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Full Length Article

Preparation and Evaluation of Lyophilized Live Attenuated Vaccine of Inclusion Body Hepatitis Hydro-pericardium Syndrome (IBH-HPS) against Challenge in Broiler Chickens

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Abstract

The current study was conducted to prepare and evaluate the efficacy of live attenuated vaccine of IBH-HPS in broiler birds in Pakistan. The IBH-HPS virus was successfully adapted after three blind passages (Vero cell line) and the 4th passage was fully adapted as it gave cytopathogenic effect (CPE) in 48 h post infection in Vero cells. The 9th passage was determined for attenuation in broiler birds. The attenuated virus was subjected to pathogenecity testing and used as vaccine candidate in broiler chickens. Two hundred and fifty, day-old commercial broiler chicks were purchased from local market, divided into 5 groups and offered feed and water *ad libitum.* At the age of 15 days, 50 birds in each group were administered with 9th passage live attenuated lyophilized vaccine, the 3rd passage ß-propiolactone inactivated vaccine (prepared in the laboratory), oil emulsified inactivated vaccine (UAF-Angavac), commercial vaccine (Bio-Angara, Sana Laboratories, Pakistan), and normal saline, respectively as negative control. The 9th passage live attenuated lyophilized vaccine protected 100% birds from mortality, clinical signs and high antibody titre was observed. On the other hand, ß-propiolactone inactivated vaccine gave 70% protection. The oil emulsified inactivated vaccine gave 80% protection but lower weight gain was observed. The commercial vaccine gave 60% protection. In negative control 70% mortality was recorded. The results revealed that the live attenuated lyophilized vaccine showed a higher protection against lethal challenge of IBH-HPS in broilers. © 2015 Friends Science Publishers

Keywords: Inclusion body hepatitis hydro pericardium syndrome; Vero cell line; Live attenuated vaccine; Cytopathogenic effect

Introduction

Avian adenoviruses are known to cause many disease conditions in chickens of all age groups like respiratory infections, hepatitis and problems in egg productivity (Fadly and Winterfield, 1973). Among these manifestations, the egg drop syndrome in layers and Inclusion body hepatitis along with hydropericardium syndrome (IBH-HPS) in broilers (Grgic *et al.,* 2011) are frequently encountered. IBH-HPS is caused by fowl adenovirus serotype-4 in broiler chickens, which mainly affects age group of 3 to 6 weeks old.

In the last few decades, IBH-HPS has been reported in almost every country around the world (Saifuddin and Wilks, 1991; Goodwin, 1993; Philippe *et al.,* 2005; Gomis *et al.,* 2007). This disease is known as 'Angara Disease' in Pakistan, after its first outbreak at Angara Goth, near Karachi (Akhtar, 1994). In India, the disease was named as 'Leechy disease' or 'Litchi disease' (Manzoor *et al.,* 2013), due to its peculiar appearance of the heart floating in pericardial fluid, which appeared similar to the deshelled leechy (litchi) fruit or IBH-HPS (Abdul-Aziz and Al-Attar, 1991; Balamurugan *et al.,* 2002). In Pakistan the disease was quite destructive and caused the death of 100 million broiler birds in less than two years (Cowen *et al.,* 1996). The disease also caused huge economic losses in South Asian region, owing to mortality, reduced productivity and immune suppression (McFerran and Adair, 2003). Similarly, the disease is widely spread and cause losses to broiler industry in many countries, including India, Iraq, Kuwait, Mexico, Peru, Russia, Japan, Central and South America, Ecuador and Chile (Kataria *et al.,* 2013).

The consistent and predominant gross lesion at necropsy of affected birds is hydropericardium (Anjum *et al.,* 1989). The liver is friable, mottled, swollen and discolored had multiple regions of focal necrosis, pin point or ecchymotic hemorrhages. Microscopic lesions are most commonly seen in the liver, with coagulative necrotic areas and infiltration of mononuclear cells. Histopathological studied revealed intra nuclear inclusion bodies basophilic (INIB) in hepatocytes (Alemnesh *et al.,* 2012). In the bursa of Fabricius, spleen and thymus cyst formation have been observed in some cases (Nakamura *et al.,* 2003).

Good hygiene, bio-security and immunization of the

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broiler birds have paramount importance to control this fatal disease. The liver organ formalin inactivated vaccine has been widely used to control IBH-HPS. The autogenously prepared vaccines had impure virus and other pathogens. Moreover, the dose and dosage of these vaccines were not evaluated and mentioned. Therefore, present study was carried out to prepare and evaluate IBH-HPS attenuated vaccine to overcome the losses caused by this devastating disease.

Materials and Methods

Procurement of IBH-HPS Virus

IBH-HPS virus was procured from Institute of Microbiology, University of Agriculture, Faisalabad, which was already isolated and purified (Accession number: DQ 264728) (Mansoor *et al.,* 2009). The sequenced virus was fowl adenovirus serotype-4.

Cells and Media

Vero cell line was established in the laboratory from frozen ampoule stored at -196^{o}C imported earlier from the Centre for Applied Microbiology and Research (CAMR), (ECACC 84113001, ECACC, Salisbury, Wilt-shire) (Rasool and Hussain, 2006). Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum was used as growth medium and with 5% fetal bovine serum as maintenance medium.

Adaptation and Attenuation of IBH-HPS Virus

The purified and characterized IBH-HPS virus was taken and a healthy confluent monolayer of Vero cells was infected. Briefly, a total of 0.3 mL of virus having $10^{5.31}$ EID_{50} was inoculated and the cell culture flasks were incubated at 37^{o}C in the presence of 5% CO_2 in a CO_2 incubator and observed for cytopathogenic effects. The culture fluid of each passage of IBH-HPS virus was harvested by three freeze thaw cycles and clarified by centrifugation. The IBH-HPS virus was serially passed on Vero cells for attenuation.

Pathogenicity Testing of IBH-HPS Virus

Pathogenicity of attenuated IBH-HPS virus was tested in 80 broiler birds of 15 days old, reared in the Animal House of Institute of Microbiology. The total number of broilers were randomly divided into 10 groups (A, B, C, D, E, F, G and H) each comprising 10 birds. The original virus and passage number 4, 5, 6, 7, 8 and 9 was inoculated in group A to G with dose 0.2 mL through sub-cutaneous route (s/c), respectively.

Group H was inoculated with normal saline as negative control. The birds were examined daily for clinical signs and mortality. At the day 7 and 14 post inoculation, three birds from each group were randomly selected for slaughtering. The gross and pathological lesions on liver, heart and kidneys were observed (Nakamura *et al.,* 1999).

Preparation of Vaccines

The 9th passage was filtered through 0.2 µm filter and lyophilized. The purified IBH-HPS virus was used for the preparation of oil adjuvanted inactivated vaccine using ISA-207 montanide oil (SEPPIC, France). The 3rd passage of IBH-HPS virus was taken and inactivated by adding beta-propiolactone (Fellows Medical, Mich). The IBH-HPS virus was titrated by calculating plaque forming units (pfu/mL) and infectivity titre through tissue culture infective dose 50 ($TCID_{50}$). The commercial vaccine (Bio-Angara) was purchased from local market. All the prepared vaccines were tested for sterility on different microbiological media. The stability of the vaccines was also checked before administration to birds (Mansoor *et al.,* 2010).

Evaluation of Vaccines

The efficacy of different vaccines, including lyophilized live cell culture attenuated, cell culture adapted inactivated, oil adjuvanted inactivated and commercial IBH-HPS vaccines was evaluated through experimental trials. A total number of 250 day old broilers birds were purchased from local market and divided into 5 groups each having 50 birds. Group 1 (G1) was vaccinated with lyophilized live attenuated vaccine through drinking water, group 2 (G2), group 3 (G3) and group 4 (G4) injected subcutaneously with cell culture adapted inactivated vaccine, oil adjuvanted vaccine and commercial vaccine, respectively. Group 5 (G5) with normal saline served as negative control. At 3, 7, 14 and 21 days post vaccination, 5 birds from each group were randomly selected, slaughtered and the organ to body weight ratios were calculated. Gross lesions were recorded on liver, heart and kidneys. The livers were collected and subjected to histopathology (Hussain *et al.,* 2012).

Humoral Immune Response

The blood samples from five birds in each group were collected at 0, 7, 14 and 21 days post-vaccination, before slaughtering, in sterilized test tubes and the serum was separated. The humoral immune response was tested by measuring IBH-HPS antibodies titre in each group by enzyme linked immunosorbent assay (ELISA) using Trop-Bio ELISA kit (Australia). The geometric mean titre was calculated and compared in all vaccinated groups with control negative group.

Cell mediated Immune Response

The Cell mediated immune response was evaluated *in-vivo* in all the groups by injecting phyto-haemagglutinin-P (PHA-P) in wattle of the birds. A 100 µL of PHA-P was injected intradermally in 5 birds of each group at 14 days post-vaccination, and the swelling of the wattle was

measured after 24 and 48 h post-injection with constant tension caliper. The thickness was compared with control negative group (El-Safty *et al.,* 2006).

Challenge Protection Assay

The remaining birds in each group were challenged with 10^5 embryo infective dose 50 (EID_{50}) of IBH-HPS virus orally at 21 days post-vaccination. Clinical signs, mortality and symptoms were observed twice daily for 21 days.

Statistical Analysis

Data collected were analyzed through geometric mean titre using descriptive one way analysis of variance (ANOVA) at significance level of $P < 0.05$. The efficacy of all vaccines was compared by Tukey's Test.

Results

Adaptation of IBH-HPS Virus in Vero Cell Line

The Vero cell line attained its normal growth in two successive passages after revival, and a healthy monolayer was formed in 36 h in DMEM. The morphology of normal Vero cells was fibroblast like. The monolayer was intact in passage 1 and passage 2 up to 6 days post infection (P.I.). In the third passage, the changes in monolayer were observed at 96 h P.I. The complete and clear cytopathogenic effects (CPEs) were observed in passage 4 at 48 h P.I. The CPEs were clear and consistent up to 9th passage observed at 48 to 72 h P.I.

Pathogenecity Analysis of IBH-HPS Virus

The pathogenicity of procured IBH-HPS virus reduced considerably after serial passages on Vero cells. The liver to body weight ratio in group treated with passage 9 virus was non-significantly comparable to un-inoculated control group. A maximum hydropericardium was observed in passage 4 group, while it was absent in passage 8 and 9 (Table 1). Hence, the 9th passage was non-pathogenic to broiler birds and attenuated. This passage indicated a TCID50 (log10) of 6.70/mL and plaque forming unit/mL 10^6 (pfu/mL). The 9th passage was lyophilized and its efficacy as live attenuated lyophilized vaccine was evaluated in broilers.

Response of Vaccines in Broilers

At 14 days of post-vaccination, no apparent untoward reaction was observed in all groups. The results of Mean liver to body weight ratio at 3, 7, 14 and 21 days post-vaccination (P.V.) are presented in Table 2.

The liver to body weight ratio was maximum in commercial vaccine immunized group (G4), followed in order by cell culture adapted inactivated vaccine immunized group (G2), oil adjuvanted vaccine immunized group (G3) and lyophilized live attenuated vaccine immunized group (G1). There was negligible difference between control group (G5) and (G1) at ($p<0.05$).

Histo-pathologically, no changes in the liver of birds in group 1 were observed as compared to other groups. The changes observed during histopathology of livers of birds in other group included the presence of intra-nuclear inclusion bodies, degenerative changes, infiltration of heterophils, lymphocytes necrosis and atrophy.

For the evaluation of humoral immune response, the results of ELISA antibody titres were observed and geometric mean titres were calculated. The ELISA titre was negligible at day zero (0) in all groups under trial. The overall geomean antibody titre was maximum in G1 followed in order by G3, G2 and G4 (Fig. 1). In contrast, the antibody titre was significantly lower in control group (G5).

The cell mediated immune response was also checked in all the groups under trial of different vaccines. Maximum swelling was observed in group 1 immunized with lyophilized live attenuated vaccine followed in order by group 3 immunized with oil adjuvanted vaccine, group 2 immunized with cell culture adapted inactivated vaccine, group 4 immunized with commercial vaccine.

Protective Efficacy of Vaccines

The remaining birds in all immunized groups were challenged with virulent IBH-HPS virus through oral route. All the birds in group 1 survived up to 7 days (100% efficacy) and had no clinical signs and gross lesions. However, the protective efficacy of cell culture adapted inactivated vaccine (G2), oil adjuvanted vaccine (G3) and commercial vaccine (G4) was 70%, 80% and 60%, respectively. The 70% birds in control group were died after lethal challenge of virulent IBH-HPS virus.

Discussion

The formalin inactivated liver homogenate is vaccine used extensively in controlling IBH-HPS in broilers (Kumar *et al.,* 1997; Anjum, 1990; Ojkic and Nagy, 2003; Alvarado *et al.,* 2007). This vaccine has many drawbacks, including (1) unpredictable immune response provoked by the vaccine, (2) impurity and the vaccine may contains other viruses and bacteria (Mansoor *et al.,* 2010). The above results revealed that the lyophilized live attenuated vaccine was safer, immunogenic and easy to administer. The live attenuated vaccine provoked a high antibody titre for longer time. The IBH-HPS virus was attenuated on Vero cell line after three blind passages. The 9th passage was completely attenuated determined through pathogenicity testing and used for the preparation of vaccine. The 9th passage was titrated by plaque assay before lyophilization and the virus titre was 10^6 pfu/mL. The infectivity of the attenuated 9th passage was also checked and gave TCID50 (log10) of 6.70/mL.

Table 1: Evaluation of mean liver to body weight ratio and the development of hydropericardium in experimental broiler birds by different passage number of IBH-HPS virus

Group Name	Mean liver to body weight ratio		Hydro-pericardium	
	7 days post-inoculation	14 days post-inoculation	7 days post-inoculation	14 days post-inoculation
A	6.3	7.5	++++	++++
B	6.3	7.4	++++	++++
C	6.0	7.1	++++	++++
D	6.1	6.5	++	+++
E	5.7	6.1	++	+++
F	5.2	5.6	_	_
G	4.9	5.5	_	_
H	5.1	5.5	_	_

Group A, inoculated with 0.2 mL of purified IBH-HPS virus subcutaneously (s/c); Group B, inoculated with 0.2 mL passage # 4of IBH-HPS virus (s/c); Group C, inoculated with 0.2 mL passage # 5 of IBH-HPS virus (s/c); Group D, inoculated with 0.2 mL passage # 6of IBH-HPS virus (s/c); Group E, inoculated with 0.2 mL passage # 7 of IBH-HPS virus (s/c); Group F, inoculated with 0.2 mL passage # 8of IBH-HPS virus (s/c); Group G, inoculated with 0.2 mL passage # 9of IBH-HPS virus (s/c); Group H, Negative control.
++++ = 10-15 mL water in pericardial sac, +++ = 5-8 mL, ++ = 3-4 mL and - = no water

Table 2: Mean liver to body weight ratio ± SE in different groups vaccinated with four different types of vaccines

Age in Days	Group 1	Group 2	Group 3	Group 4	Group 5
3	5.14±0.0509^{c}	5.74±0.0927^{ab}	5.56±0.0927^{b}	5.960.0400^{a}	5.14±0.0509^{c}
7	5.48±0.0663^{c}	5.96±0.0509^{b}	6.14±0.0509^{b}	6.84±0.0812^{a}	5.64±0.0927^{c}
14	5.78±0.0583^{d}	6.26±0.0509^{bc}	6.480.1280^{b}	7.4±0.0707^{a}	6±0.03162^{cd}
21	6.12±0.0583^{d}	6.38±0.0374^{bc}	6.5±0.0316^{b}	7.9±0.0707^{a}	6.2±0.0707^{dc}

(Tukey's HSD alpha<0.05)
Group 1, birds immunized orally with 0.2 mL of lyophilized live attenuated vaccine; group 2, birds immunized subcutaneously with 0.2 mL cell culture adapted inactivated; group 3, birds immunized with subcutaneously with 0.2 mL oil adjuvanted vaccine (UAF-Angavac); Group 4, birds immunized subcutaneously with 0.2 mL of commercially formalized liver organ vaccine (SANA Lab, Pakistan); group 5, birds kept as PBS-inoculated control
^{a,b,c,d} Mean liver to body weight ratio with different superscripts indicate statistical significance ($p<0.05$) between different vaccinated groups

Vero cell line, high infective virus titre coincides well with the appearance of CPEs.

Altogether, the indigenous IBH-HPS virus was adapted and attenuated on Vero cell line (Continuous cell line) without prior passages to embryonated eggs or chicken embryo fibroblasts. Moreover, higher yield of attenuated was also produced on Vero cell line. There was neither any clinical signs nor any death was observed in lyophilized live attenuated vaccine immunized group (G1). The gross lesions score was also zero in case of G1. Mild lesions marked as (1) found in group 3 (G3) and moderate lesion score (2) was observed in cell culture adapted inactivated vaccine group 2 (G2), but a severe lesion score (4) was observed in group 4 (G4) immunized with commercial vaccine. Histo-pathologically, the livers of birds in group 1 had no change as compared to other groups. There were mild to severe changes in the hepatocytes of group 3, group 2 and group 4, respectively. Similar observations were observed by other workers upon experimental trials of cell culture vaccines (Roy *et al.,* 1999). The present study revealed that the live attenuated vaccine can be used for saving the broiler birds from this devastating disease in Pakistan.

Conclusion

The 9th passage in Vero cell line was non-pathogenic to broiler birds and attenuated. The lyophilized live attenuated vaccine prepared from the passage 9 significantly induced antibody titres and cell mediated immune responses. The protective efficacy of this vaccine, in terms of mortality, clinical signs and gross lesions, against lethal challenge of virulent IBH-HPS virus was also 100%. However, the field trials in commercial broiler chicks are necessary before recommending this lyophilized live attenuated vaccine for future use against this devastating disease. Above all, the study will pave the way for researchers to work and find out the possibility of the development of oral vaccines for other diseases.

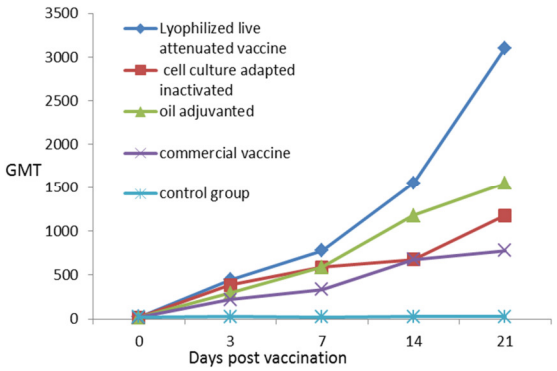

Fig. 1: Geometric mean of ELISA antibody titres (GMT) of different groups of birds immunized with different vaccines

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