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Assessing the immunogenicity of an inactivated monovalent vaccine in the endangered African wild dog (*Lycaon pictus*)

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ABSTRACT

Captive held African wild dogs (*Lycaon pictus*) were vaccinated with a commercially available inactivated canine parvovirus (CPV) vaccine licensed for use in domestic dogs. Serological responses were recorded by testing for viral antibodies with the haemagglutination inhibition test (HAI) following a vaccination schedule involving two initial inoculations a month apart and an annual booster. Vaccines were delivered by hand injection or remotely (i.e. by dart). All naïve dogs vaccinated with Parvac® did seroconvert as determined by canine antibody titres $\geq$ 1:80 in one or more samples collected after inoculation. Duration of immunity (DOI) within the first year persisted for approximately 98 days only. To enable greater immunological protection revaccination should occur more frequently than once in a calendar year. No significant differences in the seroprotection rate were observed when comparing route of administration.

1. Introduction

Parvoviruses have been observed in species from six carnivore families (Felidae, Canidae, Procyonidae, Mustelidae, Ursidae and Viveridae) with all described as having very similar clinical signs [1]. Canine parvovirus (CPV) has existed in three forms and was first detected in wild wolves (*Canis lupus*) in northeast Minnesota during 1973 [2]. In free ranging wildlife populations this virus can persist in a diverse range of conditions and is not only an important cause of juvenile mortality but also contributes to the maintenance of small populations in fragmented landscapes [3,4]. Epidemiological studies recognise that small low density populations are at greater risk to spill over transmission from higher density hosts (e.g. jackals, domestic dogs) emphasising the need to develop effective approaches to mitigate disease risk [5,6]. Species such as the African wild dog (AWD) [3], Ethiopian wolf [7], and Island fox [8], which typically live at low densities, are likely end points for infection transmission from other species with this increasing their risk of localised extinction. For such species the effect of infectious disease has been responsible for both dramatic die-offs and local extinctions [9–11]. Disease has been categorised as a significant conservation area with its management not only important for small naturally occurring populations but also for those involved in conservation programs such as translocation, captive management and reintroduction [12,13].

Vaccination programs designed for endangered species are aimed at reducing the effects that large outbreaks have on populations by increasing an individual's ability to resist or cope with a disease's harmful effects [14,15]. The success of these programs are however significantly influenced by the proportion of the population that are inoculated, the efficaciousness of the utilised vaccine and an appropriate monitoring program [16]. In the Action Plan for the African wild dog attention is drawn to the usefulness of vaccines and their protocols, stating that they should be explored in captive bred animals or those that are unsuitable for release [17]. Reducing transmission rates or controlling disease in wildlife populations is generally limited by the availability of specific vaccines, which are mostly untested for use in wildlife species [18], while the delivery of such preventative measures in field settings is surrounded by logistic, financial, and ethical complications [19].

Research relating to CPV and managing its risk through surveillance and treatment protocols is limited in AWD populations. This is not assisted by the small number of studies reporting a post vaccination antibody response. From published articles all have combined this pathogen in a bi or multivalent vaccine [20–22] with there being no study that has assessed the immune response of an inactivated vaccine. Expanding basic research on vaccine use

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in the AWD helps to establish more appropriate vaccination schedules, particularly as the use of inactivated vaccines is a valuable option in the management of adverse vaccine reactions and the recrudescence of a live vaccine [23]. It can also assist in determining which preventive approaches are more likely to succeed under field conditions. In AWD populations, little known about the regulatory role that CPV plays in population persistence.

This study examined the immunogenicity of the inactivated monovalent vaccine Parvac® (Zoetis) in the AWD. This vaccine is commercially available in Australia for use in domestic dogs and is known to be safe for use in pregnant bitches and those individuals that are at high risk or otherwise immunodeficient [24]. Parvac® is frequently used in captive populations of non-domestic canid species to guard against CPV [25]. However, information pertaining to duration of immunity hasn't been described. The primary objectives were to (i) assess immunogenicity and the duration of immunity and (ii) evaluate the effect of route of administration.

2. Methods

2.1. Experimental animals

The study site was Monarto Zoo (MZ) in South Australia, which is an open-range wildlife park occupying approximately 1,000 ha. At the commencement of the study it housed (separated by sex) the largest number of AWDs in the Australasian region (19 males and 5 females) [26].

Eighteen male dogs from four cohorts respectively aged 5, 6, 7 or 11 years were used in the trial. This group of animals had been displayed long term in the main AWD exhibit but were separated into two groups after nine months of the study. Increased aggression between cohorts coincided with the breeding season which resulted in one fatality of the study group (ID. *A69114*).

Generally, dogs held at MZ were not vaccinated against any infectious disease due to its remote location and lower disease transmission risk. At the beginning of the study all animals were regarded as being healthy with experimental procedures performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes [27]. Ethics approval was gained through the Flinders University Animal Ethics Committee (Project no. E338).

2.2. Vaccine administration and schedule

At the commencement of the study all dogs were divided randomly into three treatments groups each consisting of six individuals. Groups included a control where animals were initially given a placebo (water for injection), while dogs in the second and third groups were respectively vaccinated by hand injection and darted in the hind leg using Parvac® (CPV-2, containing adjuvant Thiomersal (0.01%), Lot no. 7922-09602) [28]. The recommended dosage rate for this vaccine is 1 ml for dogs of all ages with the administration route being either subcutaneous or intramuscular [28]. The use of a blow pipe (length 105cms) discharged at approximately 1 m was utilised for all animals in the darted group, except for the annual vaccination of *A69115* where a Dan-Inject (model JM) dart rifle was employed. This animal was darted within 20 ms using 5–7 Bar of pressure. During inoculation 3 ml syringes (Telinject USA Inc) were used with a 1.5 mm diameter and 20 mm length needle.

The frequency of immunisations followed the recommended protocol for naïve animals [29]. These vaccination guidelines state that there should initially be two inoculations approximately one month apart followed by an annual booster at 12 months. Table 1 shows the schedule of administration with monitoring of antibody titre levels occurring for all groups during the intervening months as well as after the annual booster. Dogs *A59172* and *A59175* received a third booster to explore what influence an extra dose had on the vaccine's immunogenicity.

Table 1
Vaccination schedule and program for monitoring antibody titre levels.

Day	Group 1 (n = 6)	Group 2 (n = 6)	Group 3 (n = 6)
0	Placebo	Parvac delivered by hand	Parvac delivered remotely
33	Monitoring only	Parvac delivered by hand	Parvac delivered remotely
68	Monitoring only	Monitoring only	Parvac delivered to A59172 and A59175 only
131	Monitoring only	Monitoring only	Monitoring only
278	Monitoring only	Monitoring only	Monitoring only
369	Monitoring only	Parvac delivered by hand	Parvac delivered remotely
404	Monitoring only	Monitoring only	Monitoring only
446	Monitoring only	Monitoring only	Monitoring only
777	–	Monitoring only	Monitoring only

2.3. Blood sample collection

Antibody monitoring occurred nine times across a 25 month period. Bloods taken on day 0 determined baseline titre levels prior to vaccination, while samples collected on day 777 were done opportunistically as seven vaccinated dogs were being transferred to another zoo and required pre-shipment health checks. Sampling was performed by venepuncture from either the cephalic or saphenous vein with a minimum of 2.5 ml of whole blood collected. All samples were centrifuged on the day of collection and placed into a screw top container and stored at −80 °C until required for laboratory analysis.

2.4. Determination of parvovirus antibody titres

A hemagglutination inhibition assay (HAI) was performed by a commercial laboratory service (Biobest, UK) to determine the antibody response. In domestic dogs antibody titre levels greater than 1:80 are considered to be protective [20,30]. For computational purposes, titres of '<1:20′ or similar were respectively assigned a value for the preceding dilution e.g. 1:10. Seroprotection was defined as pre-vaccination HAI antibody titres $\leq$ 1:10 and a post vaccination titre of $\geq$1:80, or when the post-vaccination titre had at least a numerical four-fold increase when using double dilutions.

2.5. Statistical methods

To assess immunogenicity three primary end points were examined including the geometric mean response of titres (GMT), geometric mean fold increase (gMFI) and seroprotection rate. As HAI antibody titres were reciprocals the lowest tested dilution factor was incorporated in a $\log_2$ transformation [31]. Immunogenicity values were analysed using Excel [32] and Graph Pad Prism [33] with statistical significance defined as P values < 0.05.

To evaluate whether the HAI titres of the vaccinated animals differed significantly from those in the control group the Mann-Whitney *U* test was used on data collected one month after the initial inoculation to two months following the annual booster (days 33–446). The Friedman test with Dunn's multiple comparison was applied to the same period focusing only on vaccinated animals to examine the differences in antibody titres across time. A Wilcoxon

matched pair's signed rank test assessed GMTs at specific time intervals to compare against baseline values within each treatment group.

To determine whether the route of administration influenced the number of dogs that developed protective immunity, the pooled vaccination results were separated into two treatment groups (subcutaneous hand injection, SC: darted, intramuscular, IM). Immunological values were calculated for each time (day 33–777) along with a Fisher's exact test. It was hypothesised that the ratio of seropositive to seronegative dogs would be the same at each sampling event for animals vaccinated by hand injection and those darted.

3. Results

Dogs were monitored daily for changes in behaviour and health. Initial baseline antibody titre values were examined identifying only one dog that did not have a HAI titre value of <1:10. This animal (*A49160*) had a protective titre value of 1:80 leading to a baseline imbalance and was subsequently excluded from further statistical analysis.

3.1. Immune response to inoculation

Comparisons in immunogenicity values for control and vaccinated dogs were made from samples collected during days 33–446. The Mann-Whitney *U* test showed vaccinated dogs had a significantly higher immune response than control animals, $U\ (114) = 129.5$, $p = <0.0001$. Fig. 1 shows the geometric mean values for control and vaccinated animals being 6.13 ($CI_{95\%}$ 5.43–7.07) and 59.01 ($CI_{95\%}$ 43.86–81.85) respectively.

GMTs for dogs in the control group showed little variability with titres ranging from 5 to 14 ($CI_{95\%}$ 5.2–38.6). The largest fold change in the GMT for this group was observed on days 131 and 278 (Table 2) with titre values of the latter being 2.8 times the baseline value. A Wilcoxon matched pairs signed rank test indicated only moderate differences in titre values at days 0 and 278 ($z = 1.5$, $p = 0.125$). No dog from this treatment group was considered to have protective immunity throughout the study.

Dogs that were vaccinated had GMTs that ranged from 17.6 ($CI_{95\%}$ 11.7–26.5) to 363 ($CI_{95\%}$ 183–720). The Friedman test evaluated the response of antibody titres across days 33–446 (Fig. 2) finding significant differences over time ($F_{r\ (6)} = 50.02$, $p < 0.05$). A 6.6 fold increase in immunological values was evident one month following the initial inoculation with 18% of animals considered to be seroprotected. At day 68 geometric mean titres peaked with 91% of the dogs having antibody titres above 1:80. The geometric mean ratio (GMR) between treatment groups showed a fold increase of 72 in the titres of vaccinated dogs compared to control animals.

The gMFI decreased by 87% at day 131 with the seroprotection rate also falling to 45%. This declining trend in the seroprotection rate and GMT continued till the annual booster where no animal was found to have protective immunity. Within the first 12 months protective immunity was primarily induced over a period of 98 days (days 33–131).

The second significant rise in antibodies was observed in the month following the annual booster with the largest gMFI of 19.3 recorded. GMTs at day 404 reached 340.8 ($CI_{95\%}$ 231.4–501.9) with all vaccinated animals considered to be seroprotected. There was a geometric mean fold decrease of 50% in titres 42 days later with all but one individual (*A59175*) sustaining a titre level deemed to be protective (91%).

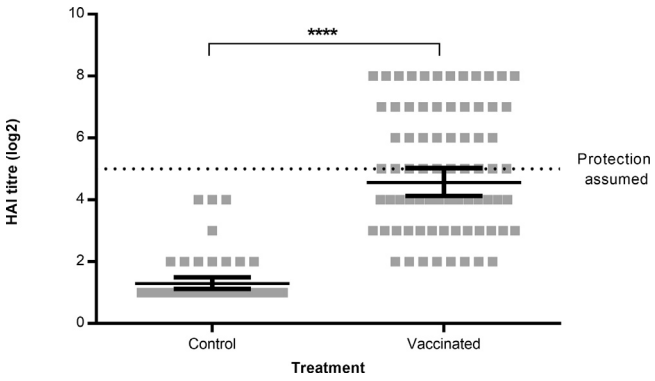

Fig. 1. HAI titre values from control animals and dogs vaccinated with the Parvac® vaccine. Protection assumed at log transformed HAI titre value of 1:80.

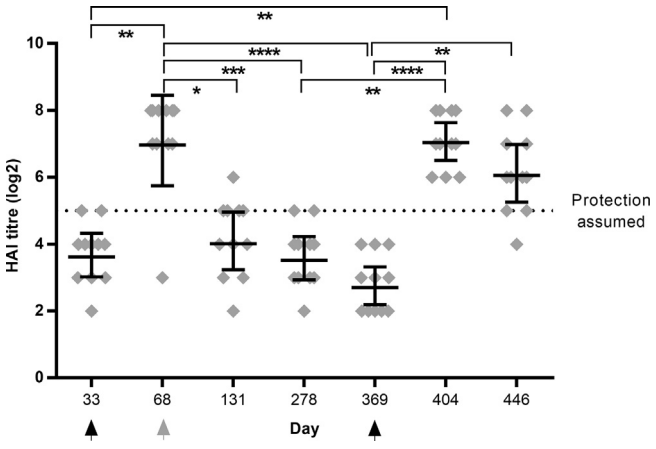

Fig. 2. Change in antibody titres for vaccinated animals over time. Arrows indicate booster vaccinations (black: all dogs; grey: only two dogs).

Table 2
Immunogenicity values for control and vaccinated dogs.

Treatment	Statistic	Day							
		0	33	68	131	278	369	404	446
Control	N	6	6	6	6	6	5	5	5
	GMT (SD)	5.0 (1.0)	5.0 (1.0)	5.0 (1.0)	7.94 (1.4)	14.14 (2.6)	5.0 (1.0)	6.6 (1.46)	7.6 (2.53)
	gMFI*	–	1.0	1.0	1.6	1.8	0.4	1.3	1.1
Vaccinated	GMT (SD)	5.0 (1.0)	33.1 (1.87)	363.0 (2.77)	45.4 (2.25)	31.9 (1.90)	17.6 (1.83)	340.8 (1.78)	181.5 (2.38)
n = 11	Seroprotection Rate (%)	–	18	91	45	18	0	100	91
	gMFI*	–	6.6	11.0	0.13	0.69	0.57	19.3	0.5
Geomean ratio (GMR)		1.0	6.6	72.6	5.7	2.2	3.5	51.7	23.9
Geomean fold ratio (gMFR)		–	6.6	11.0	0.08	0.4	1.8	15.3	0.5

* Fold change across time interval.

3.2. Comparing route of administration

One month on from the initial inoculation saw similar fold increases in titres with the GMT of hand injected animals and darted dogs respectively being 35.6 ($CI_{95\%}$ 15.2–83.4) and 30.3 ($CI_{95\%}$ 18.9–48.6). A nominal change in the GMR was observed at day 68 with there being a 2.1 fold increase in titres for hand injected animals compared to those that were darted (Table 3). Differences in the maximum seroprotection rate were observed only in the first year with just one darted dog (*A69113)* not achieving a protective titre.

Dogs vaccinated by hand had the highest fold increase in the GMT after the annual booster with GMTs being 359.2 ($CI_{95\%}$ 207.7–621.1) and 320 ($CI_{95\%}$ 135.3–756.7) for darted dogs. The greatest declines in antibody titres were from dogs that were darted with GMTs waning by 60%. Opportunistic sampling more than a year after the annual booster (day 777) showed 50% of individuals (n = 6) still had protective immunity. In these animals DOI in the second year was sustained for a minimum of 373 days. Dogs vaccinated by hand were at this time found to have titres four times higher than animals that were darted. Conducting the Fisher's exact test showed no significant differences in the ratio of seropositive or seronegative animals in each of the treatment groups across each time period. Fig. 3 shows the change in GMTs across the study period for all treatment groups.

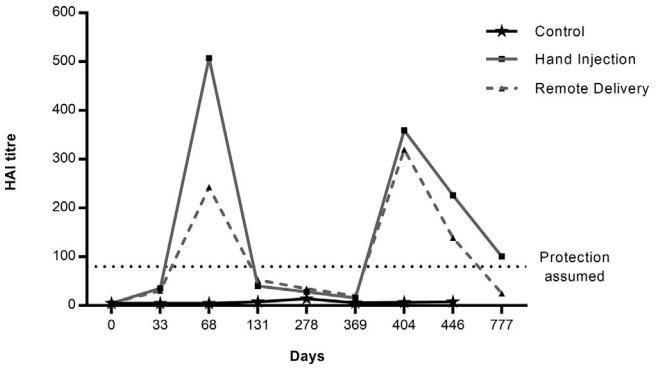

Fig. 3. Geometric mean response for respective treatment groups.

4. Discussion

The findings from this study indicate that the commercially available inactivated Parvac® vaccine did stimulate an adequate humoral response in *Lycaon*, which was protective. No negative post vaccination reactions were observed throughout the study implying that this vaccine is safe for use in this canid. Data pertaining to DOI in inactivated vaccines is relatively limited with even less information for 'off-label' species [34]. The primary response to the vaccine allowed immunity to persist for approximately 98 days. From day 131 to the annual booster at day 369 immunity fell below the protective level. This would have left individuals susceptible to the disease for approximately 8 months of the first year. A similar result was observed in bush dogs (*Speothos venaticus*), which were repeatedly vaccinated with an inactivated feline parvovirus (panleukopenia) vaccine with titres being undetectable after 3 months [35].

Following the annual booster, there was a rapid anamnestic response with all dogs becoming seropositive in the subsequent month. Protective immunity persisted in most animals for at least 77 days. Sampling more than a year after revaccination showed half of the sampled animals had sustained a protective titre. This suggests that subsequent inoculations can provide protective immunity for a longer time before declining to a level where an individual is sensitive to the disease.

Serology results showed no statistical differences in the seroprotection rate when comparing the two modes of delivery. The immune response of an inactivated CPV vaccine can be affected by administration route [36]. Results of this study showed there was greater variability observed in the GMT between treatment groups, potentially indicating that delivery approach may influence long term protection.

Individual antibody titres of *A69115* were examined for potential differences in the response after being inoculated remotely. The observed trend in waxing and waning of titres was not dissimilar to other darted dogs. Dogs *A59172* and *A59175* who received a third booster at day 68 had a less pronounced decline in antibody titres between days 131 and 278. As the sample size was small these results were only reviewed qualitatively.

Natural exposure to CPV cannot be discounted in this study as serological results for *A49160* revealed a baseline imbalance in

Table 3
Immunological values for differing modes of delivery.

Treatment	Statistic	Day								
		0	33	68	131	278	369	404	446	777
Hand Injected	N	6	6	6	6	6	6	6	6	3
	GMT (SD)	5.0 (1.0)	35.6 (2.25)	508.0 (1.43)	40.0 (1.86)	28.3 (1.79)	15.9 (1.76)	359.2 (1.68)	226.3 (2.07)	100.8 (2.88)
	Seroprotection Rate (%)	–	33	100	33	17	0	100	100	67
	gMFI*	–	7.1	14.3	0.1	0.7	0.6	22.6	0.6	0.1
Remote Delivery	N	5	5	5	5	5	5	5	5	3
	GMT (SD)	5.0 (1.0)	30.3 (1.46)	242.5 (4.2)	52.8 (2.86)	34.8 (2.14)	20.0 (2.0)	320.0 (2.0)	139.3 (2.80)	25.2 (2.90)
	Seroprotection Rate (%)	–	0	80	60	20	0	100	80	33
	gMFI*	–	6.1	8.0	0.2	0.7	0.6	16.0	0.4	0.1
Geomean ratio (GMR)		1.0	1.2	2.1	0.8	0.8	0.8	1.1	1.6	4.0
Geomean fold ratio (gMFR)		–	1.2	1.8	0.4	1.0	1.0	1.4	1.4	1.0
Fisher's Exact Test†		–	0.455	0.455	0.567	1.000	1.000	1.000	0.455	0.400
Rate Difference (%)		–	3.9	1.3	0.6	0.8	nil	1.0	1.3	5.0
Rate Ratio		–	6.1	4.3	0.3	0.8	0.8	1.8	4.3	11.7

* Fold change across time intervals.
† $\alpha = 0.05$.

antibody titres at the commencement of the trial. Positive cut-off values used by laboratories to detect exposure to CPV have been reported as 1:10 (ELISA tests) [37]. This would also suggest that the increase in titres found at day 278 in the control group could potentially be attributed to natural exposure despite no clinical signs being observed. Blood collection at day 278 occurred mid-autumn where temperatures in the preceding months were above average [38]. Seasonality has been noted to increase the risk of CPV with a greater incidence observed during warmer months [39–42].

Commercial vaccines commonly include the original CPV-2 antigen which has since been completely replaced by three alternative antigenic types; 2a, 2b and 2c [43]. In Australia the new CPV-2a is the predominant antigen in dog populations [44], while in southern Africa the CPV-2b variant is most widespread [45,46]. Serological analysis has found that there are substantial differences in the cross-neutralizing activity between the various heterologous virus types [47]. Utilising a CPV-2 vaccine can lower and shorten immunity against the alternative variants with only sub-optimal protection achieved [47]. There is some suggestion that the use of certain vaccines that target specific antigen types could better complement that which is present in a given environment or that a polyvalent vaccine should be developed [48,49].

Monitoring post vaccination responses in exotic canids is important as there is limited information relating to antibody production, ability to seroconvert and sustained immunity. Such research assists in determining the safety of vaccines as some preparations have either been ineffective or have reverted to virulence resulting in morbidity and mortality. Evaluating a serological response against a measure of protection, such as that for domestic dogs, can minimise the use of challenge studies. In the case of this vaccine there was no publicly available information pertaining to DOI in domestic dogs for comparison purposes. With the AWD being Endangered a direct challenge would have been unethical increasing the reliance of immunological values to assess vaccine efficacy. Erring on the side of caution due to the absence of a challenge Böhm, Thompson [50] increased the cut-off titre value which assumed protective immunity. This approach has merit when vaccinating endangered canid species as Spencer and Burroughs [21] noted that there are potential differences in immune responses between members of the Canidae family with reference to CPV.

The discontinuous periods of immunity during this trial suggest that the given vaccination schedule was inadequate. To maintain a longer interval of protective immunity in the first year an additional booster should be given at four months with biannual revaccination occurring thereafter. The use of multiple boosters in a given year for this endangered species is advocated, especially when utilising inactivated vaccines [22]. Parvac® could also be used in an attempt to prevent more animals from becoming infected during periods of high risk, thereby limiting the geographic spread of CPV through a barrier or suppressive vaccination strategy [51]. Parvac® could also be used as a primer for attenuated vaccines, thereby reducing the chance that it may revert to virulence and cause clinical disease [52].

The success of a disease management approach for wildlife species can be challenged by factors relating to the practicality of some control methods due to cost, confirmation of a vaccine's efficacy, and effective surveillance and monitoring programs. Resources involved in immobilising animals and the logistics in locating them can impose additional financial costs. Hence, the administration of Parvac® is more practical in captive populations and less likely for free ranging packs due to the dogs' ranging behaviour and the recommendation for biannual vaccination.

Zoological institutions provide a controlled environment in which to conduct safe and rigorous trials. For the AWD and many threatened species the testing of vaccines in captive held animals has significantly aided conservation efforts. For example, Frankfurt Zoological Society was involved in administering an inactivated rabies vaccine (Madivak, Hoechst) to AWDs to assess seroprevalence. This vaccine was shown to induce an antibody response and was subsequently given to dogs in the Serengeti [53]. Whereas, a trial at the Wildlife Conservation Society's Bronx Zoo involving a recombinant canary-pox vectored vaccine compared delivery approaches (oral vs. IM injection) identifying significant disparity in titre values for canine distemper [54]. To assist with the logistical challenges of administering vaccines to AWDs, an oral vaccination technique involving a modified live vaccine (SAG-2) presented in chicken heads was tested at De Wildt Cheetah and Wildlife Centre and separately Lion Park, South Africa [55]. The antibody levels achieved did suggest adequate protection was conferred with this approach extrapolated and modified for use in free ranging Ethiopian wolves [56]. While more broadly, the Los Angeles Zoo in conjunction with the California Condor Recovery Team assessed an experimental DNA vaccine for West Nile virus in captive condors determining it was a viable option for the conservation of free ranging populations; and similarly but more controversially an oral vaccine for Ebola was tested in chimpanzees at the University of Louisiana Lafayette's New Iberia Research Center to assist in mitigating the risk to wild gorillas and chimpanzees in north west Africa [57]. These studies demonstrate the usefulness that captive populations have in developing methods to control infectious disease, while for some species (e.g. Black-footed ferret, Prairie dog) they have been essential to prevent extinction [58]. Understanding which preventive medicine approach is likely to succeed helps inform scientists on strategies that best reduce disease risk and improves conservation outcomes.

5. Conclusion

CPV occurs globally and is an emerging and re-emerging pathogen that has a broad range of host species. To decrease the risk of infectious disease, particularly in endangered wildlife species, validating a vaccine's efficaciousness is needed. This study demonstrated that Parvac® did elicit an adequate humoral response which constituted protective immunity. The DOI was however limited but with an adapted vaccination schedule there is scope for protective immunity to be extended. The seroprotective rate was not affected by mode of delivery though monitoring of individual titres over a longer period could better assist with this determination. This research expands on the limited information available regarding CPV and the African wild dog, whilst also providing an insight into the practical use that this vaccine has for captive and free ranging populations. Future research will assess maternal antibody decay in pups which is a known cause of vaccination failure.

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Declaration of interest

The authors confirm that there are no known conflicts of interest associated with this publication. No funding provider had any involvement in the preparation of this manuscript.

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