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Original Research Article

Preparation of inactivated canine distemper vaccine using different inactivators

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ABSTRACT

Development of environmental, safe and protective vaccines against infectious pathogens remains a challenge. In consequence of its high morbidity and mortality rates canine distemper is one of the most important diseases of young dogs. The object of the present study is to develop a selected method for preparation of an inactivated canine distemper vaccine. This method involved exposure of the virus to different concentrations of binary ethyleneimine (BEI), beta propiolactone (ßPL) and hydrogen peroxide (H_2O_2). Complete virus inactivation was obtained with BEI (0.003M) for 6 hours, ßPL (1/5000) for 4 hours and H_2O_2 at a concentration of 3% rapidly inactivated a Vero cell adapted canine distemper virus strain within 3 h of exposure without affecting its antigenicity or immunogenicity. The safety, immunogenicity and potency induced in four groups of puppies were evaluated using the three prepared experimental batches of inactivated canine distemper vaccine. These results revealed that no residual infectious virus was detected in H_2O_2 inactivated CD vaccine that proved to be safe and effective when compared with the same virus harvest that inactivated with the classical inactivating agents as BEI and βPL. Thus, an alternative inactivation method, such as H_2O_2 is able to maintain the integrity of the virus protein may be essential for improving the potency of inactivated canine distemper virus vaccine produced sufficient level of antibodies which measured by serum neutralization test (SNT) and was protected when challenged with virulent CD virus strain. These findings reinforce the idea that H_2O_2 can replace BEI and βPL as inactivating agents for canine distemper virus to reduce time and cost of inactivation process.

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1. Introduction

Canine distemper (CD) is one of the most serious viral diseases causing the highest fatality among dogs and other wild carnivores. The disease is highly contagious and is characterised by respiratory signs, fever and death with severe morbidity and high mortality. It is caused by a virus belong to family Paramyxoviridae (Greene, 2006). CDV transmission between dogs is primarily through aerosolised respiratory excretions and it is likely that a similar route is followed in foxes (Williams, 2001). Modified live canine distemper virus vaccines are currently available in many countries. They do provide protective immunity to CD, but such vaccines may revert to virulence, may cause immunosuppression following vaccination, and have been shown to cause mortality in non-canine species.

The CDV vaccines, currently used in dogs are all live attenuated vaccines, which are not recommended for wild live animals, because of the danger of insufficient

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attenuation and spread to other wild animal species (Appel, 1978). There is therefore a real need for an efficacious, inactivated canine distemper virus vaccine, which eliminates the serious problems associated with modified live canine distemper virus vaccines. The use of inactivated vaccines is considered a safer approach. The CDV vaccine did induce protective immunity in dogs against challenge with a virulent CDV strain (De vries, et al. 1988). In the process of inactivation, the pathogen's ability to propagate in the vaccinated host is destroyed but the viral capsid remains intact, such that it is still recognized by the immune system. Inactivation of CD virus has been achieved using a variety of agents such as formalin, β-propiolactone (Della-Porta and Snowdon, 1979), and binary ethyleneimine (Hsieh, et. al 2006). Currently, the inactivating agents used in CD vaccine production for dogs is beta propiolactone (βPL) and binary ethyleneimine. Beta propiolactone (βPL) is a very expensive chemical and has been shown to produce squamous-cell carcinomas, lymphomas and hepatomas in mice. Additionally, studies show that beta propiolactone can produce an immune complex reaction when combined with other components of the viral vaccine (Crashi, 2007). Binary ethylenimine (BEI) which is used as inactivating agent is very hazardous since it attacks nucleic acids and proteins. Thus, there is a need to find alternative inactivating agents which are not expensive, non-toxic and easily available. Hydrogen peroxide (H_2O_2) is a strong oxidizing agent which is proved to have a broad spectrum anti-microbial activity. Hydrogen peroxide shown the ability in maintaining immunogenic epitopes, for example the immunogenicity and adverse effects of vaccine consisting of a purified pertussis toxin inactivated with hydrogen peroxide was evaluated. Also, mice vaccinated with H_2O_2-inactivated lymphocytic choriomeningitis virus (LCMV),

H_2O_2-inactivated Vaccinia virus or H_2O_2-inactivated West Nile virus showed high virus-specific neutralizing antibody titers and were fully protected against lethal challenge. Also, the investigational vaccine, called HydroVax-001 which is H_2O_2-inactivated West Nile virus entered phase I clinical trials in May, 2015 after successful preclinical studies. Together, these studies demonstrate that H_2O_2-based vaccines are highly immunogenic, provide protection against a range of viral pathogens in mice and represent a promising new approach to future vaccine development. In this study demonstrates that influenza viruses inactivated with H_2O_2 retain immunogenicity and are able to both detect humoral and elicit cellular immune responses in vitro, which could reduce the need to handle live viruses in the laboratory (Jennifer, et al., 2014). This study aimed to prepare and evaluate the efficacy of a locally prepared inactivated canine distemper vaccine through evaluation of the use of three different inactivators including binary ethyleneimine, β propiolactone and hydrogen peroxide without adversely affecting its antigenicity and immunogenicity.

2. Material and methods

2.1. Viruses and Cells:

Synder-Hill canine distemper-Vero cell culture adapted strain of canine distemper virus of titer $10^{7.8}$ $TCID_{50}$/ml. Standard Snyder Hill CDV strain was propagated on embryonated chicken egg with a titer 10^5 EID50/ml, and were maintained frozen at −70°C until used (Guirguis, 1991), were used in the challenge test of the vaccinated dogs. African green monkey kidney (Vero) cells were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Osman, et. Al. 1991), the viruses and cells were provided by Dept. of Pet Animal Vaccine Research, Veterinary Serum and Vaccine Research Institute. Cairo. Egypt. CD virus was titrated in the corresponding Vero cell cultures using the microtitre technique (Trimarchi et al., 1996) and the virus titer was calculated (Reed and Muench, 1938).

2.2. Experimental animals:

Puppies (4-6 weeks old) were proved to be seronegative to canine distemper were used in the present study. Dogs in our study were considered susceptible to challenge with CDV. All of them were screened for using safety, specific-neutralizing CD antibodies and challenge. They were apparently healthy free from external and internal parasites.

2.3. Vaccine preparation:

Vero cells were infected with Snyder-Hill canine distemper virus at a MOI of 0.1. The titer of virus harvested was $10^{7.8}$ $TCID_{50}$/ml. The virus was inactivated by binary ethylenimine at final concentration of 0.001, 0.002 and 0.003 % M (WHO, 1973). The inactivation by Beta-Propiolactone was at final concentrations ranged from 1:5000, 1:10000 and 1: 20000 (Gledel, et. al. 1968) and that of hydrogen peroxide was at 1%, 2% and 3% (Asmaa, et. al. 2016).; Each batch was incubated overnight at 4°C and then for 1,2,3,4,5,6 and 7h at 37°C, then adjuvanated with aluminum hydroxide at a volumetric ratio of 1: 4 (Rivera and conlha, 2003).

2.4. Animal immunization and challenge:

Puppies were randomly divided into 4 groups with (4 animals/ group) and were inoculated with each prepared inactivated CD vaccines via three groups, respectively. The last group was kept as a control. The vaccinated and unvaccinated dogs were challenged with the Snyder Hill CDV strain that was diluted at 1:20 in serum-free Dulbecco modified Eagle medium and administered intranasal at dose of 2mL(Wayne, et al., 2015).Then, the puppies were observed for clinical sings of canine distemper for 21 days.

2.5. Serological assay:

Sera were tested for CDV antibodies by means of a serum neutralization test (SNT) (Rossiter, et al. 1985). It was collected from vaccinated dogs with the three prepared inactivated CD vaccines every week till one month then monthly for 6 months. The mean SN titer was expressed as the log_{10} of the final serum dilution which protected 50% of wells as calculated by (Reed and Muench, 1938).

2.6. Evaluation of the prepared inactivated CD vaccines:

The prepared vaccines were subjected to examination of its sterility, safety via Vero cell line and puppies, moreover, the potency and challenge following the recommendations of (FAO, 1994 and European pharmacopoeia, 2001).

3. Results and discussion

Canine distemper was placed beside rabies as diseases causing the highest fatality among dogs and other wild carnivores (Appel and Montali, 1994). The disease is characterized by respiratory, gastro-intestinal and central nervous manifestations (Craig, 1998). The disease was recognized among infants causing severe respiratory diseases, fever and death (Smith and Lauffer 1962) and in adult human making a chronic inflammatory bone disorder "Paget's disease" (Cartwright et al., 1993 and Reddy et al., 1996). The use of inactivated morbillivirus vaccine has had little success in inducing protective immunity (Appel, 1978 and Gillespie, 1974), However we have recently shown that a subunit ISCOM CDV vaccine did induce protective immunity in dogs against challenge with a virulent CDV strain (De vries, et al. 1988). Depending on the safety of inactivated vaccines in addition to their easy to be given at any age, such vaccines become of choice as recorded by(Olson et al., 1988). The prepared inactivated CD vaccines were found to be free from foreign contaminants (aerobic and anaerobic bacteria, fungi and mycoplasma), safe in both Vero cell culture and dogs where there was no adverse reaction or any reaction at the vaccination site. Current methods used for inactivation process in vaccines production involve the use of chemical agents such as phenol, formaldehyde, beta-propiolactone or binary ethylenimine to chemically modify the antigenic material of the virus. Binary ethylenimine (BEI) is used as inactivating agent for animal vaccine which is very hazardous since it attacks nucleic acids and proteins, where it suppresses the infectivity of viruses by cross link of the nucleic acid chains, leaving their antigenic structure and viral protein unaffected; hence it does not interfere with their antigenicity (Mowat and Rweyemamu, 1997 and Tizard, 2000). The results of the present study illustrated that the inactivation process of CD virus was done by using BEI of 0.003M concentration at 37˚C till complete inactivation which was obtained after 6 hours as shown in Table (1), similar findings were recorded by (Koteb and Douad, 2004 and Guirgius, 2004), conforming that complete inactivation which was obtained after 7 hours. (Mondal, et al., 2005), who stated that BEI at a lower concentration (0.4 mM) was able to inactivate the rabies virus within 30 and the routine concentration (1.6 mM) of BEI is effective in inactivating rabies virus within 18 hours.

Table (1): Inactivation of canine distemper virus using binary ethylenimine (BEI) at 37ºC.

Periods of virus inactivation / hours	Virus titer (Log10 TCID50/ml) with different molarities of BEI		
	0.001	0.002	0.003
Pre-inactivation	7.8	7.8	7.8
1	7.3	7.0	6.3
2	6.8	6.2	4.8
3	6.2	5.4	3.3
4	5.7	4.6	1.8
5	5.2	4.0	0.3
6	4.7	3.2	0
7	1.7	0	0

The recommended inactivating agent used in rabies vaccine production is beta-propiolactone (βPL), but it is a very expensive chemical and proved to be potential carcinogen. In vitro studies, it effects on nucleic acids extracted from Escherichia coli that showed that βPL produces complexes between DNA and proteins. Many studies in different experimental animals showed that βPL caused tumors at various tissue sites, and by different routes of exposure. A study showed that oral exposure to βPL caused squamous cell carcinomas in rats. When topically administered in mice and guinea pigs, skin tumors have been reported. Upon subcutaneous injection; local tumors have been observed at injection site. Also lymphomas and hepatomas have been observed in mice after intraperitoneal injection with βPL. Inhalation of βPL developed nasal cancer in experimental rats. The present obtained results revealed that complete inactivation of CD virus was obtained after 4 hours, as shown in Table (2), the using of 3 concentrations of ßPL induced complete

inactivation of canine distemper virus in final concentration ranged from 1:5000, 1:10000 and 1: 20000 at 4; 6 and 7 hours respectively, where the virus inactivation by ßPL is the result of alkyinlation of imidazol functional groups in the viral nucleic acid (Fishbein et al., 1970). Where the rabies virus was inactivated brain tissue with 1/4000 ßPL for one hour at 37˚C, while using of 1:1000 ßPL for inactivating rabies virus after 6 hours at 37˚C (Gledel et al., 1968).

Table (2): Inactivation of canine distemper virus using beta-propiolactone (ßPL) at 37ºC.

Periods of virus inactivation / hours	Virus titer (Log10 TCID50/ml) with different concentrations of βPL		
	1/5000	1/10000	1/20000
Pre-inactivation	7.8	7.8	7.8
1	4.6	5.7	6.6
2	2.4	4.5	5.7
3	1.1	3.6	4.3
4	0	2.3	3.5
5	0	1.2	2.1
6	0	0	1.2
7	0	0	0

The required time to obtain complete inactivation of rabies virus; in addition to its origin; depends on the concentration of ßPL and the process temperature. Also; as an important point of view; it was stated that ßPL acts as nucleic acid denaturant and does not denaturant the virus protein but cross link nucleic acid (plotkin et al., 2004 and OIE, 2004). Thus, there is a need to find alternative inactivating agents which are not expensive, non-toxic and easily available. This study aimed to investigate the ability of hydrogen peroxide to irreversibly inactivate canine distemper virus without affecting its antigenicity or immunogenicity in pursuit of finding safe, effective and inexpensive alternative inactivating agents. Hydrogen peroxide is a strong oxidizing agent which is proved to have a broad spectrum anti-microbial activity. Hydrogen peroxide shown the ability in maintaining immunogenic epitopes (Abd-ELghaffara, et. al., 2016). From the obtained results were found that H_2O_2 (3%) rapidly inactivated a Vero cell adapted canine distemper virus strain within 3hours of exposure without affecting its antigenicity or immunogenicity as shown in Table (3), that is similar findings were stated by (Abd-ELghaffara, et. al., 2016), confirming that H_2O_2 3% rapidly inactivated a Vero cell adapted fixed rabies virus strain designated as FRV/K within 2 hours of exposure.

No residual infectious virus was detected and the H_2O_2 inactivated vaccine proved to be safe and effective when compared with the same virus harvest inactivated with the classical inactivating agents as BEI or βPL. The results of serum neutralization test (SNT) as in Table (4), which revealed that detectable levels of the neutralizing CD antibodies appear from the first week post vaccination and reached good levels within four weeks. These levels of antibodies protected animals against challenge with virulent strain came in agreement with (CFR, 1997) that recommended serum neutralizing titer not less than 1:50 (1.7 log10) for the CD. The obtained results also agreed with (Sprino and Harris, 1983; Guirguis, 1991; Miyamoto et al., 1995) who reported that dogs were considered immune to canine distemper if their antibody titer was higher than 30.

Table (3): Inactivation Process of canine distemper virus using hydrogen peroxide at 37ºC.

Periods of virus inactivation process/ hours	Virus titer (Log10 TCID50/ml) with different concentrations of H_2O_2		
	1%	2%	3%
Pre-inactivation	7.8	7.8	7.8
1	6.6	5.7	4.6
2	5.7	4.5	2.4
3	4.3	3.6	0
4	3.5	2.3	0
5	2.1	0	0
6	0	0	0
7	0	0	0

The potency test of CDV, it was found that full protection (100 %) was obtained in all vaccinated dogs upon challenge with the corresponding virulent strain, while non vaccinated dogs showed typical symptoms of CD (Ocular and nasal discharge, fever then recovered) as shown in Table (5). From mention results we can claim that hydrogen peroxide has a potential for being used as an inactivating agent for the canine distemper virus without adversely affecting its antigenicity and immunogenicity, reducing time and cost of inactivation process. These results indicated that all currently used single vaccines

were safe and potent inducing good levels of specific antibodies (Sprino and Harris, 1983). Finally, it could be concluded that, the hydrogen peroxide H_2O_2 –inactivated canine distemper (CD) vaccine that proved to be safe, potent and effective when compared with the same virus harvest that inactivated with the classical inactivating agents as BEI and βPL.

Thus, we have reinforce the idea that H_2O_2 can replace BEI and βPL as inactivating agent for canine distemper virus to reduce time and cost of inactivation process and at the most important point of using it the challenge safe for environment.

Table (4): Neutralizing antibody titers in vaccinated puppies with different batches of inactivated CD vaccines.

Periods post-vaccination	Neutralizing antibody titers (log10/ml)		
	CD- BEI*	**CD-ßPL****	**CD- H_2O_2*****
0 WPV	0.00	0.00	0.00
1WPV	0.9	1.2	1.2
2WPV	1.2	1.5	1.7
3WPV	1.2	1.7	1.8
4WPV	1.5	1.9	1.9
2MPV	1.8	2.4	2.4
3MPV	2.1	3.0	2.4
4MPV	2.1	2.7	2.8
5MPV	1.8	2.7	2.6
6MPV	1.8	2.4	2.5

*CD vaccine inactivated with BEI. **CD vaccine inactivated with ßPL. *** CD vaccine inactivated with H_2O_2
WPV: Week post vaccination. MPV: Month post vaccination.
Antibody titers expressed in log10, protective serum neutralizing antibody 1.5

Table (5) Potency of prepared inactivated CD vaccines.

Type of tested vaccine	No. of vaccinated puppies	Challenge virus	No. of survival puppies	Observed signs
(i) CD – BEI	4		4	
(ii) CD – β PL	4	Synder- Hill virulent CDV	4	No clinical signs
(iii) CD – H_2O_2	4		4	
Control (Non- vaccinated)	4	Synder Hill virulent CDV	0	Ocular and nasal discharge, fever , then recovered

4. Conclusion

The present results reflect that use different inactivators (BEI, βPL and H_2O_2) for canine distemper virus vaccine produces sufficient level of antibodies, that time of inactivation process by H_2O_2 less than another inactivators and also the cost, that we have reinforce the idea that H_2O_2 can replace BEI and βPL as inactivating agent for canine distemper virus and at the most important point of using it the challenge safe for environment.

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