ENHANCING THE BEI- INACTIVATION RATE OF EQUINE HERPESVIRUS-1 BY FORMALDEHYDE

Fatma F.Warda*;Maha,R. Abd El-Fadile* and Ibrahim , M.M.**

*Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Postal No: 11381 P.O. Box 131.
E –mail: Svri @ idsc.gov.eg

**Central Laboratory for Quality Control of Veterinary Biologics

ABSTRACT

In order to control Equine herpes viral disease (EHV-1),completely inactivated vaccines was produce based on the use of effective inactivator and good adjuvant, so this study was carried out to determine the optimal inactivation protocol for the locally isolated strain of(EHV-1) by using alternative protocol(combination of different concentration from BEI and formalin) in comparison to the usual(EHV-1) inactivation protocol with Binary ethyeneimine (BEI) (0.008M) alone giving complete inactivation within 18-24hr(time –consume),which revealed inactivation rate 0.57 log_{10} $TCID_{50}$\2hr while the alternative protocol at concentration of (0.005\M BEI -0.0006% formalin) and (0.004\M BEI -0.001% formalin) giving complete inactivation in 8hr (time saved) with inactivation rate $2log_{10}TICD_{50}$\2hr .Virus infectivity was carried out on VERO cell line and chorioallantoic membrane of embryonated chicken SPF eggs. From these three different concentration of different compared inactivated viral fluid Alhydragel vaccines were prepared tested for safety, and potency in mice as murine model and measure immunogenicity by using ELISA test. Finally we concluded that the best ,safe and highest potent immunogenic with shorter inactivation time was the alternative protocol of (0.005\M BEI -0.0006% formalin).

INTRODUCTION

Equine herpesvirus-1 (EHV-1) is an important ubiquitous viral pathogen of both domestic and wild horse that induce serious economic losses as sporadic or stormy abortion in pregnant mares, early neonatal death, respiratory disease in young foals and myloencephalopathy **(Reed and Toribio, 2004 and Patel and Heldens, 2005).** Equine herpesvirus- 1 (EHV-1) is a virion of family Herpesviridae, subfamily Alphaherpesvirnea, genus Varicellovirus. The virus has 9 serotype from 1-5 belong to domestic horse but from 6-9 are belonging to wild life **(OIE 2012**).

Vaccination in conjunction with good animal management is the best way to prevent and control outbreaks of this disease especially inactivated vaccine in non endemic areas. Classical EHV1 vaccine virus is inactivated by binary ethelenemine which causes complete viral inactivation within 24hr with final concentration 0.008M (**Nehal et at, 2006**).

Adjuvants are modulators when used in combination with specific antigen enhance the immune response. Alhydragel enhance the immune response by slow release of the viral antigen (**Eman et al, 2005**).

Using of formalin alone as inactivator may alter the viral immunogenicity through denaturation structural deformity and binary consume long inactivation time (24hr) and danger of carcinogecity (**Brown1968**).

The aim of this study is the determination of lowest optimum BEI-formalin mixture concentration giving the shortest inactivation period to decrease the long inactivation period and to enhance the immunogenicity required for EHV-1 inactivated vaccine.

MATERIAL AND METHODS

1-Virus :

Freeze dried locally isolated EHV-1 at its second Vero cell passage (VEp_2) **(Magda et al., 2013)** was supplied by Veterinary Serum and Vaccine Research Institute (VSVRI) and used for experimental vaccine preparation.

2-Antisera

Freeze dried rabbit anti EHV-1 & 4 sera were kindly supplied by Dr. Jennet Wellington, Research follow , Dept. of Biological Science , Maquairia , Univ. , NSW Australia and used for virus identity.

3-Animals:

Sixty Albino Swiss mice 4-6 weeks old, were supplied by VSVRI and used in evaluation of safety, potency and immunogenicity of the prepared experimental vaccine preparations (**Slater et al, 1993 and Awan et al, 1990)**

4-Specific pathogen free embryonated chicken eggs (SPF-ECE):

SPF-ECE were obtained from Koum Oshiem Farm, Fayoum, Egypt and used for EI virus propagation, infetivity titration and detection of the residual infective virus in the inactivated fluids through chorioallantoic membrane (CAM) according to **(Tyerell and valentine, 1957**)

5-African green monkey kidney cells (Vero):

Vero cells were maintained and grown in Eagle's minimum essential media supplemented with 10% newly born calf serum, penicillin sodium 100 IU/ml and streptomycin 100 mg/ml and used for EHV-1 propagation, titration, and vaccine preparation.

6-Aluminum hydroxide gel:

It was obtained from Honil Limited, London, United Kingdom, Lot No. 3238402 used as adjuvant for the inactivated virus for preparation of experimental vaccine.

7-Formaldehyde solution 37-41%:

Analytic reagent grade (Fisher Chemical Company) formaldehyde 37-41% and was used in combination with BEI for inactivation of EHV-1. Its reaction was stopped by adding sodium bisulphate at final concentration 2% according to **(Farid, 1979**).

8-Binary ethyleneimine (BEI):

0.1M binary ethyleneimine (Aldrich chemical Co. LTD) was used as virus inactivator according to **Bahnemann (1990)** and its reaction was stopped by sodium thiosulphate at final concentration 2%.

9-Identity test:

Identity of the selected vaccine seed virus EHV-1VE P_2 was carried out by virus neutralization test using reference antiserum against EHV-1&4 **(Bernhardet, 1993 and OIE 2012).**

10-Titration of EHV-1:

Titration of EHV-1 was carried out on Vero cells using the microtiter technique and the titer was expressed in $log_{10}TCID_{50}$/ml according to **Reed and Muench (1938).**

11-Preparation of viral suspension:

EHV-1 seed virus of VE$\mathbf{p_2}$ was propagated on Vero cells for three successive passages (VEp_2) was titrated.

12-Virus inactivation:

1-A portion of the prepared viral suspension with a titer of 7.0 $\log_{10}$ $TCID_{50}$/ ml was inactivated by BEI at a final concentration 0.008M at 37°C for 24 hours with continuous stirring **(Nehal, 2006).** Sodium thiosulphate at final concentration 2% was added to stop the action of BEI alone.

2-Different binary-formalin concentration mixtures were added to individual portions of the virus suspension and the toxic action of residual inactivators were neutralized and tested in the target host (ECE) according to **(Soliman et al, 2013).** The prepared virus fluid was inactivated by different BEI and formalin mixture as tabulated in table (1).

Table (1): Used formulae of BEI and formalin mixture

Formula number	Used BEI and formalin concentrations	
	Formalin	BEI
1	0.0001	0.004
2	0.0001	0.005
3	0.0001	0.006
4	0.0002	0.004
5	0.0002	0.005
6	0.0002	0.006
7	0.0003	0.004
8	0.0003	0.005
9	0.0003	0.006
10	0.0004	0.004
11	0.0004	0.005
12	0.0004	0.006
13	0.0005	0.004
14	0.0005	0.005
15	0.0005	0.006
16	0.0006	0.004
17	0.0006	0.005
18	0.0006	0.006
19	0.001	0.004
20	0.001	0.005
21	0.0000	0.008

Samples were collected at intervals with suitable stoppage solution according to each inactivator and subjected for determination of the end point of complete virus inactivation **(Soliman et al, 2013)**

13-Vaccine formulation:

Each of the three chosen inactivated EHV-1 suspensions including formulae 17 (inactivated by mixture of BEI0.005 and formalin 0.0006); 19 (inactivated by mixture of BEI 0.004 and formalin 0.001) and 21 (inactivated with BEI alone) was mixed separately with 20% of Alhydrogel solution as adjuvant and stirred on a magnetic stirrer to obtain a homogenized solution to 24h at 4^{o}C to ensure virus adsorption to alhydrogel molecules. The PH was adjusted to 7.5 then thiomersal was added as vaccine preservative at final concentration 0.001% and distributed in sterile vials (2ml/vial).

14-Vaccine quality control

14.1-Sterility:

This test was performed on the inactivated virus suspension as well as the final products. Samples from them were cultured on different media to exclude bacterial, fungal and mycoplasma contaminations (**OIE, 2012)**

14.2-Safety:

14.2.1-Detection of residual virus activity:

This test was performed on the inactivated virus fluid just after inactivation process to insure complete virus inactivation. Undiluted inactivated EHV-1 was inoculated on the chorioallantoic membrane (CAM) of embryonated chicken eggs (ECE) 11-13 days then incubated at 37ºC for five days with daily examination, pock lesion shouldn't be detected on CAM **(Doll, et al 1956 and Nehal, 2006).**

14.2.2--Safety test in animals:

It was performed on final vaccine product, 3 Group of pregnant mice, was Inoculated subcutaneously (S/C) with 0.2 ml of the prepared vaccines**(Slater et. al, 1993**)

- All groups of mice were kept under observation in a good hygienic condition for two weeks.

14.3-immunogenicity and Potency test:

14.3-1-In mice:

Sixty (sero-negative) mice were divided into 4 groups (15 mice/group) were inoculated subcutaneously (S/C) with 0.2ml of inactivated EHV-1 vaccine adjuvant with Alhydragel (Each of the 4 prepared experimental EHV-1 vaccines was inoculated in a mice group) **(Slater et. al, 1993).** All mice were kept under observation under hygienic condition for two weeks.

Group (A) inoculated with vaccine prepared from formula-1 (0.005/M binary-0.0006% formalin).

Group (B)) inoculated with vaccine prepared from formula-2 (0.004/M binary-0.001% formalin).

Group (C) inoculated with vaccine prepared from formula 3 (0.008/ M binary only**).**

Group (D): was kept as a control under the same conditions of the experiments.

After one week the first three groups were inoculated with a booster dose, 2 weeks later five mice from each group were bled to collect serum samples to detect specific antibodies by indirect

ELISA. All mice groups were challenged by I/N 10 days post inoculation by 45micron of living EHV-1(log_{10} 0.7 $TCID_{50}$/ml). at least 2 mice from each group were sacrificed at intervals time (3rd-5th -7th - 9th days) post challenge and 10% of liver and lung suspension of scarified mice were inoculated on CAM of ECE to detect the role of the vaccine in the reduction of virus circulation and excretion.

15-Indirect Enzyme linked immunosorbent assay (Solid phase ELISA):

Indirect ELISA (Single-dilution) was carried according to **Crab**b **and Studdert (1993)** and **Sugiura et al. (1997).**

RESULTS AND DISCUSSION

Infectious disease control programs in conjugation with vaccination are important in maximizing the health, productivity and performance in horses (**Nehal et al, 2013 and Debra and Mureen, 2007**).

Inactivated pathogen vaccines are the most common form of equine vaccine in current use preserving their immunogenicity. Inactivated vaccines are biologically safe because they have, in theory no residual virulence **(Debra and Maureen 2007**).

Viral inactivation and its safety are the most critical issue in the production steps of inactivated vaccine, viral inactivation methods include chemical treatment as formaldehyde, binary ethelen imine and beta propiolactan **(Barteling and Woortmeizer, 2002; Martn et al., 2011 and Culbertson et al.,1956**).

This current study is a trail for determination of the optium inactivation protocol for the locally isolated EHV-1strain using combination of binary and formalin in different concentrations and provided opportunity to compare and evaluate the immunogenic capacity of formalin and BEI inactivated vaccines. Firstly, the locally isolated EHV-1 seed virus was propagated on Vero cells for further passages. Titer of the vaccine stock virus was 8.5 log10 TCID50/ ml which is recommended for preparation of inactivated vaccine (**Mumford and Bates, 1984**).

It should be noted that in the micro-titer system undiluted and 10 fold diluted samples were causing a CPE – like effect but this was due to the toxicity of formaldehyde and no virus could be propagated from these cups .according to (**Simon, et al 2004)**

Table (2) extracts that BEI-formalin mixture caused complete inactivation in shorter time than BEI alone which consume 24hr inactivation period in agreement with **Barteling et al (2004**) and clarified the best BEI-fomalin mixture concentration which was found to be 0.005\M BEI-0.0006% formalin and (0.004\M BEI-0.001% formalin and also this agree with S**oliman et al (2013).**

The tabulated results in table (3&4) and figure (1) illustrate the inactivation curve of EHV1 with different inactivation chosen formulae showing that BEI alone at concentration 0.008M induced complete inactivation in 24hr with inactivation rate 0.57 log10 TCID50/2hr. This results agree with those of **Nehal (2006) and Bahnemann (1990**) but inactivated virus with the two different chosen BEI-formalin mixture (alternative protocol) in 8hr (time-saved) with inactivation rate 2log10 TICD50/2hr agree with **Ali et al .(2009).**

Concerning the vaccine safety, there is no residual virulent virus in each of the inactivated viral fluids which was proved by absence of pock lesion on CAM in ECE as recommended by **OIE (2012)**. Moreover all inoculated mice showed neither abortion nor undesirable local or systemic reaction (roughness, loss of weight, nervous signs,death, hypersensitivity) as recommended by **Kirisawa et al (1995) and OIE (2008**).

The results of potency and immunogenicity of the prepared vaccines in mice are demonstrated by the obtained data in table (5) and figure (2) showing the ELISA serum antibody titers of different inoculated mice groups revealing that mice of group (A) which inoculated with (0.2ml) of inactivated EHV-1 vaccine inactivated with (BEI 0.005M –formalin0.0006% mixture "formula-1") exhibited the highest mean ELISA antibody titer which (1136); group (B) which inoculated with (0.2ml) of inactivated EHV-1 vaccine inactivated with(BEI 0.004M – formalin0.001% mixture "formula-2") exhibited the lowest mean ELISA antibody titer (656) and group (C) which inoculated with (0.2ml) of inactivated EHV-1 vaccine inactivated with binary alone "formula-3") showed medium mean ELISA antibody titer (710) while group (D) remained as control negative in agreement with **Bahnemann (1990) and Nehal (2006)**.

Table (6) demonstrated the duration of viral reisolation from challenged mice which the significant shorter time of inoculated mice than in control group, as in the 7^{th} day post challenge 75% of the virus was reisolated in control group but in group A and B 0% of the virus was reisolated and 25% of the virus reisolated in case of group C this result with ,in the control group 25% of the virus reisolated at 9^{th} day post challenge and this agree with **Kirisawa et al .(1995**) who stated that virus recovery from lung of infected mice occur within 9 days.

In conclusion it was clear that inactivation of EHV-1 by BEI-FA together (BEI 0.005M– formalin0.0006% mixture "formula-1") has provided a very fast inactivation process with in(8hr) without affecting the antigenic structure of the virus which will limit proteolytic destruction of antigen and increase antigen yields. It is expected that by cross-linking activity of FA the stability of the antigen (and of vaccines) and the endurance of the immune response will be favorably influenced.

Table (2): Inactivation time of EHV-1 using different inactivation protocols

Formula number	Used BEI and formalin concentrations		Inactivation time /hr
	Formalin	BEI	
1	0.0001	0.004	28
2	0.0001	0.005	26
3	0.0001	0.006	26
4	0.0002	0.004	24
5	0.0002	0.005	22
6	0.0002	0.006	22
7	0.0003	0.004	20
8	0.0003	0.005	18
9	0.0003	0.006	18
10	0.0004	0.004	16
11	0.0004	0.005	14
12	0.0004	0.006	12
13	0.0005	0.004	12
14	0.0005	0.005	12
15	0.0005	0.006	10
16	0.0006	0.004	10
17 (Formula-1)*	0.0006	0.005	8
18	0.0006	0.006	8
19 (Formula-2)**	0.001	0.004	8
20	0.001	0.005	8
21 (Formula-3)***	0.0000	0.008	24

***Formula 1**:(group17,inactivated by mixture of BEI0.005 and formalin 0.0006).

****Formula 2**:(group19, inactivated by mixture of BEI 0.004 and formalin 0.001)

*****Formula 3**:(group 21, inactivated with BEI alone).

Table (3): Inactivation kinetics of EHV-1 by different inactivation chosen protocols at 37^0C and PH 8.

Formula No.	EHV-1 titer (log10 $TCID_{50}$/ml) on hours post starting of the inactivation(hr)												
	***0hr**	**2hr**	**4hr**	**6hr**	**8hr**	**10hr**	**12hr**	**14hr**	**16hr**	**18hr**	**20hr**	**22hr**	**24hr**
1*	7.0	5.0	3.0	1.0	0	0							
2**	7.0	4.8	2.9	2.1	0	0							
3***	7.0	5.8	5.3	5.0	4.8	4.3	3.8	3.3	2.8	2.3	1.8	1.0	0
untreated virus	7.0	7.0	6.8	6.8	6.4	6.4	6.1	6.0	5.9	5.8	5.6	5.5	5.5

***Formula (1):** (0.005\M binary-0.0006% formalin)

**** Formula (2):** (0.004\M binary-0.001% formalin)

*****Formula (3):** 0.008/ M binary onl**y**

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Table (4): EHV-1 Inactivation rate (log/2hr) by Binary and best alternative protocol (Binary-formalin mixture of EHV-1 expressed by $log_{10}TCID_{50}$/ml

Formula No.	Inactivation rate
Formula (1&2)	2.0log/2hr
Formula (3)	0.57log/2hr

Formula (1&2): (0.005\M binary-0.0006% formalin) **Formula (3):** 0.008/ M binary only

Table (5): EHV-1 ELISA antibody titer of mice inoculated with inactivated EHV-1 vaccine on two weeks post vaccination.

Mice No.	Group(A)	Group(B)	Group(C)	Group(D)
1	1300	600	650	0
2	1200	680	700	0
3	1100	650	700	0
4	1050	650	750	0
5	1000	700	750	0
Mean antibody titer	1136	656	710	0

Group (A): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 1)

Group (B): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 2)

Group (C): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary alone (formula 3)

Group (D): left as control

Table (6): EHV -1 re-isolation from inoculated mice after challenge

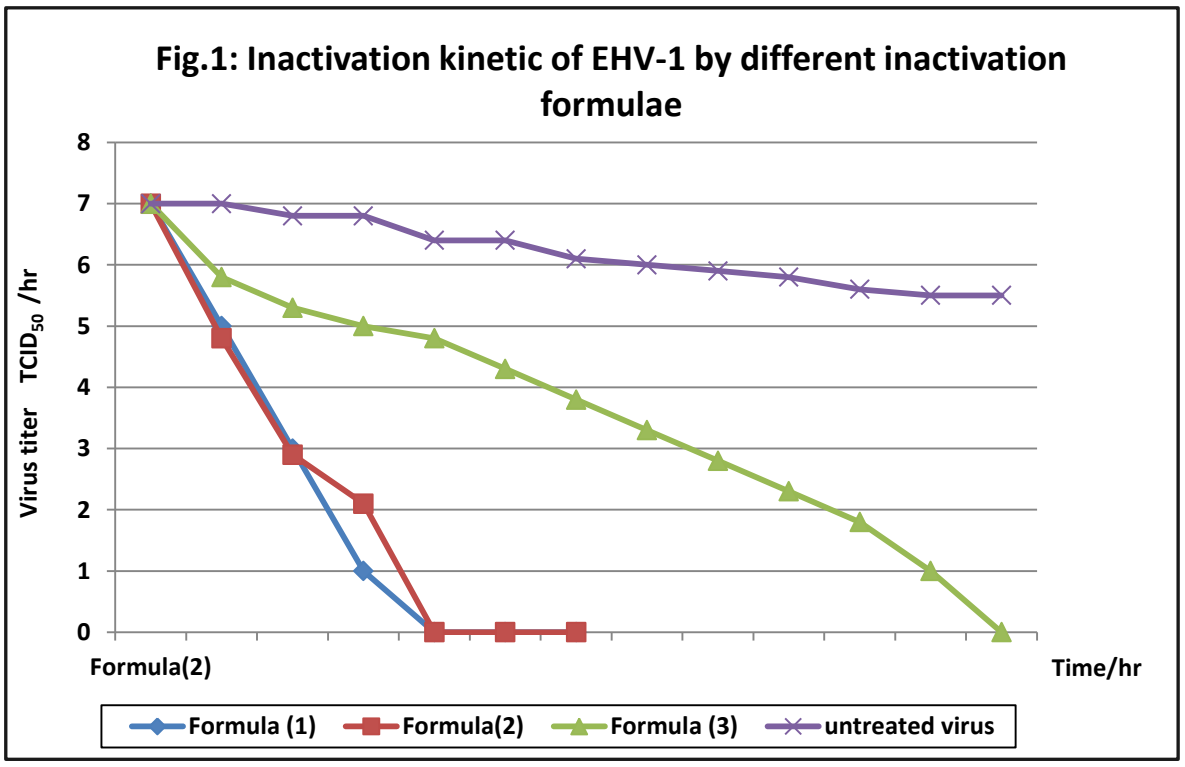
Animal group	Days post challenge	% of virus re isolation
Group (A)	3	100
	5	75
	7	0
	9	0
Group (B)	3	100
	5	75
	7	0
	9	0
Group (C)	3	100
	5	75
	7	25
	9	0
Group (D)	3	100
	5	100
	7	75
	9	75

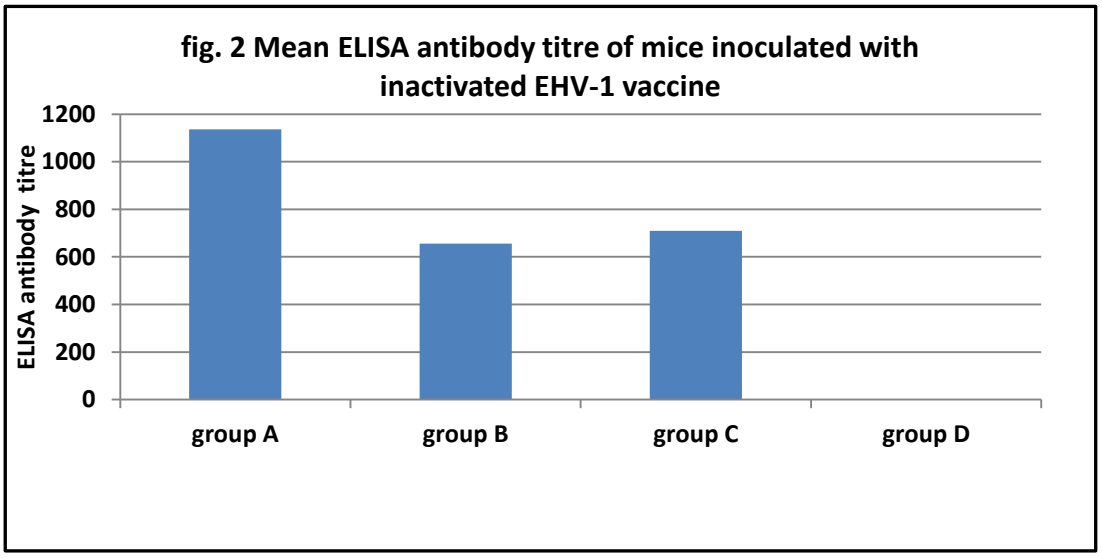
Group (A): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 1)

Group (B): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 2)

Group (C): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary alone (formula 3)

Group (D): left as control





Group (A): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 1)

Group (B): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary –formalin mixture (formula 2)

Group (C): inoculated with 0.2ml of inactivated EHV-1 vaccine inactivated with binary alone (formula 3)

Group (D): left as control

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تحفيز تثبيط فيروس بالبينارى بإضافة الفورمالدهيد

فاطمة فاضل وردة ,مها رأفت عبد الفضيل , ماجد إبرهيم منير

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**المعمل المركزى للرقابة على المستحضرات الحيوية البيطري

الملخص العربى

للوقاية من مرض الإجهاض المعدي الفيروسي للخيول يتم إنتاج لقاحات مثبطة باستخدام مثبط فعال ومحفزجيد .

أجريت هذة الدراسة لتحديد أفضل بروتوكول لتثبيط العترة المحلية المعزولة لفيروس هربس الخيول باستخدام بروتوكول بديل يتكون من مزيج من تركيزات مختلفة من مادة بينارى إيثيلين إيمين مادة الفورمالين و مقارنته بالبروتوكول المعتاد استخدامه (بينارى عند تركيز ٠.٠٠٨) مول لمدة ٢٤ ساعة بمعدل تثبيط($0.57\ \log_{10}$ $TCID_{50}$) لكل ساعتين (مستهلك للوقت), بينما البروتوكول البديل عند تركيزيه (بينارى ٠.٠٠٥ مول و الفورمالين ٠.٠٠٠٦%) ' (بينارى مول٠.٠٠٤ و الفورمالين ٠.٠٠١%) أعطى تثبيط كامل بعد ٨ ساعات (موفر للوقت) بمعدل تثبيط ($2\ \log_{10}$ $TCID_{50}$)لكل ساعتين .تم قياس عيارية و قدرة الفيروس علي إحداث عدوى فى البيض المخصب الخالى من المسببات المرضية بالحقن فى الغشاء اللقانقى(CAM) . تم تحضير ثلاث لقاحات من السائل الفيروسى المثبط باستخدام الثلاث تركيزات السابق ذكرها بإضافة الجل كمادة محفزلكل منها على حدى . تم إجراء إختبارات الاما ن و النقاوة للقاحات المحضرة والفاعلية فى الفئران . تم قياس الكفاءة المناعية بإستخدام إختبارر الاليزا وعلى هذا يمكن القول أن البروتوكول البديل(بينارى ٠.٠٠٥ مول و الفورمالين ٠.٠٠٠٦%) ان الافضل و الامن و الاعلى كفاءة مناعية و الاكثر توفيراً للوقت .

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