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VALIDATION OF TREATMENT OF COMBINED AVIAN ENCEPHALOMYELITIS AND FOWL POX VACCINE WITH CHLOROFORM ON TITRATION OF AVIAN ENCEPHALOMYELITIS VIRUS IN CHICKEN EMBRYOS

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ABSTRACT

Validity including conductivity and specificity of Avian Encephalomyelitis (AE) virus infectivity titration of combined AE-Fowl Pox (FP) vaccine in 5-7 day-old embryonated chicken eggs (ECEs) has been faced almost with nonspecific embryos mortality due to FP virus infectivity. The present study was performed to establish whether or not the treatment of such vaccine with chloroform to eliminate the pathogenicity of FP virus before titration guarantees a validated accurate AE virus titer. The study goal was achieved throughout statistical analyzed titration data of AE virus infectivity of three commercial batches of AE vaccine and seven commercial batches of combined AE-FP vaccine in chicken embryos before and after treatment with chloroform. The results obtained from titration tests showed that treatment of AE vaccine batches with chloroform had no significant effect on its infectivity titers comparing with those titers which exhibited by the vaccine batches before treatment, and demonstrated that treatment of combined AE-FP vaccine batches with chloroform significantly affected the conductivity and specificity of AE virus infectivity titration. The present study proved the validity of using chloroform for treatment of combined AE-FP vaccine before AE virus titration, and using of distilled water for reconstitution of AE-FP vaccines instead of normal saline solution before dilutions made for titration.

INTRODUCTION

Avian Encephalomyelitis (AE) is an infectious viral disease of poultry in young chickens, turkeys, pheasant and quail. It is characterized by clinical signs of central nervous system disorder with high morbidity and variable mortality. Therefore, vaccination of breeders is an important preventive measure to control the disease in progenies **(AL-aboratory Manual for the Isolation, Identification and characterization of Avian Pathogens, 1998)**. Live AE

vaccine of attenuated strain 1143 is mostly used to control the disease worldwide **(Calnek *et al.,* 1961)**.The need to titrate the infectivity of AE vaccine virus as a batch release potency test makes an accurate method for virus infectivity titration is very important. One system for assay of virus is to inoculate embryonated chicken eggs (ECEs) (obtained from a susceptible flock) via the yolk sac when 5-7 days of age, allow embryos to hatch, and observe chicks for signs of disease during the first few days **(Bülow, 1965)**. On the other hand, infectivity titer of Fowl Pox (FP) vaccine virus propagated in chicken embryos is assessed by inoculation via the Chorion-allantoic Membrane (CAM) in 10-12 day old developing chicken embryos from specific pathogen free-flock, and infectivity titer of fowl pox vaccine virus propagated in cell cultures is determined by inoculation of primary cell cultures of chicken embryo fibroblasts, and 5-7 day old ECEs after inoculation, embryos are examined for pock lesions on CAMs, and cell cultures are examined for cytopathic effect **(Cox,1980, Senne, 1998, Tripathy and Reed,1998).** Intra-yolk inoculation of five to seven days old ECEs with a combined AE + FP vaccine causes few early embryonic deaths due to the pathogenic effect of FP virus in such young embryos **(Tripathy and Reed, 1998)** and actually this interferes with titration of AE virus. So, it is necessary to eliminate the fowl pox virus of the combined AE-FP vaccine before titration of AE virus. To achieve this goal, one of the following two methods is recommended to be used; (1) neutralization of FP virus with monospecific antiserum or (2) inactivation of FP virus with chloroform. The use of monospecific antiserum to neutralize the virus is strongly indicated. But if neutralization is not properly achieved due to difficulty to neutralize the cell associated-virus such in case of FP virus in the combined AE-FP vaccine, the treatment with chloroform may be illustrated most suitable. Chloroform is a lipid solvent that inactivates the enveloped viruses such as fowl pox virus **(Randall *et al.,* 1964)** and not affects the naked (non-enveloped) virus such as AE virus **(Bülow, 1964, Hoekstra, 1964).** Treatment with chloroform could be used for eliminating the live enveloped virus vaccine (Newcastle disease, infectious bronchitis, Marek's disease, laryngeotrachitis, and fowl pox vaccines) before testing for possible contamination with non-enveloped viruses (AE and infectious bursal disease viruses, and avian adenovirus 1), **(Egyptian Standard Regulations for Evaluation of Veterinary Biologics,2009**).Consequently, the present study was undertaken to validate treatment of combined AE-FP vaccine with chloroform before titration of AE virus throughout infective dose fifty in ECEs assay (titration) of different

batches of commercial AE vaccine and combined AE-FP vaccine before and after chloroform treatment.

MATERIAL AND METHODS

Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECE):

They were obtained from SPF Egg Farm, Koum Osheim, Fayoum Dist., Egypt and it was used as follows:

1. Five to seven day old SPF-ECEs were used for titration of AE virus in AE vaccine (Three batches) and combined AE+FP vaccine (seven batches) before and after treatment with chloroform.
2. Ten to twelve day old SPF-ECEs were used for titration of FP virus in combined AE+FP vaccine (Seven batches).

Vaccines:

- Samples of three batches of commercial live attenuated AE vaccine (3 vials per batch).
- Samples of seven batches of commercial live attenuated combined AE-FP vaccine (3 vials per batch).

Chloroform:

Lot#STBG25067V meets analytical specification of DAB9, BP, 99-99.4% (GC) Sigma-Aldrich Company.

Treatment of AE vaccines with chloroform before inoculation into SPF-ECEs for titration:

Each 1000 doses vaccine vial was reconstituted in 10 ml of saline solution, or in distilled water (in some experiments). Equal volumes of chilled chloroform and reconstituted vaccine pool (3 vials/pool/ vaccine batch) were thoroughly mixed for 2 minutes then centrifuged at 3000 rpm for 30 minutes at 4^{o}C. The upper layer (aqueous phase) of the centrifuged mixture was separated (harvested) and mixed well before preparation of its serial ten-fold dilutions and inoculation into five to seven day old SPF-ECEs via yolk sac for titration **(Egyptian Standard Regulations for Evaluation of Veterinary Biologics, 2009).**

Embryo Infective Dose fifty assay (titration):

1. Infectivity of AE vaccine virus was tested by titration in ECEs as follows:

Serial ten-fold dilutions between 10^{-1} and 10^{-6} of the vaccine (before and after treatment with chloroform were prepared in sterile saline, pH 7.2 containing antibiotics, and dilutions 10^{-2}

through 10^{-6} were inoculated into five to seven day-old SPF-ECEs via yolk sac (Ten eggs/ dilution). fifteen ECEs of the same age and source were maintained as negative controls. Eggs were incubated at 37ºC for 12 days with daily candling. Embryonic deaths during the first 48 hours after inoculation were disregard. On day 12 of the incubation, the surviving eggs of each dilution and control eggs were kept in separate containers and allowed to hatch. Three days later, dead embryos and embryos that unable to hatch were examined for evidence of the vaccine strain infection such as the muscular atrophy that could be caused by the Van Roekel strain of AEV and the hatched chicks for clinical signs of AE (paralysis and ataxia). Dead embryos, un-hatched eggs, paralyzed and ataxic chicks were counted as positive evidence of the vaccine virus in each group **(American Code Federal Regulations, 2016**). In a valid test, at least 75 % of negative control embryos should be hatched with no evidence of AEV infection. EID_{50} per dose was calculated according to **Reed and Muench (1938)**.

2. Infectivity of FP vaccine virus was tested by titration in ECEs as follows:

Serial ten-fold dilutions (10^{-1} through 10^{-6}) of the vaccine were prepared in sterile saline, pH 7.2 containing antibiotics, and dilutions 10^{-2} through 10^{-6} were inoculated into 10-12 day-old SPF-ECEs via CAM, (5 eggs/dilution).The tested eggs were incubated at 37ºC for 7 days with daily candling. Embryonic deaths occurring in ECEs during the first 24 hours were disregard, and embryos that die later and those that survive up to the day 7 of incubation were examined individually for FPV pock lesions on CAMs **(American Code Federal Regulations, 2016)**. EID_{50} per dose was calculated according to **Reed and Muench (1938**).

Statistical analysis:

Titration data generated by AE virus of three batches of AE vaccine and seven batches of combined AE-FP vaccine before and after treatment with chloroform were subjected to statistical analysis to evaluate the significance of the differences recorded between the titers before and after treatment. The method for assessment of statistical significant and non-significant was T-Student Test **(Sendecor, 1971)**.

RESULTS

Table (1) demonstrates the results of titration of AE vaccine virus in three batches of AE vaccine and seven batches of combined AE-FP vaccine in ECEs before and after treatment with chloroform, titers of 3.6, 3.8 and 3.4 Log_{10} EID_{50} per dose and 3.1, 3.7 and 3.3 Log_{10} EID_{50} per dose were scored by batches E1, E2, and E3 of AE vaccine before and after treatment with chloroform respectively, and titers of 3.0, 3.7, 3.9, 5.3, 4.3, 3.9, and 4.1 Log_{10}

EID_{50} per dose and 1.7, 3.2, 3.0, 3.7, 3.3, 3.0, and 3.0 Log_{10} EID_{50} per dose were scored by batches EP1, EP2, EP3, EP4 EP5, EP6 and EP7 of combined AE-FP vaccine before and after treatment with chloroform respectively. The subtracted values of AE virus titers (EID_{50} per dose) before and after treatment of AE vaccine batches E1, E2, and E3 with chloroform were 0.5, 0.1, and 0.1 respectively, before and after treatment of combined AE-FP vaccine batches EP1, EP2, EP3, EP4 EP5, EP6 and EP7 chloroform were 1.3, 0.5, 0.9, 1.6, 1.0, 0.9, and 1.1 respectively. Thereafter, FP virus infectivity titers of batches EP1, EP2, EP3, EP4 EP5, EP6 and EP7 of the combined AE-FP vaccine before treatment with chloroform were $\geq$4.2, 3.6, 3.6, $\geq$4.2, $\geq$4.2, 3.8, and 4.0 Log_{10} EID_{50} per dose respectively as illustrated by (Table 2) with the relationship between these infectivity titers of FP vaccine virus and subtracted values of AE virus titers (EID_{50} per dose) before and after treatment of combined AE-FP vaccine batches with chloroform. Pattern of embryonic deaths after inoculation of 5-7 day-old ECEs with combined AE-FP vaccine and AE vaccine batches before and after treatment with chloroform was studied. Embryonic deaths began at 4^{th} day post inoculation (DPI) and continued to 11^{th} DPI with combined AE+FP vaccine batches, while no deaths were recorded by chloroform treated combined AE-FP vaccine batches as well as AE vaccine batches. Embryonic deaths scored by the lowest dilutions (10^{-2}, 10^{-3} and 10^{-4}) were higher and faster than that scored by the highest dilutions (10^{-5} and 10^{-6}) of combined AE-FP vaccines (un-tabulated results).

Also, as shown by (Table3) AE vaccine batch (E1) and combined AE-FP vaccine batch (EP2) reconstituted with normal saline solution and treated with chloroform, and reconstituted with distilled water and treated with chloroform were exhibited AE virus infectivity titers of (3.1 and 3.2 Log_{10} EID_{50} per dose) and (3.6 and 3.7 Log_{10} EID_{50} per dose) respectively. Statistical analysis of titration data generated by AE virus of three batches of AE vaccine and seven batches of combined AE-FP vaccine before and after treatment with chloroform showed no significant differences ($P \leq 0.05$) between AE virus titers of the three batches of single AE vaccine pre–treatment and post-treatment with chloroform, and significant differences ($P \leq 0.05$)between AE virus titers of the seven batches of combined AE-FP vaccine pre–treatment and post-treatment with chloroform.

DISCUSSION

Strict batch by batch release potency test for the live viral poultry vaccines (the vaccine virus infectious dose fifty titer assay in its corresponding embryonated eggs or cell cultures) is routinely undertaken by Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, and Cairo Dist., Egypt. The necessity for elimination of one or more of the vaccine viruses of combined live viral poultry vaccine might be mentioned as a point of definite practical disadvantage for titration of such type of vaccines. This study was carried out to gain statistical data of indeed privilege on influence of FP virus on titration of AE virus in combined AE-FP vaccine batches in five to seven day-old SPF-ECEs, and validation of treatment of combined AE-FP vaccine batches and AE vaccine batches with chloroform **(Egyptian Standard Regulations for Evaluation of Veterinary Biologics, 2009**) on the infectivity titer of AE virus in chicken embryos. This particular aspect has been covered throughout, (1) titration of three commercial batches of the live AE vaccine before and after treatment with chloroform to get the answer on the question of logic, how far the process of treatment of AE vaccine with chloroform is statistically affected the vaccine virus titers. The results obtained from the experiment declared that no significant statistical differences had recorded between the virus titers before and after treatment with chloroform of the three batches of AE vaccine; the virus infective titers were decreased between 0.1 and 0.5 Log_{10} EID_{50} per dose after treatment with chloroform, and (2) titration of seven commercial batches of combined AE-FP vaccine before and after treatment with chloroform to assess statistically the degree of interference of FP virus of combined AE-FP vaccine with AE virus infectivity titration in ECEs; the results obtained from the experiment concluded that significant statistical differences had recorded between the virus titers before and after treatment with chloroform of the seven batches of AE vaccine; the virus infectivity titers increased between 0.5 and 1.6 Log_{10} EID_{50} per dose without treatment with chloroform, and there was a relationship between the high scores of FP virus infectivity titers and AE virus infectivity titers of combined AE-FP vaccine. Infectivity of AE virus was shown to remain unaffected by treatment with chloroform **(Butterfield *et al.*, 1969**). Pattern of embryonic deaths after inoculation of five to seven day-old ECEs with combined AE-FP vaccine and AE vaccine batches before and after treatment with chloroform summarized that embryonic deaths were recorded in chicken embryos inoculated with combined AE+FP vaccine batches, while no

deaths were observed by chloroform treated combined AE-FP vaccine batches as well as single AE vaccine batches. This pattern of embryonic deaths which accompanied the inoculation of ECEs with combined AE-FP vaccine batches attributed to the pathogenic effect of fowl pox strain of the vaccine on five to seven days old ECEs (**Cox, 1980, Senne, 1998, Tripathy and Reed, 1998)**. So, embryonic deaths scored by the lowest vaccine dilutions (10^{-2}, 10^{-3}, 10^{-4}) were higher and faster than that scored by the highest dilutions (10^{-5}, 10^{-6}). On the other hand, it's expected that intrayolk inoculation of single AE vaccine batches on five to seven day old ECEs did not cause any embryonic deaths along the observation period as the age of the inoculated ECEs is the age of choice for AE virus **(Hoekstra, 1964 and Bülow**, **1965)**. Moreover, absence of embryonic deaths of the inoculated ECEs with chloroform treated combined AE-FP vaccine batches at the same age (5-7 days old) indicates that chloroform inactivates the fowl pox strain of the vaccine consequently it eliminates the pathogenic effect of these chloroform treated vaccine batches **(Bülow**,**1964**). Also, results demonstrated by (Table 3) appears possible improvement of AE virus titers of AE vaccine and AE-FP vaccine by reconstitution with distilled water instead of the normal saline solution before treatment with chloroform. Distilled water seems to be disrupt the lyophilized tissue flaks of the vaccine as well liberate moreofthecell associated virus.In conclusion,undoubtedly, titrationof Avian Encephalomyelitis (AE) virus of combined AE- Fowl Pox (FP) vaccine in five to seven day-old Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECEs) without elimination of FP vaccine virus seemed to be not accurate due to the embryonic deaths that caused by FP virus, so that It is necessary to eliminate FP virus of the combined AE-FP vaccine by treatment with chloroform before going for titration of AE virus in ECEs.

Table (1): AE virus-Egg infective dose fifty (EID_{50}) titers of AE vaccine and Combined AE-FP vaccine batches before and after treatment with chloroform.

Type of vaccine	Batch code	AE virus titer per dose (- log_{10})		AE virus titer difference* (- log_{10})
		Before treatment with chloroform	After treatment with chloroform	
AE vaccine	E1	3.6	3.1	0.5
	E2	3.8	3.7	0.1
	E3	3.4	3.3	0.1
AE-FP vaccine	EP1	3.0	1.7	1.3
	EP2	3.7	3.2	0.5
	EP3	3.9	3.0	0.9
	EP4	5.3	3.7	1.6
	EP5	4.3	3.3	1.0
	EP6	3.9	3.0	0.9
	EP7	4.1	3.0	1.1

***Subtracted values of AE virus titers EID_{50}/dose before and after treatment with chloroform.**

Table (2): Relationship between FP virus titer (EID_{50}) and AE virus titers difference of combined AE-FP vaccine batches before and after treatment with chloroform.

Type of vaccine	Batch Code	AE virus titer difference * (- log_{10})	FP virus titer (EID_{50}) per dose (- log_{10})
AE-FP vaccine	EP1	1.3	$\geq$ 4.2
	EP2	0.5	3.6
	EP3	0.9	3.6
	EP4	1.6	$\geq$ 4.2
	EP5	1.0	$\geq$ 4.2
	EP6	0.9	3.8
	EP7	1.1	4.0

*** Subtracted values of AE virus titers EID_{50}/dose before and after treatment with chloroform.**

Table (3): AE virus-Egg infective dose fifty (EID_{50}) titers of AE vaccine and combined AE-FP vaccine reconstituted with normal saline solution or distilled water and treated with chloroform.

Type of vaccine	Batch code	AE virus titer(EID_{50}) per dose (- log_{10})		
		Treated with chloroform		Untreated with chloroform
		Reconstitution with saline	Reconstituted with distilled water	
AE vaccine	E1	3.1	3.6	3.6
AE-FP vaccine	EP2	3.2	3.7	3.7

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تقييم معالجة لقاح الأرتعاش الوبائى وجدرى الطيور المركب بالكلوروفورم على كفاءة معايرة فيروس الأرتعاش الوبائى فى أجنة الدجاج

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الملخص العربى

تقييم خصوصية وقوة عترة فيروس الارتعاش الوبائى (AE) الموجودة فى اللقاح المركب لفيروس جدرى الطيور AE-FP بقياس عيارية فيروس AE وذلك عن طريق حقن هذا اللقاح على أجنة البيض الخالى من المسببات المرضية عمر 5-7 أيام. وقد تبين أن هذه الطريقة تواجه مشكلة وهى حدوث نفوق غير متخصص للبيض المستخدم فى معايرة عترة AE بسبب التأثير الممرض لعترة فيروس FP الموجود فى اللقاح. وقد اجريت هذه الدراسة لإستبيان ما إذا كان معالجة هذا اللقاح بمادة الكلوروفورم للتخلص من التأثير الممرض لعترة فيروس FP قبل إجراء المعايرة طريقة تصلح للاستخدام فى المعايرة وتعطى نتائج دقيقة. وقد تحقق هذا الهدف بفضل من الله تعالى وذلك عن طريق التحليل الاحصائى لبيانات عيارية عدد ثلاثة تشغيلات من اللقاح الاحادى AE وسبعة تشغيلات من اللقاح المركب AE-FP وذلك بعد حقنهم على أجنة البيض قبل وبعد معالجتهم بمادة الكلوروفورم. وقد أظهرت نتائج اختبارات المعايرة أن معالجة اللقاح الاحادى AE بالكلوروفورم ليس له تأثير معتبر على عيارية اللقاح مقارنتاً بعيارية اللقاح قبل المعالجة . كذلك أظهرت النتائج أن معالجة اللقاح المركب AE-FP بمادة الكلوروفورم تؤثر تأثيراً معتبر على عيارية فيروس AE للقاح مقارنتاً بعيارية عترة AE قبل معالجة اللقاح. وقد أثبتت الدراسة الحالية صلاحية استخدام مادة الكلوروفورم لمعالجة اللقاح المركب AE-FP قبل معايرة عترة AE للقاح وكذلك يفضل حل اللقاح فى الماء المقطر بدلاً من محلول الملح الطبيعى قبل إجراء التخفيف المتسلسل للقاح ومعايرته.