Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 1, 144–151
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0094

Original article

REDUCTION OF VIRAL LOAD OF AVIAN INFLUENZA A VIRUS (H9N2) ON SPF EGGS AND CELL LINE BY GAMMA IRRADIATION

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Summary

Javan, S., F. Motamedi-Sedeh & M. Dezfulian, 2021. Reduction of viral load of avian influenza A virus (H9N2) on SPF eggs and cell line by gamma irradiation. *Bulg. J. Vet. Med.,* **24**, No 1, 144–151.

Avian influenza A H9N2 viruses are circulating in domestic poultry worldwide. In this research a low-pathogenic AIV H9N2 was multiplied on MDCK cell line and SPF eggs and irradiated by a Nordian gamma cell instrument. Irradiated and non-irradiated AIV samples were titrated by $TCID_{50}$ and EID_{50} methods, respectively. Haemagglutinin antigen was analysed by Haemagglutinin test. Infectivity of irradiated virus samples was determined by cell culture and egg inoculation methods. The virus titration decreased as the dose of gamma radiation increased. AIV proliferation on cell culture can be inactivated by gamma irradiation at a lower dose of gamma-ray (20 kGy) than the virus inactivation on embryonated eggs (30 kGy). The safety test showed complete inactivation of AIV on allantoic fluid with gamma-ray doses: 30 kGy and 20 kGy for virus on MDCK cells after four blind cultures.

Key words: avian influenza, EID_{50}, gamma irradiation, $TCID_{50}$, virus titration

INTRODUCTION

Influenza A viruses are the only orthomyxoviruses, which infect birds naturally. Many species of birds have shown to be susceptible to infection with influenza A viruses (OIE, 2015). Avian influenza virus (AIV) subtype H9N2 was first reported in 1966 and isolated from the turkeys in the United States (Homme *et al.*, 1970). Many avian species are susceptible to this infection across large geographical areas. AIV subtype H9N2 is low pathogenic and can cause huge economic losses in the poultry industry including reduced egg production and decreased growth rate. Moreover, it can sometimes cross the species barrier and cause human infections, which has raised public health concerns (Homme *et al.*, 1970).

Vaccination is necessary for preventing AIV outbreaks and to avoid economic losses. Embryonated eggs were used to produce avian influenza vaccine, but this

method has some disadvantages. Antigenic variations after continuous passages, the necessity of large amounts of embryonated eggs and the possibility of contamination by an exogenous virus are some of them. Therefore, use of animal cell culture technique to produce influenza virus is more ideal, economic and reproducible (Khalili *et al.*, 2015). The use of immortalised cell lines (such as MDCK and VERO) has been investigated several times for vaccine production. These cell lines have been found to produce consistently high viral titres (Nunnally *et al.*, 2015). The inactivation of viruses via gamma irradiation is a reliable method to produce a safe inactivated vaccine against pathogenic viruses. There is a considerable body of evidence to suggest that radiation-inactivated vaccines are safer as well as a more effective and feasible alternative for vaccine efficacy (Chu *et al.*, 2009). The viral proteins (especially structural proteins) of irradiation-inactivated viruses are left in their native conformation thereby greatly enhancing immunogenicity and efficacy. The irradiated viruses indeed are similar to native viruses in many ways but cannot cause disease and therefore should elicit a more potent immune response (Chu *et al.*, 2009).

In this research, AIV subtype H9N2 was multiplied on MDCK cell line and embryonated eggs and then inactivated by gamma irradiation in order to evaluate and compare its inactivation power in both propagation systems.

MATERIALS AND METHODS

Virus multiplication on SPF eggs and cell culture

The isolated AIV strain A/Chicken/IRN/Ghazvin/2001 (0.1 mL per each egg) was inoculated on the embryonated SPF chicken eggs of 9–11 days, and incubated at 37 °C for 2–5 days according to Salehi *et al.* (2018). The allantoic fluid was harvested after 72 hours, and centrifuged 1500 ×g at 4 ºC, 10 minutes, to separate RBC and other cells. The infectious viral titre was determined using 10-day-old embryonated SPF eggs. Then EID_{50} (eggs infectious dose 50) was calculated according to Reed and Muench and OIE Manual (IAEA, 2010; Salehi *et al.*, 2018). The EID_{50} is a biological method to determine the amount of infectious virus in a sample by determining the highest dilution of the sample that can infect 50% of the embryonated chicken eggs.

The single-layer MDCK cells (Madin-Darby canine kidney cells) were grown by EMDM medium and 10% FCS and then inoculated with the AIV subtype H9N2 (1 mL per each 75 cm^2 cell culture flask) after 48 h. The cells supernatant was harvested after 48–72 h, the viral titration obtained by $TCID_{50}$ micro-method based on the appearance of CPE (cytopathic effect) on MDCK cell line and calculated according to Reed and Muench mathematical method.

Gamma irradiation and inactivation of virus samples

Gamma radiation was used for the inactivation of avian influenza A subtype H9N2 viruses multiplied on MDCK cell line and SPF eggs. AIV was irradiated by a Cobalt60 irradiator; the Gammacell 220 (MDS Nordion, Ottawa, Canada), at a dose rate of 2.07 Gy/s and activity of 8677 Ci to inactivate virus infectivity and damage influenza virus genomic RNA in Nuclear Science and Technology Research Institute, Tehran, Iran. Gamma-ray doses of 5, 10, 15, 20, 25, and 30 kGy were administered for the frozen infected allantoic fluid with AIV. In addition, 2, 5,

10, 15, and 20 kGy doses were used to irradiate the frozen infected MDCK cell supernatant and three sample replicates were irradiated for each dose. The titres of irradiated AIV samples were first obtained by EID_{50} and $TCID_{50}$ methods, and then dose/response curves were drawn using Origin software for both virus stocks (irradiated infected allantoic fluid and cell supernatant), separately. The D_{10} value (dose of gamma radiation which can decrease one logarithmic cycle of virus population) and optimum dose of gamma rays for AIV inactivation were calculated according to the dose/response curve for both of the virus stocks (Reed & Munch, 1938; Motamedi-Sedeh *et al.*, 2015). Finally, the safety test to confirm complete virus inactivation was performed by four blind passages of inactivated AIV on MDCK cells and embryonated SPF eggs, separately (Lombardo & Smolko, 1990; Motamedi-Sedeh *et al.*, 2015).

Haemagglutinin antigen assay

Haemagglutination test was conducted to measure the HA titre of the irradiated and non-irradiated virus suspension on MDCK cells and in embryonated SPF eggs. HA antigen of irradiated and non-irradiated viral samples was analysed by haemagglutinin test (HA test) according to the WHO pattern method. HA test was done as described, briefly; 50 µL of PBS was added to wells 2 through 12 a U-bottom micro-plate, and 100 µL of each tested virus to the first well. Two-fold serial dilutions made by transferring 50 µL from the first well to successive well 11 and discarded the final 50 µL from well 11. Well 12 contained only PBS as RBC control. Fifty microliters of 1% SPF chicken RBC suspension were added to each well on the plate. Then the plates were mixed and incubated at room temperature for 30 min. The titres of viruses were recorded after 30 min by tipping plates and reading RBC buttons that stream. The HA titration endpoint is considered as the highest dilution of virus suspension which causes complete haemagglutination. The HA titre is Log of the virus dilution reciprocal in the last well with complete haemagglutination (Motamedi-Sedeh, 2009).

RESULTS

Virus titration on MDCK cell lines and on SPF chicken eggs for irradiated and non-irradiated virus samples obtained by $TCID_{50}$ and EID_{50} methods is shown in Table 1. According to the increasing dose of gamma radiation, virus titration gradually decreased.

D_{10} value and the optimum dose for complete inactivation of AIV subtype H9N2 which multiplied on MDCK cells calculated by dose/response curve (Fig. 1) were 3.94 and 17.74 kGy, and for inactivation of virus samples in allantoic fluid suspension (Fig. 1) were 3.36 and 29.52 kGy, respectively.

The results of safety test for gamma-irradiated AIV subtype H9N2 samples displayed complete inactivation with following gamma-ray doses: 30 kGy for

Table 1. Virus titration by EID_{50} and $TCID_{50}$ methods (Salehi *et al.*, 2018)

Dose of irradiation (kGy)	0	2	5	10	15	20	25	30
$TCID_{50}$ /mL	$10^{4.5}$	$10^{3.5}$	$10^{2.5}$	$10^{1.5}$	$10^{0.5}$	10^{0}	–	–
EID_{50} /mL	$10^{8.77}$	–	$10^{5.47}$	$10^{4.83}$	$10^{4.17}$	$10^{2.33}$	–	–

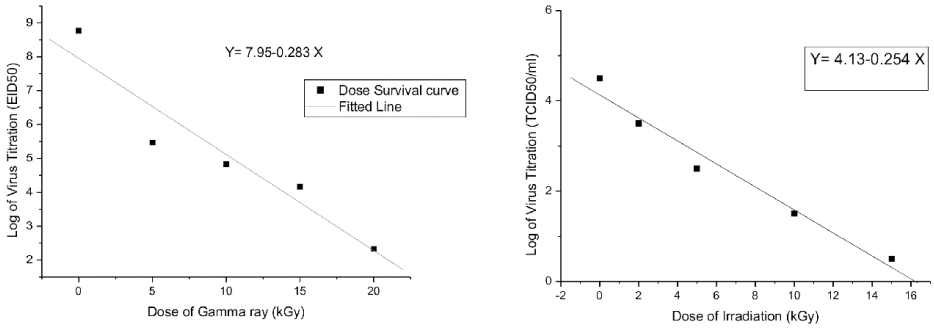

Fig. 1. Dose/response curves for inactivation of AIV subtype H9N2. Left: Virus on MDCK cells; right: Virus on infected allantoic fluid.

Table 2. Results of safety test for gamma irradiated avian influenza A subtype H9N2 virus (on eggs and MDCK cell line)

Dose of Gamma radiation (kGy)	Passage number							
	First passage		Second passage		Third passage		Fourth passage	
	On egg	MDCK cell	On egg	MDCK cell	On egg	MDCK cell	On egg	MDCK cell
20	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
30	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
35	Negative	–	Negative	–	Negative	–	Negative	–

Table 3. The results of haemagglutinin antigen assay for irradiated and non-irradiated avian influenza A subtype H9N2 virus samples

Gamma radiation dose (kGy)	Virus dilution										
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Negative control
0	+	+	+	+	+	+	+	+	–	–	–
2	+	+	+	+	+	+	+	+	–	–	–
5	+	+	+	+	+	+	+	+	+	–	–
10	+	+	+	+	+	+	+	+	–	–	–
15	+	+	+	+	+	+	+	+	–	–	–
20	+	+	+	+	+	+	+	+	–	–	–
30	+	+	+	+	+	+	+	+	–	–	–

AIV, in allantoic fluid (without any multiplication on eggs after four blind cultures) and 20 kGy for multiplication on MDCK cells (after four blind cultures on cell line) (Table 2).

Haemagglutinin antigen assay showed that HA antigenicity of gamma-irradiated virus samples at 0 to 30 kGy did not change (Table 3).

DISCUSSION

The viral vaccine might be the only way to control viral diseases successfully in the absence of alternative therapies. Gamma irradiation is one of the most effective methods of sterilisation that could be applied to ensure biological safety of food products, pharmaceuticals, and for inactivation of dangerous pathogens to enable their safe handling. Although only a few veterinary vaccines have been produced using ionising radiation attenuation, it is worth re-examining the potential of the process. Also, a number of recent investigations suggest that radiation technique could provide a viable alternative to other attenuation techniques, particularly with pathogens where recombinant vaccines have not yet delivered effective products (Viljoen *et al.*, 2012).

At present, the production of the effective cell-culture-based influenza vaccine has become the goal of numerous vaccine producers. The cell-culture-based production of influenza virus in comparison of virus multiplication on embryonated eggs is more economical, convenient and reproducible with stable quality. Also, the mass production is easy to carry out, and the antigenicity is closer to that of natural strains; therefore the immune responses are quite reliable (WHO, 2013). In the vaccine production process however, the production of influenza virus based on the cell-culture also has disadvantages including low viral load (WHO, 2013). In the current research, we have optimised lowest inactivation dose of gamma irradiation for the H9N2 influenza virus that can be used as vaccine candidate. Interestingly, our results show dichotomy for irradiation dose of inactivation of virus grown in cell cultures (20 kGy) compared to virus produced in embryonated eggs (30 kGy).

Irradiation of aqueous material by gamma radiation or an electron beam produces highly reactive unstable intermediates such as hydroxyl radicals, hydrogen atoms, and hydrated electrons. These highly reactive intermediates can cause chemical changes in the aqueous system and within microorganisms, resulting in damage to the organisms in the system (Lu *et al.*, 2017). Also, ionising radiation has indirect effects on water molecules which make free radicals such as OH° and H°. The action of the hydroxyl radical (OH°) must be responsible for an important part of the indirect effects on viruses. These indirect effects can be reduced by drying or freezing of living organisms. Living cells are less sensitive to radiation at subfreezing temperatures than at ambient temperatures. This is attributed to a decrease in water activity at subfreezing temperatures. In the frozen state, the diffusion of radicals is very much restricted (Aparecida da Silva Aquino, 2012).

Therefore, in this research, we used frozen virus suspension for reducing intermediates concentration. The data on the inactivation of viruses by ionising radiation indicate that a single ionisation is sufficient to inactivate a single virus particle. The inactivation data also show that the inactivation dose is related to the particle size of the viruses and its genome (Lu *et al.*, 2017). Gamma irradiation inactivates viruses without any change in the conformation of the viral proteins, therefore it preserves immunogenicity and thereby the vaccine efficacy (Reed & Munch, 1938; Lombardo & Smolko, 1990). The reduction of virus titre due to

inactivation would be less for irradiation than for chemical treatment. The irradiated viruses look like native viruses in the aspect of antigenic characteristics, but cannot cause disease and therefore should elicit a more potent immune response (Motamedi-Sedeh *et al.*, 2008). This could impact the cost of manufacture and result in dose-sparing in relation to virus production. Irradiation would also speed up the production processes and be useful in dealing with emergency situations as well as in providing better quality control and assurance in terms of standardisation and immunogenicity of vaccine strains (Chu *et al.*, 2009). The Manual on radiation sterilisation of medical and biological material of the International Atomic Energy Agency indicates that disrupting the haemagglutinating activity of influenza virus requires exposure to higher than 200 kGy gamma radiation (IAEA, 1973; Taghipour, 2004). Mullbacher *et al.* (1988) reported that gamma-irradiation is more effective than UV-irradiation at destroying infectivity of Influenza-A virus and it causes less damage to the antigenic structure, at least some of internal proteins than UV irradiation (Alsharifi *et al.* 2009; 2010). In this research, both cell culture and embryonated eggs methods were used for virus production and then gamma irradiation, for producing inactivated AIV antigen, which killed the viruses without any changes in HA antigenicity. Therefore the irradiated inactivated AIV antigen can be a good candidate to produce avian influenza vaccine. As the results show, viral load on embryonated eggs is higher than in cell culture. Therefore, if the vaccine producers would like to use cell culture technique for proliferation of AIV, it is necessary to optimise the conditions of influenza virus multiplication on cell culture.

ACKNOWLEDGEMENTS

The authors express their gratitude to Dr. Iraj Khalili from Razi Vaccine and Serum Research Institute of Iran. We express our gratitude to Department of Nuclear Science and Applications, Animal Production and Health Section, International Atomic Energy Organization (IAEA), VIC, Vienna, Austria for the support (IAEA Coordinated Research Project, CRP No. 22126). The authors are especially grateful to Dr. Hurmann Unger and Viskam Wijewardana.

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Paper received 18.06.2019; accepted for publication 14.08.2019