsitano E, Tempesta
266 M, Buonavoglia C. A real-time PCR assay for rapid detection and quantitation of canine parvovirus
267 type 2 in the feces of dogs. *Vet Microbiol*. 2005; 105:19-28.

268 [8] Decaro N, Elia G, Martella V, Campolo M, Desario C, Camero M, Cirone F, Lorusso E, Lucente
269 MS, Narcisi D, Scalia P, Buonavoglia C. Characterisation of the canine parvovirus type 2 variants
270 using minor groove binder probe technology. *J Virol Methods*. 2006; 133:92-99.

271 [9] Decaro N, Elia G, Desario C, Roperto S, Martella V, Campolo M, Lorusso A, Cavalli
272 A, Buonavoglia C. A minor groove binder probe real-time PCR assay for discrimination between
273 type 2-based vaccines and field strains of canine parvovirus. *J Virol Methods*. 2006; 136:65-70.

274 [10] Decaro N, Martella V, Elia G, Desario C, Campolo M, Buonavoglia D, Bellacicco
275 AL, Tempesta M, Buonavoglia C. Diagnostic tools based on minor groove binder probe technology

276 for rapid identification of vaccinal and field strains of canine parvovirus type 2b. J Virol Methods.
277 2006; 138:10-16.

278 [11] Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics.
279 Bone Marrow Transplant. 2013; 48:452-458.

280 [12] Decaro N, Crescenzo G, Desario C, Cavalli A, Losurdo M, Colaianni ML, Ventrella G, Rizzi
281 S, Aulicino S, Lucente MS, Buonavoglia C. Long-term viremia and fecal shedding in pups after
282 modified-live canine parvovirus vaccination. Vaccine. 2014; 32:3850-3853.

283 [12] Decaro N, Buonavoglia C. Canine parvovirus-A review of epidemiological and diagnostic
284 aspects, with emphasis on type 2c. Vet. Microbiol. 2012; 155, 1-12.

285 [13] Freisl M, Speck S, Truyen U, Reese S, Proksch AL, Hartmann K. Faecal shedding of canine
286 parvovirus after modified-live vaccination in healthy adult dogs. Vet. J. 2017; 219:15-21.

287 [14] Bouvet J, Cariou C, Poulard A, Oberli F, Cupillard L, Guigal PM. Compatibility between a
288 rabies vaccine and a combined vaccine against canine distemper, adenovirolosis, parvovirolosis,
289 parainfluenza virus and leptospirosis. Vet. Immunol. Immunopathol. 2018; 205:93-96

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Table 1. Haemagglutination inhibition antibody titers in pups orally vaccinated with a commercial modified live CPV-2b vaccine.^a

Dog	T0	T15	T30
A1	80	10240	ND
A2	80	10240	ND
A3	80	10240	ND
A4	80	1280	ND
A5	40	5120	ND
A6	40	10240	ND
A7	80	10240	ND
A8	80	10240	ND
A9	160	1280	ND
A10	80	5120	ND
A11	80	2560	ND
C1	20	5120	ND
C2	160	40	1280
C3	20	10240	ND
Median	80	7680	ND
IR	[50;80]	[3200; 10240]	ND

^a Antibody titres are expressed as the reciprocal of the highest serum dilution able to cause haemagglutination inhibition. T, day post-vaccination; ND, not determined; IR, interquartile range.

Table 2. Real-time PCR results for CPV DNA in rectal swabs of pups orally vaccinated with a commercial modified live CPV-2b vaccine.^a

Dog	T0 ^a	T4	T8	T15	T19	T23	T30
A1	Neg	Neg	^b 1.82×10 ⁶	1.23×10 ⁴	1.57×10 ³	Neg	Neg
A2	Neg	Neg	2.95×10 ⁴	6.84×10 ³	4.37×10 ³	Neg	Neg
A3	Neg	5.70×10 ²	1.57×10 ⁷	8.28×10 ³	1.84×10 ³	Neg	Neg
A4	Neg	3.70×10 ²	5.56×10 ⁴	1.52×10 ⁴	3.31×10 ³	Neg	Neg
A5	Neg	Neg	6.64×10 ⁵	5.56×10 ⁴	3.51×10 ³	Neg	Neg
A6	Neg	Neg	8.63×10 ⁵	4.20×10 ⁴	3.40×10 ³	Neg	Neg
A7	Neg	Neg	2.69×10 ⁶	1.20×10 ⁴	3.30 ×10 ³	Neg	Neg
A8	Neg	Neg	1.40×10 ⁶	6.13×10 ³	1.69×10 ³	Neg	Neg
A9	Neg	3.70×10 ²	4.68×10 ⁴	6.71×10 ³	5.29×10 ³	Neg	Neg
A10	Neg	3.60×10 ²	4.40×10 ⁵	7.82×10 ³	3.11×10 ³	Neg	Neg
A11	Neg	4.75×10 ³	7.37×10 ⁴	9.06×10 ⁴	4.66×10 ³	Neg	Neg
C1	Neg	Neg	3.89×10 ³	2.56×10 ⁴	1.70×10 ³	Neg	Neg
C2	Neg	Neg	Neg	Neg	3.80×10 ²	3.29×10 ⁵	5.31×10 ³
C3	Neg	3.10×10 ²	4.86×10 ⁴	8.63×10 ³	1.57×10 ³	Neg	Neg
Median	ND	ND	2.57×10 ⁵	1.03x10 ⁴	3.20x10 ³	ND	ND
IR	ND	ND	[4.72×10 ⁴ ;1.58×10 ⁶]	[7.08×10 ³ ;2.30×10 ⁴]	[1.69×10 ³ ;3.48×10 ³]	ND	ND

^a Results are expressed as CPV-2 DNA copy numbers 10 µl⁻¹ of template.
T, day post-vaccination; Neg, negative; ND, not determined; IR, interquartile range