Damanhour Journal of Veterinary Sciences 10 (1), (2023) 25-30

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

E-ISSN 2636-3003 | ISSN 2636-3011

Validation of Lyophilized-Attenuated Single and Combined Canine Hepatitis, Canine Distemper, and Canine Parvovirus Vaccines

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Abstract

Through this study, we attempted to prepare and evaluate lyophilized attenuated single and trivalent vaccines of Canine Distemper (CD), Canine Adeno-1 (CA-1), and Canine Parvo (Cp type 2a) viruses. All such preparations were found to be free from foreign contaminants, safe in mice and puppies, and potent, providing vaccinated puppies with good levels of specific antibody titers against the three viruses as estimated by serum neutralization test (SNT) and indirect immune sorbent assay (ELISA). Such antibodies were found to be stable up to 12 months post-vaccination, supporting their use to protect puppies against the three virus infections with the preferable use of the trivalent vaccine, which saves effort and avoids stress factors of vaccinated puppies.

Keywords: Canine distemper; Canine Adeno-1; Canine Parvo; single vaccines; combined vaccines; Serum neutralization; ELISA

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Email: asmaagamal223@yahoo.com
P ISSN: 2636-3003
EISSN: 2636-3011
DOI: 10.5455/DJVS.2021.80072.1039
Received: June10, 2021; Received in revised form: June19, 2021; Accepted: August 25, 2023
Editor-in-Chief:
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1. Introduction

Canine distemper (CD), infectious canine hepatitis (ICH), and canine parvo enteritis (CPI) are viral diseases affecting dogs, resulting in huge losses, especially among high breeds. Canine distemper is a highly contagious viral disease affecting domestic and wild dogs and large cats **(Ikeda et al., 2001)**. The disease affects several body systems, including the gastrointestinal and respiratory tracts and the spinal cord and brain, with high fever, eye inflammation and eye/nose discharge, coughing, vomiting, diarrhea, appetite loss, and nose and footpads hardening. The causative agent is a single-stranded RNA virus of the family Paramyxoviridae transmitted via inhalation **(Deem et al., 2000).** In domestic dogs, the acute generalized form has a high mortality rate, with a period depending on the animal's age and immune status and virulence of the infecting strain of the virus **(Deem et al., 2000** and **Andreas et al., 2015).** Prevention of canine distemper depends on the puppy's vaccination at 6-8 weeks of age and then getting the "booster shot" every 2-4 weeks until they are 16 weeks of age **(Marvista Vet, 2012).**

Infectious canine hepatitis (ICH) is a contagious disease of dogs with signs varying from a slight fever and congestion of the mucous membranes to severe depression, leukopenia, and coagulation disorders. ICH is caused by a non-enveloped DNA virus, canine adenovirus 1 (CAV-1), antigenically related only to CAV-2 **(Carter and Wise, 2006)**. Urine, feces, and saliva of infected dogs are the primary sources of infection. ICH signs vary from a slight fever to death, with a mortality rate ranging from 10% to 30%, and is highest in very young puppies. The disease has an incubation period of 4–9 days starts with biphasic fever of >104°F (40°C) lasting 1–6 days and short duration. Other signs include anorexia, thirst, conjunctivitis, serous ocular and nasal discharges, and occasionally abdominal pain and vomiting. Subcutaneous edema of the head, neck, and trunk may be observed. In most acute cases, there is a notable absence of icterus. Severely infected dogs may develop convulsions from forebrain damage; paresis resulting from brain stem hemorrhages and ataxia and central blindness have also been described **(Kate, 2013)**. ICH vaccination is recommended at the time of canine distemper vaccinations. Additional control measures include isolation, overcrowding prevention, and other co-infections **(Jane, 2014).**

Canine parvovirus is a highly contagious virus that affects all dogs, and unvaccinated puppies younger than four months old are the most at risk. It affects gastrointestinal tracts and is spread by direct dog-to-dog contact and contact with contaminated feces, environments, or people; kennel surfaces, food and water bowls, collars and leashes, and the hands and clothing of people handling infected dogs. Signs of parvovirus include lethargy, loss of appetite, abdominal pain and bloating, fever or low body temperature (hypothermia); vomiting; and severe, often bloody, diarrhea. Persistent vomiting and diarrhea can cause rapid dehydration, and damage to the intestines and immune system can cause septic shock **(Ingy, 2018** and **Khodeir et al., 2021).**Vaccination and good hygiene are critical components of prevention **(Lamm and Rezabek, 2008).** The causative agent is canine parvovirus, which belongs to the genus *Parvovirus* and family *Parvoviridae.* It is a single-stranded negative-sense DNA having a size of 5.2 Kb **(Nandi and Manoj Kumar, 2010)**.

Vaccination plays a vital role in preventive medicine and will continue to be a mainstay for promoting animal health. The

combined vaccine is considered a core vaccine, which means all dogs should receive it regardless of their lifestyle to protect them against highly contagious viruses that cause severe disease and have high fatality rates. There are bivalent and trivalent canine vaccines that succeeded in protecting dogs against infectious viral diseases **(Khodeir et al., 1998, Edries et al., 1999, Zeinab et al., 2003,Taguchi et al., 2012, Wilson et al., 2014,**and **Anon, 2020)**.

Recent isolates of CD and CP viruses related genetically to the currently present viruses were recorded in Egypt **(Ingy, 2018** and **Khodeir et al., 2021)**. The current work was directed to validate single and trivalent CD, CIH, and CP vaccines to determine the best one that provides vaccinated dogs with the highest protective immunity against the three diseases.

2. Materials and Methods

2.1. Viruses

Vero cell culture-adaptedcanine distemper virus Snyder Hill strain **(Guirguis, 1991)** and canine adeno-1 virus **(Khodeir et al., 2003-I)** and MDCK-adapted canine parvovirus type 2a **(Ingy, 2018)** were used for vaccine-preparations and serological tests.

2.2. Experimental design

Mice: Forty Swiss Albino weaned mice of about 25 g body weight were used to test the safety of the prepared vaccines.

Puppies: Forty native breed puppies of about 3-5 months age free from CA; CD and CP antibodies as screened by serum neutralization test were used in the present work where 10 of them were used to test vaccine safety while the other 30 puppies were used to test vaccine potency.

2.3. Cell culture

African green monkey kidney line (Vero) and Madin Darby canine kidney (MDCK) cell lines were used to propagate CA and CD viruses and CPV for vaccine preparations and serum neutralization tests. These cell lines were passaged and maintained using Minimum Essential medium supplemented with 10% newborn calf serum: 100 µg of streptomycin and 100 IU of penicillin G sodium/ml.

2.4. Virus titration

Virus titration was carried out using the microtiter technique (**Ferreira, 1976),** where serial tenfold dilutions of the virus were prepared in Hank's balanced salt solution (10^{-1} to 10^{-10} dilutions). The test used Vero cells for CA and CD viruses and MDCK for CPV. The virus titer was expressed as $\log_{10}$ $TCID_{50}$/ml of the original inoculums using the formula of **Reed and Meunch (1938).**

2.5. Vaccine preparations

To prepare live attenuated single vaccines (either CD; CA, or CP); a stabilizer composed of 5% lactalbumin hydrolysate and 2.5% sucrose was added to the titrated and sterility-tested virus suspension in the ratio of 1:1 then dispensed in neutral sterile vials (2.5ml/vial) and subjected to freeze-drying (lyophilization) process according to **Guirguis (1991)** and **Attyat (1994)** then subjected to the lyophilizing process on Teflon lyophilize apparatus **(Wang and Zhang, 2007 and Zhou et al., 2007).** The trivalent vaccine was prepared by mixing the three virus suspensions in equal volumes where each 1ml contains not less than three$\log_{10}$ $TCID_{50}$ of each virus, followed up as in the case of single vaccines.

2.6. Quality control testing of the prepared vaccines

2.6.1. Sterility test

The prepared vaccines were tested for the presence or absence of foreign materials according to the standard procedures of **FAO (1994)** using thioglycolate, soyabean casein digest, sabouraud, mycoplasma solid, and liquid media.

2.6.2. Safety test

In mice: According to **Jane Cooper (2008),** five vaccine vials were reconstituted and pooled in normal saline, where 0.03ml of each vaccine was inoculated intraperitoneally in each of the 8 weaned Swiss Albino mice. Immunized mice with another eight non-inoculated mice were observed for ten days.

In puppies: Each of 2 puppies was inoculated S/C with ten times the recommended doses (as one shot to test the vaccine safety) of each vaccine formula, leaving two puppies without inoculation as test control and kept under daily clinical observation for ten days according to **Amani et al (2002)** and **Zeinab et al (2003)**.

2.6.3. Potency test

For potency testing, 30 puppies were divided into five groups (6 puppies/ group) as follows:

- Group-1 vaccinated S/C with CD live attenuated vaccine using a dose 3$\log_{10}$ $TCID_{50}$/ puppy.
- Group-2 vaccinated S/C with CA-1 live attenuated vaccine using a dose 3$\log_{10}$ $TCID_{50}$/ puppy.
- Group-3 vaccinated S/C with CP-2a, live attenuated using a dose 3$\log_{10}$ $TCID_{50}$/puppy.
- Group-4 vaccinated S/C with the trivalent CD, CA-1 and CP live attenuated vaccine
- Group 5 was kept without vaccination as a control.

Vaccination of puppies with the trivalent vaccine was carried out using a dose containing 3$\log_{10}$ $TCID_{50}$ of each virus.

Each puppy group was housed in a separate kennel, receiving a balanced diet and adequate water.

Serum samples were obtained from all puppy groups at one-week intervals up to 4 weeks post-vaccination then at two months intervals up to 12 months post-vaccination to follow up the levels of induced immunity by the prepared vaccine formulae.

Depending on the animal welfare and to avoid the spread of virulent viruses, we did not carry a challenge test on vaccinated puppies and the results were evaluated depending on the results of applied serological tests, especially SNT, to evaluate vaccine potency.

2.7. Serum neutralization test (SNT)

SNT in Vero and MDCK cell culture was performed using the micro-technique method as described by **Ferreira (1976)** in flat bottom tissue culture microtiter plates for monitoring of canine hepatitis; canine distemper and canine parvo antibody titers in vaccinated puppies using two-fold dilutions of inactivated serum samples and virus suspension containing 100 $TCID_{50}$. Infected cultures and normal controls were kept at 37˚C with daily microscopic examination. The endpoint of neutralizing antibody titers was expressed as the reciprocal of the final dilution of serum inhibiting the CPE, according to **Singh et al (1967)**.

2.8. Indirect ELISA

The indirect method of ELISA was used to estimate CA, CD, and CP antibodies as a sensitive and simple method for the quantitative determination of antibodies according to the combined methods of **Hubschle et al (1981)** and **Voller et al (1976).**

3. Results and Discussion

Application of quality control testing on the prepared single and trivalent CD; ICH and CP vaccines revealed that all of them were free from aerobic and anaerobic bacteria, fungi, and mycoplasma and safe, inducing no local or systemic abnormal post-vaccinal reaction neither in mice nor in puppies coming in agreement with **CFR (1997)**.

The results of SNT and ELISA demonstrated in **Tables 1**and **2** showed that vaccinated puppies with single CD (group-1) and with the trivalent CD, CA-1, and CP vaccine (group-4) exhibited detectable specific CD antibodies by the first week (2&2≤ by SNT

and 0.70&0.71 log_{10} by ELISA respectively) recording their peaks (64 by SNT &2.5 log_{10} by ELISA in both groups) by the 2nd month and remained stable up to 12 months post-vaccination while the control group (group-5) still seronegative to CD antibodies. Such titers of CD antibodies can protect animals against challenges with virulent viruses, as (1997) concluded that completed recommended serum neutralizing titer not less than 1:50 (1.7 log_{10}) for the CD. These results also agreed with **Guirguis (1991), Miyamoto et al (1995), and Khodier et al (1998),** who reported that dogs were considered immune to canine distemper if their antibody titer was higher than 30. Also, these findings came in agreement with those of **Heerden et al. (2002),** who stated that the attenuated live CD vaccine resulted in seroconversion in all vaccinated dogs and protective concentrations of CD antibodies were present in all wild dogs for at least 451 days.

Tables 3 and **4** show the mean SNT and ELISA titers of CA-1 antibodies in puppies' group-2 and 4 (vaccinated with single CA-1 and trivalent CD; CA-1 and CP vaccines, respectively). These Tables indicate that these puppies responded well to these vaccines, having detectable specific CA-1 antibodies by the first week (2&4≤ by SNT and 0.50& 0.51 log_{10} by ELISA in group-2 and 4 respectively), recording their peaks (128 by SNT and 2.50 log_{10} by ELISA in both groups) by the 2nd month and remained unchanged up to 12 months post-vaccinationnation. In this respect, modified live vaccines induce spent-bodies bodies that persist for long periods, conferring complete protection against ICH infection **(Twark and Dodds, 2000; Soma, 2002; Khodeir et al., 2003-II**, and **Mohammadi et al., 2011).** The unvaccinated puppy group remained free from CA-1 antibodies through the experimental period.

Regarding puppy vaccination with single attenuated CP and trivalent CD, CA-1, and CP vaccines, the results of SNT and ELISA tabulated in **Tables 5** and **6** it was found that single CP vaccine induced specific CP antibodies in vaccinated puppies within the first week 4 and 2 by SNT and 0.90 and 0.88 log_{10} by ELISA in group-3 vaccinated with the single vaccine and group-4 vaccinated with the trivalent vaccine respectively. Peak titers of such antibodies (128 by SNT and 2.50 log_{10} by ELISA) were recorded by the 2nd month post-vaccination in both groups, with negative results among the unvaccinated group (Group-5). These findings came to be supported by **Attyat (1994),** who prepared successfully live attenuated CP inducing high, long-lasting immunity in vaccinated puppies and the fact demonstrated that the CPV-2 vaccines are still effective in inducing protection against CPV-2 variants (**Yule et al., 1997 and Spibey et al., 2008**).

Table 1. Mean canine distemper serum neutralizing antibody titer in vaccinated puppy groups

Periods post-vaccination	Mean CD serum neutralizing antibody titer* in puppy groups		
	Group-1	Group-4	Group-5
Pre-vaccination	0	0	0
1WPV**	2	2≤	0
2WPV	4	8	0
3WPV	8	8	0
4WPV	16	32	0
2MPV***	64	64	0
4MPV	64	64	0
6MPV	64	64	0
8MPV	64	64	0
10MPV	64	64	0
12MPV	64	64	0

Group-1 was vaccinated with a single CD live attenuated vaccine.
Group 4was vaccinated with the trivalent CD, CA-1, and Cp-2a live attenuated vaccine.
Group 5 was kept without vaccination.
*Mean CD serum neutralizing antibody titer = the reciprocal of the final serum dilution which neutralized and inhibits 100$TCID_{50}$ of CD virus
**WPV= week post-vaccination ***MPV= month post-vaccination

Table 2. Mean canine distemper ELISA titer in vaccinated puppy groups

Periods post-vaccination	Mean CD ELISA antibody titer (log10) in puppy groups		
	Group-1	Group-4	Group-5
Pre-vaccination	0.03	0.02	0.04
1WPV*	0.70	0.71	0.03
2WPV	0.93	0.72	0.02
3WPV	1.70	1.01	0.04
4WPV	2.00	1.9	0.03
2MPV**	2.50	2.50	0.03
4MPV	2.50	2.50	0.03
6MPV	2.54	2.51	0.01
8MPV	2.50	2.50	0.02
10MPV	2.50	2.51	0.03
12MPV	2.50	2.50	0.03

Group 1was vaccinated with a single CD live attenuated vaccine.
Group 4was vaccinated with the trivalent CD, CA-1, and Cp-2a live attenuated vaccine.
Group 5 was kept without vaccination.
*WPV= week pos-vaccination **MPV= month post-vaccination

Table 3). Mean CA-1 serum neutralizing antibody titer in vaccinated puppy groups

Periods post-vaccination	Mean CA-1 serum neutralizing antibody titer* in puppy groups		
	Group-2	Group-4	Group-5
Pre-vaccination	0	0	0
1WPV**	2	4≤	0
2WPV	4	4	0
3WPV	16	16	0
4WPV	64	64	0
2MPV***	128	128	0
4MPV	128	128	0
6MPV	128	128	0
8MPV	128	128	0
10MPV	128	128	0
12MPV	128	128	0

Group-2 vaccinated with a single CA-1 live attenuated vaccine.
Group-4 was vaccinated with the trivalent CD, CA-1 and Cp-2a live attenuated vaccine.
Group-5 was kept without vaccination.
* Mean CA-1 serum neutralizing antibody titer = the reciprocal of the final serum dilution which neutralized and inhibits $100TCID_{50}$ of CA-1 virus
**WPV= week post-vaccination ***MPV= month post-vaccination

Table 4. Mean CA-1 ELISA titer in vaccinated puppy groups

Periods post-vaccination	Mean CA-1 ELISA antibody titer (log10) in puppy groups		
	Group-2	Group-4	Group-5
Pre-vaccination	0.02	0.03	0.02
1WPV*	0.50	0.51	0.02
2WPV	0.73	0.72	0.03
3WPV	1.10	1.20	0.02
4WPV	1.50	1.50	0.01
2MPV**	2.50	2.50	0.02
4MPV	2.53	2.50	0.03
6MPV	2.51	2.52	0.01
8MPV	2.50	2.50	0.01
10MPV	2.51	2.50	0.01
12MPV	2.50	2.51	0.02

Group-2 vaccinated with a single CA-1 live attenuated vaccine.
Group-4 was vaccinated with the trivalent CD, CA-1, and Cp-2a live attenuated vaccine.
Group-5 was kept without vaccination.
*WPV= week post-vaccination. **MPV= month post-vaccination

Table 5. Mean canine parvo serum neutralizing antibody titer in vaccinated puppy groups

Periods post-vaccination	Mean canine parvo serum neutralizing antibody titer* in puppy groups		
	Group-3	Group-4	Group-5
Pre-vaccination	0	0	0
1WPV**	4	2	0
2WPV	8	4	0
3WPV	16	16	0
4WPV	64	32	0
2MPV***	128	128	0
4MPV	128	128	0
6MPV	128	128	0
8MPV	128	128	0
10MPV	128	128	0
12MPV	128	128	0

Group 3was vaccinated with a single CP2a live attenuated vaccine.
Group 4was vaccinated with the trivalent CD, CA-1, and Cp-2a live attenuated vaccine.
Group 5 was kept without vaccination.
* Mean Cp serum neutralizing antibody titer = the reciprocal of the final serum dilution, which neutralized and inhibited $100TCID_{50}$ of Cp virus.
**WPV= week post-vaccination. ***MPV= month post-vaccination

Table 6. Mean canine parvo ELISA titer in vaccinated puppy groups

Periods post-vaccination	Mean CP ELISA antibody titer (log10) in puppy groups		
	Group-3	Group-4	Group-5
Pre-vaccination	0.03	0.02	0.02
1WPV*	0.90	0.88	0.11
2WPV	1.10	1.80	0.10
3WPV	1.85	1.91	0.02
4WPV	2.30	2.25	0.03
2MPV**	2.50	2.50	0.04
4MPV	2.51	2.51	0.03
6MPV	2.50	2.51	0.20
8MPV	2.50	2.51	0.01
10MPV	2.51	2.50	0.11
12MPV	2.50	2.50	0.02

Group 3was vaccinated with a single CP live attenuated vaccine.
Group 4was vaccinated with the trivalent CD, CA-1, and Cp-2a live attenuated vaccine.
Group 5 was kept without vaccination.
*WPV= week post-vaccination **MPV= month post-vaccination

From the present demonstrated results, there is no antagonizing effect between CD, CA-1, and CP attenuated viruses in single and trivalent vaccines on the immune response of vaccinated puppies to any of them. Vaccination of dogs is generally performed using multivalent vaccines, which contain CDV, CP, and CA. These findings came in agreement with what was reported before by **Khodeir et al (1998), Edries et al. (1999), and Zeinab et al., 2003),** who concluded that these vaccines induce varying levels of protective immunity and are safe either alone or in combination. Also, **Wilson et al. (2014)** concluded that a single administration of a minimum titer, a multivalent vaccine to dogs of six weeks of age is efficacious and prevents clinical signs and mortality caused by CAV-1 and CDV, leucopenia and viral excretion caused by CPV.

4. Conclusion

So, it could be concluded that the prepared single and trivalent CD, CA-1, and CP vaccines are safe and potent and can cause good specific immunity levels in vaccinated puppies. However, we prefer the use of trivalent vaccines to single ones for safe efforts and stress factors on puppies.

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