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IMMUNE RESPONSE OF FOOT AND MOUTH DISEASE VIRUS TYPE A87/IRN INACTIVATED VACCINE BY GAMMA IRRADIATION ON GUINEA PIG IN IRAN*

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Abstract – Foot and Mouth Disease (FMD) is the most contagious disease of cloven-hoofed animals, causing significant economical losses in livestock animals. In this study FMD Virus type A87/IRN was cultured and multiplied on BHK21 cells. The FMD virus was titrated by the Tissue Culture Infection $Dose_{50}$ method ($TCID_{50}$ /ml), the virus titration was $10^{7.5}$. The FMD virus samples were irradiated and inactivated by gamma ray from a ^{60}Co source at -20 °C. A safety test was done by the IBRS2 monolayer cell culture method, also antigenicity of irradiated and un-irradiated virus samples was studied by Complement Fixation Test. The Dose/Survival curve for irradiated FMDV was drawn. According to the curve and D_{10} Value factor, the optimum dose range for the inactivation of FMDV type A87/IRN and unaltered antigenicity was obtained (40-44 kGy). The inactivated virus samples by irradiation and ethyleneimine (EI) were formulated respectively as a vaccine with Al (OH) 3 gel and other substances. The vaccines were inoculated to Guinea pigs and the results of the Sero-Neutralization Test for both the normal vaccine and radio-vaccine showed protective titre after 8 months. The potency test of the inactivated vaccines was done, Protective $Dose_{50}$ Value (PD_{50} Value) of the vaccines were calculated to be 7.06 and 5.6 for the inactivated vaccine by EI and gamma irradiation respectively.

Keywords – Radio vaccine, Foot and Mouth Disease virus, gamma irradiation

1. INTRODUCTION

Foot and Mouth Disease (FMD) is the most contagious disease of cloven-hoofed animals. The symptoms blister in the mouth, especially on the tongue, occasionally on the nose (in pigs), and on the coronary bands of the hoofs. In unvaccinated herds, the mortality in adult animals is usually negligible, but it may be considered in young animals. Milk production decreases significantly and animals used for traction can become useless [1 & 2]. FMD virus is a genus of the Picornaviridae family which is called Aphtovirus. This genus contains seven serotypes: A, O, C, Asia1, and three types of South African Territory: SAT1, SAT2 and SAT3. Serotype A viruses are the most variable viruses having more than 30 subtypes [1]. FMD virus is approximately 25nm in diameter [3]. The virus is highly labile, at temperatures above 50 °C, at very low salt concentrations, or when the pH drops below six [4]. Vaccination is the most important control and the eradication strategy for animal virus diseases. The different processes for preparing vaccines against viral diseases are comprised by a sequence of steps and differ in accordance with

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particular virus and selected processes, and may be classified as follows: virus production, virus inactivation and vaccine formulation. Ionizing radiation has been recognized as a powerful and convenient technique for the processing of various materials [5]. In this study, radiation technology is incorporated in the principal steps of a viral vaccine preparation. The inactivation of viruses by ionizing radiations has been studied by Pollard, Ginoza, and Dertiger [6, 7 & 8].

Virus inactivation and safety tests are the most critical steps in FMD vaccine production. Concerning virus inactivation, the known methods are based on the chemical action of some substances such as acethylenimine, betapropiolactone, etc. In such processes, the viral suspension should be kept at room temperature or at higher temperatures for 24-48 hours [1]. Inactivation by chemical substances have some residues in the final products, also some are toxic, while others make allergic responses in the animals. It is also possible that some viruses escape during chemical inactivation methods [1]. Irradiated inactivated viruses have been reported to retain most of their antigenicity [5]. The viral inactivated vaccines by ionizing radiation are safe and are not toxic; also the virus particles cannot escape from inactivation by gamma irradiation [5, 6 & 7].

2. MATERIAL AND METHODS

a) Cell culture and virus multiplication

Foot and Mouth disease virus type A87/IRN was multiplied on BHK21 (Baby Hamster Kidney cells) by Earls media and 0.5% bovine serum which was treated by PEG 6000 at 37 $^{\circ}$C incubator without CO_2 for 12 hours. The Cytopathic Effect (CPE) was then observed on BHK21 cells as lyses and separated cells. The virus suspensions were centrifuged at 600 ×g for 15 minutes, and supernatants stored at -70 $^{\circ}$C [1 & 9].

b) Virus titration

Tissue Culture Infection $Dose_{50}$ /ml ($TCID_{50}$ /ml) refers to virus particles per ml which can produce CPE at 50 percent inoculated cells, calculated by the Reed & Meunch method [10].

c) Inactivation of FMDV by gamma irradiation and ethylenimine

In this study a gamma cell instrument Issledovapel, PX-30 model with a dose rate: 0.551 Gy/sec and activity: 3652 Ci was used. Different doses of gamma ray: 10, 20, 25.35,40,45 and 50 kGy were used for the irradiation of virus samples. For each dose of gamma ray, 10 vials (each vial containing 5 ml of the virus) were irradiated. The irradiation was done at a low temperature of about -20 C. Some of the virus samples were treated by 0.035 mol/lit ethylenimmine at 30 $^{\circ}$C over 20h for virus inactivation. Also, the inactivation was stopped by 0.04 mol/liter sodium thiosulphate [1, 5, 6 & 7].

d) Safety test and complement fixation test

Infectivity of irradiated virus samples by different doses of gamma ray was determined by cell culture methods. All of the irradiated virus samples were inoculated on IBRS2 cells; also, their titration was obtained by $TCID_{50}$ methods. The antigenecity of irradiated and unirradiated virus samples were studied by the Complement Fixation Test (CFT) [11, 12 & 13].

e) Vaccine Formulation

The inactivated virus samples were treated by 6/1000 chloroform, absorbed on AL $(OH)_3$ gel and formulated by saponin, glycine, phenol red and phosphate buffer. Therefore two kinds of the vaccines against FMD Virus type A 87/IRN were prepared, the first one which is inactivated by BEI as normal

vaccine (NV), and the second one, inactivated by gamma irradiation, as radio vaccine (RV) [1 & 14].

f) Immune response of the inactivated vaccines by gamma irradiation and ethylenimine on Guinea Pigs

In the first stage, 35 guinea pigs having 450-500 g body weight were selected and divided into 7 groups, each group containing five animals. Three routes of inoculation were used for two types of the vaccines which include: 1) subcutaneous & infra-auxillary, 2) Intra peritoneal, 3) Subcutaneous on back foot, also one control group (unvaccinated) [14-18]. Three groups were inoculated by NV; the other three groups were inoculated by RV and one group as control group (non-vaccinated).

In the second stage 30 guinea pigs in 3 groups (each group contains ten animals) were vaccinated by the radio vaccine, normal vaccine and there was an unvaccinated group. Each Guinea Pig was inoculated with 0.5 ml of the vaccines subcutaneously & infra auxillary, the booster dose was injected on the 21_{st} day and the animals were bled 14 days after the booster. The last 3 groups of guinea pigs were bled after 1, 3, 6 and 8 months (19, 20 & 21). The blood was left overnight at 4 °C and the tubes centrifuged in a bench centrifuge at 600×g, 15 minutes to separate the sera. The complement factors of the sera were inactivated at 56 °C for 30 minutes. The Sera were then tested for the presence of antibodies against the FMD virus by the serum neutralization test (SNT) [10].

g) Serum Neutralization Test (SNT)

The sera were diluted in Eagle's maintenance medium in a 2-fold dilution starting from 1:4 to 1:128. Two hundred µl (100µl/well) of the diluted sera were used in two wells of a U96 micro-plate. The SN test was done according to the Kraber protocol [17]. Any well in which the virus was neutralized and the cells remain intact was considered a positive well and other wells in which the virus was not neutralized and CPE could be shown were considered negative wells. The titre of SNT is the final dilution of serum that could neutralize the virus in the serum-virus mixture at 50% end-point.

h) Challenge study for protective response

The challenge study was done in the animal laboratory of the Nuclear Research Center for Agriculture and Medicine in Iran. The adapted guinea pigs, by FMD serotype A87/IRN at the fifth passage level, were prepared in a 5 ml suspension after clarification, passaged freshly in two guinea pigs by intra dermoplantar tunneling route, and the pads collected from the animals that showed the primary lesions were used as a source of challenge virus. Approximately 1g of pad materials was triturated in 5ml Eagl's medium to obtain a homogenous suspension, centrifuged at 3000 rpm, 15 minutes and the supernatant was collected and used as the neat virus [15]. A challenge experiment in guinea pigs was carried out following the method of Lucam et al [19]. Both types of vaccines (inactivated by Gamma-irradiation and Ethylenimine) were diluted in a carbonate-bicarbonate buffer pH=8.2 in a 2-fold dilution from 1:1 to 1:16. Each dilution of the vaccines was inoculated to each group of animal via subcutaneously & infra auxillary 0.5ml for every dose. A booster was given with the same dose of vaccine on the 21_{st} day. After two weeks 10 groups of vaccinated animals with two types of vaccines, and 2 groups of unvaccinated (control) animals, each group containing 5 animals, were challenged with a guinea pig adapted virus (sixth passage) at 100 ID_{50}(Infection $Dose_{50}$) [15]. The animals were checked after 4-5 days of the challenge test for the development of primary and secondary lesions. If the virus generalizes in the guinea pig's body, the vesicles on the un-inoculated feet and the tongue are observed for a positive reaction to the FMDV infection in guinea pigs. The observations were recorded and the Protection $Dose_{50}$ (PD_{50}) was calculated in both of the two kinds of vaccinated groups and the control groups using Karber's method [17]. The virus Generalization has been described by Lucam et al [14, 15, 17 & 19].

3. RESULT

Table 1 indicates the virus titration for irradiated and un-irradiated samples after safety test and Fig. 1 also shows the Dose/survival curve for the irradiated samples. According to Table 1 and Fig. 1, the virus titration was decreased gradually by increasing the gamma ray doses; also, the D_{10} value factor (dose of gamma ray which can decrease one logarithmic cycle of virus population) was obtained (5.3-5.88 kGy). The optimum dose range of gamma ray for FMDV inactivation (with $TCID_{50}$ /ml $=10^{7.5}$) was obtained between 40-44 kGy, so that the virus antigenecity remained unalterated. The results of the safety test for the irradiated samples with gamma ray doses: 40, 45 and 50 kGy were suitable since CPE was not visible after a blind culture on cell culture was performed on them three times.

The results of CFT for irradiated and un-irradiated samples show the antigenecity of the irradiated FMD virus from 0-45 kGy was not changed, therefore the antigenecity, which is very important for vaccine preparation, remains unchanged.

After the vaccines were inoculated subcutaneously on the back foot in two groups of guinea pigs, just two of ten animals showed antiserum titration above the protective titration (PT=1.2), and the other 8 animals were below the PT [22]. Also, the antibody titration of the vaccinated animals (based on SNT) which were inoculated intra peritoneal are in Table 2. This shows that the intra peritoneal route is not suitable for the vaccine inoculation**.** The anti FMDV sera titration of the Guinea pigs which were vaccinated subcutaneously and infra auxillary are as shown according to Table 3; This shows that the irradiated FMD vaccine can immunize guinea pigs as well as the inactivated FMD vaccine by ethylenimine. Therefore the best route of injection is subcutaneously and infra auxillary [10, 15, 20 and 22]. The PD_{50} Value was calculated by Karber's method [14 & 16]. The virus generalization in the control animals was described by Lucam et al [18]. The results of the PD50 Value for the two different vaccines are summarized in Table 4; This shows that three dilutions of the radio-vaccine (1:1, 1:2 and 1:4) can immunize the guinea pigs as well as the normal vaccine.

Table1. The result of virus titration for irradiated and unirradiated FMD type A87/IRN after safety test

Dose of irradiation(KGy)	0	10	20	25	30	35	40	45	50
Virus titration/ml ($TCID_{50}$)	$10^{7.5}$	$10^{5.5}$	$10^{3.3}$	$10^{2.5}$	10^{2}	$10^{1.5}$	0	0	0

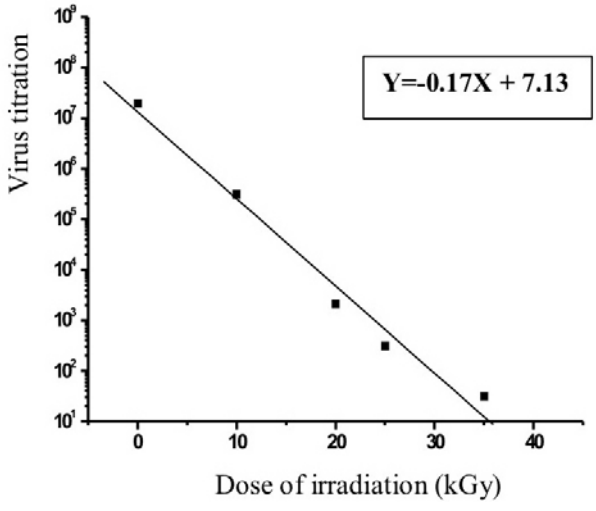

Fig. 1. Dose/Survival curve for irradiated FMDV typeA87/IRN

Table 2. The results of SNT for titration of guinea pig anti FMDV sera that were injected intraperitoneally. If the titre of SNT is more than 1.2, it is protective and less than 1.2, it is not protective [22]

Type of vaccine	No. of animals	Antiserum titration	result
Radiovaccine	2	0.9	not protective
Radiovaccine	3	=<0.6	not protective
Normal vaccine	5	=<1.2	Partially protective
Control group	5	=<0.6	not protective

Table 3. The results of SNT for titration of guinea pig anti FMDV serathat were injected subcutaneously & infra auxillary. If the titre of SNT is more than 1.2, it is protective and less than 1.2, it is not protective [22]

Duration of serum titration / Mean of anti FMD serum titration		mean	results
1 month	NV	1.8	P
	RV	1.8	P
	unvaccinated	0.6	NP
3 months	NV	1.8	P
	RV	1.8	P
	unvaccinated	0.6	NP
6 months	NV	1.74	P
	RV	1.74	P
	unvaccinated	0.6	NP
8 months	NV	1.77	P
	RV	1.74	P
	unvaccinated	0.6	NP

NV: Normal Vaccine RV: Radio-Vaccine
P: Protective NP: Not protective

Table 4. PD_{50} of the inactivated FMD vaccine

Type of vaccine	Dilution of vaccine	No of Guinea pigs	Percentage of generalization	PD_{50}
Normal vaccine	1:1	5	0	7.06
	1:2	5	0	
	1:4	5	0	
	1:8	5	60	
	1:16	5	100	
	Control	5	100	
Radio-vaccine	1:1	5	0	5.60
	1:2	5	0	
	1:4	5	0	
	1:8	5	100	
	1:16	5	100	
	Control	5	100	

4. DISCUSSION

The inactivation methods of FMDV are included: 1) Inactivation by formaldehyde 2) Inactivation by aziridines such as acetylethylenimine, ethylenimine and propylenimine. Both of them have some residues

in the final products, further some are toxic while others cause allergic responses in the animals, however it is possible that some viruses may escape during the chemical inactivation routes [1]. Irradiated inactivated viruses have been reported to retain most of their antigenicity [5 & 21]. The viral inactivated vaccines by ionizing radiation are safe and are not toxic; also the virus particles can not escape from inactivation by gamma irradiation [5, 6 & 7]. Some researchers from Argentina studied the production of some inactivated vaccines by ionizing the irradiation of viruses such as: FMD virus, Herpes Simplex virus and etc [5]. Particularly, Frescura et al have observed that the antigenicity of the type C lyophilized FMD virus, which was inactivated by gamma radiation, is kept unaltered by the Complement Fixation method [13]. In this study the optimum dose range of gamma ray for inactivation of FMDVtypeA87/IRN at -20 C, without any change in antigenicity was obtained as 40-44 kGy. Therefore, we can use the inactivated virus with the unalterated antigenicity character and good safety test results to the preparation of the inactivated radio-vaccine. We also formulated the vaccines by the Alhydrogel as adjuvant and other substances. The formulated vaccines were inoculated to Guinea pigs and the vaccinated animals were studied using the SNT method, showing the inactivated FMD vaccine by gamma irradiation can immunize guinea pigs as well as the inactivated FMD vaccine by ethylenimine. The result of the potency test was shown in Table 4, PD50 Value were 7.06 and 5.60 for the inactivated vaccine by EI and gamma irradiation respectively, therefore it can be shown that three dilutions of the radio-vaccine (1:1, 1:2 and 1:4) can immunize the guinea pigs as well as the normal vaccine [14].

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