STANDARDIZATION OF ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETERMINATION OF SEROLOGICAL RESPONSE OF CHICKENS TO HYDROPERICARDIUM SYNDROME VACCINE

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ABSTRACT

The present study was conducted to standardize the Enzyme Linked Immunosorbent Assay (ELISA) for the assessment of serological response of vaccinated chicks with avian adenovirus-4 (AAV-4). The AAV-4 was grown on chicken embryo liver cell culture after plaque purification. The stock virus was utilised to standardize ELISA and preparation of experimental oil-based hydropericardium syndrome vaccine (OBHPSV) and aqueous base hydropericardium syndrome vaccine (ABHPSV). Optimum ELISA titres (87.26) were recorded for 1:64 dilution of antigen.1:80 dilution of test serum and 1:1000 dilution of conjugate. Serological response of chicks vaccinated at different age groups was monitored using ELISA and it was inferred that vaccination at 3rd day of age, with OBHPSV and at 10th day of age with ABHPSV gave maximum titers.

Key words: Standardization, ELISA, serological response, hydropericardium syndrome vaccine

INTRODUCTION

In 1987, an un-usual malady occurred among the commercial broiler flocks in Angara Goth near Karachi, Pakistan, causing high mortality. The disease, later on spread to other parts of the country and was characterised by Hydropericardium Syndrome (HPS) (Hasan, 1988). Further investigations revealed that disease was infectious in nature. The HPS virus was identified as virulent isolate of type-1 avian adenovirus and named as PARC-1 isolate (Naeem *et al.*, 1995). An aqueous-based vaccine developed from the liver of infected birds helped overcoming the disease (Chishti, *et al.*, 1989). Subsequent investigations revealed that aetiological agent of HPS was in fact an avian adenovirus serotype-4 (AAV-4). A vaccine was later developed using cell culture propagated AAV-4 (Naeem *et al.*, 1995).

Different serological tests were earlier employed to study the presence of HPS associated AAV-4 and its specific antibodies in the affected birds (Hassan *et al.*, 1994) but the virus partially purified from liver cells of infected bird did not give good results when used in developing Enzyme Linked Immunosorbent Assay (ELISA). The present study was designed to propagate and purify the AAV-4 for developing an ELISA for the determination of antibodies against AAV-4, as described by Akhtar (1994).

MATERIALS AND METHODS

Inoculum preparation

A 20% (w/v) HPS infected liver homogenate was prepared by triturating and blending the liver in sterile phosphate buffered saline (PBS), pH 7.4. The preparation was centrifuged at 7000 rpm for 20 minutes at 4°C. The supernatant obtained was filtered through a series of whattman filters ranging from 0.8 µm to a 0.2 µm. The filtrate was stored at -20°C until used.

Purification of Avian AdenoVirus-4 (AAV-4)

To prepare chicken embryo liver (CEL) cell culture, 16-day-old embryos were used. After removing under sterile conditions, the embryo was opened and liver was taken out and placed in a solution of glucose (0.1%), NaCl (0.3%), KCl (0.2%), phenol red (0.001%), streptomycin (1mg/ml) and gentamicin (100 µg ml). The minced liver tissue was treated with 0.1% solution of warm trypsin, followed by filtration through muslin cloth. One ml foetal bovine serum was added to it and centrifuged at 300 rpm for 15 minutes at 4°C. The pellet was suspended in 5 ml Eagle's Minimum Essential Medium (E-MEM) to bring the final concentration of 10^6 cells/ml. The cell suspension was placed in 25 cm^2 plastic flask.

One ml of earlier collected liver extract material containing the AAV-4 was inoculated into the CEL

culture flask and cytopathic effects (CPE) were observed after 72 hours of incubation at 37°C. After confirming CPE the cells were freeze-thawed thrice and material was centrifuged to remove the cell debris. The supernatant was used for plaque purification. Single characteristic plaque was inoculated into a fresh flask containing CEL. By this way AAV-4 was propagated further. The cells were freeze-thawed after the appearance of CPE, centrifuged at 300 rpm for 15 minutes at 4°C and supernatant was titrated and stored at -80°C as a stock virus .

Determination of TCID $_{50}$

To determine $TCID_{50}$ of the stock virus , its 10-fold dilutions were prepared in PBS (pH 7.2) from 10^{-1} to 10^{-6}. Four rows of a 12-well plate were prepared using CEL cells and were infected with each dilution, using 25 µl of virus per well. The infectivity was checked for 48 hours and $TCID_{50}$ was calculated, as described by Reed and Muench (1938).

Preparation of vaccines

Oil-based HPS Vaccine (OBHPSV)

For vaccine production the virus was inactivated with 0.3% formaldehyde for 72 hours. The vaccine was prepared using following oil-base.

a) Virus = 100 ml
b) Mineral oil = 700 ml
c) Emulsifier = 64 ml
(lanolin anhydrous)

Sterile mineral oil and lanolin were mixed first and then virus was slowly added to it. This was blended at 15000 rpm for 15 minutes.

Aqueous-based HPS Vaccine (ABHPSV)

Virus was inactivated using 0.3% formaldehyde. Stock virus having $10^{6.4}$ $TCID_{50}$ titre was diluted in sterile normal saline to have final titre of $10^{4.4}$ $TCID_{50}$. The vaccine was inoculated at a dose rate of 0.25ml s/c (streptomycin 1mg/ml and gentamicine 100 µg/ml).

Preparation of hyperimmune serum

Hyperimmune serum against AAV-4 was raised in broiler chickens. For this purpose a group of four birds were inoculated with the above prepared oil-based vaccine at 7-day of age and a second shot was given at 21-day of age. The chickens were bled after 14 days of second vaccination and tested for the presence of antibodies against AAV-4 by AGID. The serum was stored at -20°C (Hussain *et al.*, 1996) .

Serological analysis

Agar gel precipitation test

Agar gel precipitation test (AGPT) was conducted adopting the standard protocol, as described by Crowle (1973). For this purpose, 1% noble agar was prepared in PBS (pH 7.2) and poured into petridishes. Upon solidification wells were made in it using multiwell-mould. To the middle well 50 µl of AAV-4 was added. In one of the surrounding wells 50 µl of known antisera against AAV-4 was placed, whereas unknown antisera was placed in other wells. A turbid precipitating line between the middle and surrounding wells was considered positive.

Experimental design

A total of 70 broiler chicks were equally divided into seven groups. These groups were vaccinated as follows.

Group A1 Vaccinated at day 3rd with ABHPSV
Group A2 Vaccinated at day 3rd with OBHPSV
Group B1 Vaccinated at day 10th with ABHPSV
Group B2 Vaccinated at day 10th with OBHPSV
Group C1 Vaccinated at day 17th with ABHPSV
Group C2 Vaccinated at day 17th with OBHPSV
Group D Unvaccinated/ Control

Blood samples were collected weekly upto 49 days of age and serum samples were properly labelled and stored at -20°C, until use. On 49th day, three birds from each group were challenged with $10^{5.4}$ $TCID_{50}$ of stock virus and were kept under observation for 14 days .

Enzyme Linked Immunosorbent Assay (ELISA)

Preparation of antigen for ELISA

Twenty five ml of saturated solution of ammonium sulphate was mixed drop by drop into equal volume of viral antigen. Stirring was continued at 4°C overnight. It was then centrifuged at 10,000 rpm for 30 minutes at 4°C and pellet was suspended in 5 ml PBS. To the suspension 1 ml of chloroform was added and both were mixed by shaking. It was then kept in stand for 2 minutes. That was centrifuged at 2500 rpm at 4°C for 10 minutes, chloroform settled down and antigen was collected by pasture pipette. This was used as antigen for ELISA (Mansur- ud -Din, 1994).

Standardization of ELISA

A 200 µl of purified AAV-4 viral antigen was added in each well for coating of microtitration plate in a two fold dilution from 1:2 to 1:256 using carbonate-bicarbonate-buffer (pH 9.6). Coating was done in a set of ten flat bottom microtiter plates and four rows of

each were used for each dilution of conjugate i.e. 1:500 and 1:1000. After an incubation of 24 hour at 37°C in a humid environment, plates were washed with washing buffer (Tris 0.605%, EDTA 0.037%, NaCl 0.877% and Tween 20 0.0044% pH 8) and stored at 4°C until use. Hyperimmune serum against AAV-4 was added into 100 µl well of the microtitration plate in two fold dilution from 1:10 to 1:80 using Casein buffer (0.2% casein in washing buffer Table 1). After an incubation of 1 hour at 37°C, the microtitration plates were washed with washing buffer. To these horse raddish peroxidase (HRP) labelled anti-chicken IgG was added in a concentration of 1:500 and 1:1000 in duplicate plates four rows of each respectively. After one hour of incubation at 37°C, the microtitration plates were washed with washing buffer and 100 µl of the substrate, Ortho phenylene diamine (OPD), was added to each well. After an incubation of 30 minutes in dark, microtitration plates were read with ELISA reader and absorbance (optical density) was recorded.

Hyperimmune Serum prepared was positive to AGPT and its ELISA titer was 135.07. Hyperimmune serum was prepared using the oil-based vaccines, as described by Hussain *et al.* (1996).

Enzyme linked immunosorbent assay was standardized using different dilutions of antigen, test serum (from vaccinated chicks) and conjugate dilutions (Table 1) for titration of serological response of the chicks vaccinated with OBHPSV and ABHPSV at different ages. The dilution of antigen in 1:64, dilution of test serum in 1:40 and dilution of conjugate in 1:1000 gave maximum titer and proved to be the best dilution for efficient detection of antibodies against AAV-4. The dilutions were used for further titration of serological immune response of the vaccine. Enzyme linked immunosorbent assay is very sensitive technique for the detection of antibodies as compared to all other serological tests like IHA, AGPT (Hassan *et al.*, 1994). Different serological tests e.g. IHA and AGPT had been used in the past with variable results by various workers. (Hussain *et al.*, 1996) used ELISA for detection of antibodies against Adeno virus. The antigen used was crude and results were not unvariable. In the present study AAV-4 virus was purified by picking the characteristic plaque and it was propagated on chicken embryo liver cell culture. The AAV-4 was harvested, inactivated and used for the preparation of experimental vaccines.

Table 1: ELISA mean titres using different dilutions of antigen, test serum and conjugate

Well No.	Conjugate dilution	Dilution of test serum	Dilution of antigen 1:4	1:8	1:16	1:32	1:64	1:128	1:256
A	1:500	1:10	14.11	10.30	13.48	15.41	12.90	10.10	8.16
B		1:20	16.87	9.10	14.10	14.90	12.40	9.20	7.90
C		1:40	30.10	40.10	48.08	50.10	55.10	42.10	35.83
D		1:80	20.18	32.33	37.64	36.90	24.40	22.56	19.65
E	1:1000	1:10	17.77	12.33	22.48	17.77	15.98	24.13	15.06
F		1:20	20.87	10.49	18.07	17.47	15.95	26.85	21.42
G		1:40	63.39	64.30	14.91	68.90	87.26	65.21	41.53
H		1:80	50.28	50.28	18.68	18.58	42.10	65.67	38.21

RESULTS AND DISCUSSION

The virus AAV-4 was passaged in natural host and filtered antigen was used for infecting chicken embryo liver (CEL) cell culture (Animal Sciences Institute, NARC Islamabad). The pure antigen was used for standardization of ELISA and preparation of hyperimmune sera and vaccines for titration of serological immune response with ELISA. The $TCID_{50}$ of the virus was $10^{-6.4}$ per 25 µl of stock virus The finding corroborate with that of Naeem *et al.* (1995). Two types of vaccines i.e. OBHPSV and ABHPSV were prepared with final viral titer of $10^{-4.4}$ $TCID_{50}$.

Serological response of all the groups of chickens was carried out with AGPT and the standardize ELISA technique. The antigen-antibody reaction in agar gel precipitation test showed low levels of titers which shows that it was not highly sensitive test. Titer of all the vaccinated groups is shown in Table 2.

The ELISA titers on 49th day (challenge day) of all the groups were also compared with those obtained 2 weeks later i.e. day-63 (after 14 days of challenge). The data showed that chicken given oil-based vaccine on day-3 had a mean titer of 671 on day-49 which decreased slightly after challenge to 658 on day-63. This change was found statistically non-significant (P>0.05). In the birds given oil-based vaccine on day-10. a titer of 587 on day-49 was observed which increased to 820 on day-63. This increase was. however. found statistically significant (P<0.05). On 17th day oil-based vaccinated chickens showed a titer of 724 on day 49 which increased to 926 on day-63. This increase was also found statistically significant (P<0.05) (Table 2). The chicken vaccinated with aqueous based vaccine on day-10 developed a titer of 660 on day-49 which increased to 764 upon challenge by day-63. This was also found non-significant (P>0.05). Post challenge protection levels and mortality rates were also recorded. From the challenged birds. seven out of ten birds of uninoculated control group died showing typical signs of HPS during 3-5 days post-challenge. Other birds of all the vaccinated groups showed no signs of abnormality or mortality.

It was concluded that chicks vaccinated at day 10 with ABHPSV and chicksvaccinated atday 3rd with OBHPSV developed maximum ELISA titers (Table 2). These findings are congruent with the finding of Chishti *et al.* (1989) and Ahmad *et al.* (1990). The earlier studies revealed variable protection against an oil-adjuvanted vaccine (Chishti *et al.*, 1989). This might be due to poor antigenic response of chicks to crude antigen used in vaccine manufacturing. In the present study OBHPS vaccine produced similar titers as produced by ABHPS vaccine at the initial days post inoculation. however. these titers were consistant for relatively longer period of time. i.e. 49 days. These titers were increased following challenge with AAV-4 in all the vaccinated groups. These titers are sufficient to resist any field exposure for a longer duration of time.

REFERENCES

Ahmad. I.. M.I. Malik. K. Iqbal. K. Ahmad and S. Naz. 1990. Efficacy of formalized liver origin vaccine against Angara disease in broilers. Vet. Archiv. 60: 131-138.

Table 2: ELISA titres of chicks vaccinated at different age intervals with ABHPSV and OBHPSV

Age of chicks at the time of sampling	Control	Aqueous based HPS Vaccine (ABHPS-V)			Oil based HPS vaccine (OB HPS-V)		
		Group A1	Group B1	Group C1	Group A2	Group B2	Group C2
0	118.6 ±35.4^{a}						
7	71.335 ±12.43^{b}	610.086 ±78.35^{d}			695.65 ±113.55^{f}		
14	66.30 ±1.60^{b}	827.77 ±54.87^{e}	924.46 ±77.84^{c}		751.7 ±78.5^{g}	830.97 ±79.8433^{e}	
21	6.064 ±1.496^{c}	838.45 ±63.47^{e}	756.09 ±95.81^{g}	782.74 ±68.800^{c}	674.9430 ±70.5^{k}	711.084 ±73.25^{f}	632.99 ±60.0368^{d}
28	0.10 ±0.1^{c}	709.2 ±76.01^{f}	756.71 ±101.07^{g}	681.70 ±89.66^{k}	1291.75 ±541.68^{m}	614.95 ±65.2347^{d}	755.34 ±75.4236^{g}
35	1.8925 ±0.5225^{c}	745.16 ±118.906^{g}	856.90 ±75.28^{i}	748.66 ±92.18^{g}	624.80 ±77.21^{d}	654.55 ±67.33^{k}	670.92 ±78.1414^{k}
42	3.15 ±0.450^{c}	632.98 ±45.55^{d}	745.44 ±84.3057^{g}	712.68 ±52.29^{f}	739.71 ±79.6^{g}	727.341 ±73.84^{f}	687.598 ±52.54^{f}
49	1.7275 ±0.0725^{c}	586.91 ±61.5154^{h}	660.40 ±36.7901^{k}	607.42±4 1.957^{d}	671.69 ±56.34^{k}	587.504 ±26.27^{h}	724.577 ±63.2566^{f}

Group A1 = Vaccinated on day 3
Group B1 = Vaccinated on day 10
Group C1 = Vaccinated on day 17
Group A2 = Vaccinated on day 3
Group B2 = Vaccinated on day 10
Group C2 = Vaccinated on day 17
Values having similar superscripts are non significant with each other

Akhtar, S., 1994. Hydropericardium syndrome in broiler chickens in Pakistan. Poul. Sci. J., 50 (2): 177-182.

Chishti, M. A., M. Afzal and A. H. Cheema, 1989. Preliminary studies on the development of hydropericardium syndrome of poultry. Revu Scientifique et Technique de l' office International Epizootics, 8: 799-801.

Crowle, A.J., 1973. Immunodiffusion. Academic Press. New York

Hasan, S.A., 1988. Identification of hydropericardium syndrome. Misset International Poultry, 1-54.

Hassan, N.U., M. Afzal, A. Hameed and A. R. Khan. 1994. Immune response to inactivated hydropericardium syndrome vaccine in broilers. Pakistan Vet. J., 14: 5-10.

Hussain, I., A. A. Anjum and I. H. Khan. 1996. An oil-emulsion vaccine against hydropericardium syndrome in broiler chickens. Pakistan Vet. J., 16(3): 125-127.

Mansur-ud-Din, A., 1994. Studies on the avian adenoviruses (Group-1) associated with inclusion body hepatitis. Ph.D. Thesis. Department of Biomedical and Tropical Veterinary Sciences. James Cook University of North Queensland. Australia.

Mashkoor, S., A. Hameed, K. Ahmad and M. Saleem. 1994. Improved Angara diseases vaccine for broiler chicks. Veterinars ki Archiv., 64(1-3), 27-33.

Naeem, K. M. Rabbani, M. Hussain and A. H. Cheema. 1995. Development of cell culture vaccine against hydropericardium syndrome in poultry. Pakistan Vet. J., 15 (3): 150-151.

Reed, L. J. and H. Muench. 1938. A simple method for estimating fifty percent end point. Am. J. Hyg., 27: 293-295.