

Development and Evaluation of Montanide-adjuvanted Vaccines for the Protection of Chickens Against Inclusion Body Hepatitis-Hydropericardium Syndrome

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Abstract.- Protective parameters of embryonated egg adapted, Montanide-adjuvanted, inclusion body hepatitis-hydropericardium syndrome (IBH-HPS), formalin inactivated, and commercial oil-based vaccines, were recorded and analyzed in the present study. Efficacy of the vaccines was tested on the basis of humoral and cell-mediated immune responses, challenge protection against IBH-HPS agent, mean body weight, and organ to body weight ratios. Indirect hemagglutination IHA antibody titer in terms of geometric mean titre GMT in the group vaccinated with Montanide-adjuvanted IBH-HPS vaccine (Group C) was significantly higher than the groups vaccinated with formalin-inactivated vaccine (Group A) and commercial-oil based vaccine (Group B), followed by unvaccinated challenged (Group D) and unvaccinated unchallenged control (Group E). Responsiveness to a mitogenic lectin, phytohemagglutinin-P was significantly reduced in Group A as compared to Group B, followed by Group C. Immunization with Montanide-adjuvanted (IBH-HPS) vaccine conferred protection in 95% of the chickens as evidenced by the absence of clinical signs, hydropericardium, and mortality, followed by the group vaccinated with commercial oil based and formalin-inactivated IBH-HPS vaccines. There was reduction in mean body weight and organ to body weight ratios of the bursa of Fabricius BF, spleen, and liver recorded in group A as compared to groups B and C. The results revealed that administration of Montanide-adjuvanted IBH-HPS vaccine could protect chickens more efficiently from IBH-HPSV challenge as compared to formalin-inactivated and commercial oil-based vaccines.

Keywords: Montanide-adjuvanted IBH-HPS vaccine, RPHA, IHA.

INTRODUCTION

Fowl adenoviruses (FAVs) are very heterogeneous viruses represented by five different species (A-E) and numerous serotypes (FAV-1-12) (Benko *et al.*, 2005). Even though various serotypes of fowl adenovirus (FAV) produce inclusion body hepatitis (IBH) in broiler chicks, along with hydropericardium syndrome, popularly called Litchi heart disease or Angara disease, has recently been reported in some countries in Asia and America (Shane, 1996; Abe *et al.*, 1998). IBH-HPS has caused huge economic losses to the poultry industry in Pakistan, since September 1987 when it was reported at Angara Goth, the extensive broiler

growing area in Karachi, Pakistan. IBH-HPS has been observed in broilers of either sex, aged 3 to 6 weeks or over 5 weeks of age, and occasionally in layers and breeder pullets aged up to 20 weeks. The disease is characterized by sudden onset and high morbidity, with a high mortality of up to 80% in broilers and low mortality of below 10% in layers, associated with hydropericardium (Shane, 1996).

Fowl adenoviruses (FAV 1-12) belong to group 1 and share a common group antigen with viruses isolated from geese, ducks, and turkeys (McFerran, 1997). Non-enveloped, icosahedral, FAV serotype-4 is believed to be the causative agent of hepatitis and hydropericardium syndrome in chicken (Hess *et al.*, 1999; Kumar and Chandra, 2004). The disease is characterized by the accumulation of colorless or amber-green, watery or jelly-like fluid in the pericardial sac, ranging from 3 to 20 ml in volume and with a pH of 7 (Asrani *et al.*, 1997). The pericardial fat may exhibit yellowish discoloration

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with petechial hemorrhages, and the heart appears misshapen and flabby, with its apex floating in the pericardial sac (Asrani *et al.*, 1997; Kumar *et al.*, 1997). Intranuclear inclusion bodies are present within hepatocytes. The pathologic lesions are more severe in younger birds, whereas some infected birds show no prodromal signs and died acutely within three days. The virus is transmitted horizontally to uninfected flocks through contact and vertically through embryonated eggs (McFerran and Adair, 1977).

The agent fowl adenovirus-4 causes immunosuppression by damaging lymphoid tissues. The presence of Infectious Bursal Disease (IBD) and Chicken Infectious Anemia (CIA) viruses may predispose to IBH-HPS and this may further predispose to other viral infections (Balamurugan and Kataria, 2006). Singh *et al.* (2006) reported a reduced reaction to T cell-dependent and T cell-independent antigen in FAV-1-infected animals, along with a reduced mitogenic response of peripheral blood lymphocytes obtained from FAV-1 infected chicken after PHA-P stimulation.

There has been an extensive use of formalin-inactivated, liver homogenate vaccines for the prevention and control of disease. Various vaccine formulations are being used in the field but none of them fulfill the criteria of eliciting a prompt and long-lasting immune response against natural outbreaks of IBH-HPS (Khan *et al.*, 2005). The immune response induced by these vaccines is not always consistent or predictable. Inactivated antigens require an oily formulation for the production of efficacious vaccines. In animal models, many novel adjuvants reported to be effective in enhancing antibody and/or cell-mediated immune responses have been described (Lawrence, *et al.*, 1997; Aucouturier and Ganne, 2000). In particular, the Montanide™ ISA series of water-in-oil emulsion adjuvants have shown superior efficacy with a variety of human and animal vaccines (Cox *et al.*, 2003). However, even if Montanide™ adjuvants have not previously been tested for their ability to enhance the immunogenicity of embryonated egg-adapted, IBH-HPS vaccines, Montanide™ ISA 70 (W/O) and Montanide™ ISA 206 (W/O/W) have already been demonstrated as safe and efficient in numerous

poultry disease models (Dupuis *et al.*, 2006; Belloc *et al.*, 2008).

The present study was designed to develop and evaluate embryonated egg-adapted, Montanide-adjuvanted IBH-HPS vaccine for the protection of chickens against IBH-HPSV challenge, in comparison to formalin-inactivated and commercial oil-based IBH-HPS vaccines.

MATERIALS AND METHODS

Source of antigen

Fowl adenovirus type-4, which has been characterized and sequenced by Mansoor *et al.* (2009), was obtained from the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The virus was propagated in 11-day-old, embryonated chicken eggs. Allantoic fluid was harvested and stored at -20°C until further use.

Quantification / identification of antigen

Harvested fluids were tested for qualitative and quantitative estimation of the virus on the basis of reverse passive haemagglutination test (RPHA) (Manzoor and Hussain, 2003) and Agar gel precipitation test (AGPT) (Beard, 1970).

Measurement of EID₅₀ of IBH-HPS virus

Ten fold dilution of isolated virus was made from 10⁻¹ to 10⁻⁵. The infectivity of the virus was checked in 9-day-old, live, embryonated chickens eggs (Reed and Muench, 1938).

Formalin-inactivated vaccine

Vaccine was prepared by using 10^{3.8} EID₅₀ per ml of IBH-HPSV in phosphate buffered saline and inactivated with 0.5% formalin Montanide-adjuvanted *IBH-HPS vaccine*

Montanide (ISA-206)-adjuvanted, IBH-HPS vaccine was prepared using 10^{3.8} EID₅₀ of IBH-HPS virus inactivated with 0.5% formalin, followed by homogenizing the aqueous and non-aqueous phases at 50:50 v/v.

Experimental chicks

Three hundred one-day-old broiler chicks were purchased from the market and reared in the

experimental animal house of the Institute of Veterinary Microbiology, University of Agriculture, Faisalabad. The chicks were offered commercial broiler feed and water *ad libitum*.

Experimental design

Fifteen-day-old chicks were divided into five groups (A, B, C, D, and E) of 60 chicks. Group A was vaccinated with formalin-inactivated IBH-HPS vaccine. Group B was vaccinated with commercial oil-based vaccine. Group C was vaccinated with Montanide-adjuvanted IBH-HPS vaccine. Groups D and E were unvaccinated for challenged (positive control) and unchallenged (negative control) groups, respectively. Chicks in all groups were injected subcutaneously with a dose volume of 0.3 ml. The chicks in each group (A, B, C, D, and E) were maintained in separate isolation units, which were thoroughly cleaned and disinfected before housing the birds, and the birds were offered feed and water *ad libitum*.

Antibody titration against IBH-HPSV by IHA

Blood was withdrawn from the wing vein of chickens and antibody titers against IBH-HPSV were monitored weekly by IHA test (Rahman *et al.*, 1989) for 4 weeks post-immunization. In each group, geometric mean titers (GMT) were calculated (Burgh, 1977).

The *in vivo* lymphoproliferation assay was performed by injecting 100 µg of PHA-P into the toe webs of all of the chickens in the study at 21 days of age, as described by Mahmood *et al.* (2006). The toe web between the third and fourth digits of the left foot was injected with 100 µg of PHA-P dissolved in 100 µl of PBS. The right foot was injected in the identical manner to that of the left foot with 100 µl of PBS to serve as control. The toe webs were measured using a constant tension caliper at 48 and 72 h after PHA-P injection. The data were expressed as the PHA-P-mediated swelling minus control swelling in millimeters in all groups.

Evaluation of protection against HPSV challenge

At 7 days post-vaccination, 20 chickens from each group were orally inoculated with $10^{4.8}$ mean embryonic infective dose (EID₅₀) of HPSV (Reed and

Muench, 1938). Clinical signs and mortality were recorded daily and all challenge survivors were slaughtered at day 3 post-challenge. The remaining unchallenged chickens in each group were maintained for later serum antibody assay until 28 days post-immunization.

Mean body weight and organ to body weight ratios

On days 7, 14, 21 and 28 post-infection, five birds selected at random from each group were weighed individually and then slaughtered. The BF, spleen, and liver of these birds were weighed separately and the ratios of these organs to body weight were calculated. Gross lesions in the lymphoid and various other organs were recorded (Shivachandra *et al.*, 2003).

Statistical analysis

The data were analyzed by one way ANOVA and Tukey's test (Steel and Torrie, 1984).

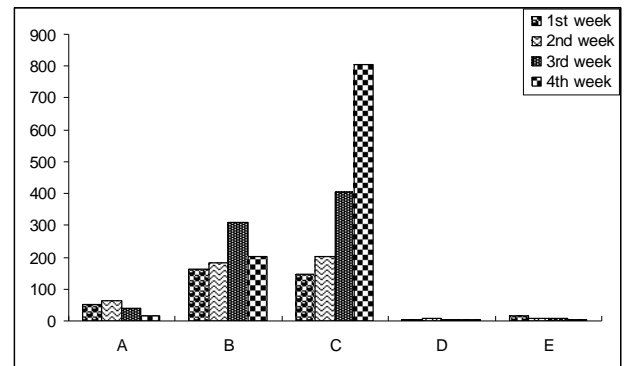


Fig. 1. Cumulative comparison of GMT of all groups

RESULTS

Detection of antibodies to IBH-HPSV

In the pre-vaccination stage, 10 birds were randomly selected and serum samples revealed IHA antibody titers against IBH-HPSV ranging from 8 to 16. This indicated that there were maternal antibodies present in the chicks, but these antibodies were not at a protective level, because unvaccinated birds later died after challenge (Hussain *et al.*, 1999). The serum samples were examined by IHA for antibody titer to IBH-HPSV at 7, 14, 21, and 28 days of age. As shown in Figure 1, the antibody titer to IBH-HPSV in chickens vaccinated with

Montanide-adjuvanted vaccine increased from 1 to 4 weeks after post-immunization as compared to all other groups. At 3-4 weeks post-immunization, the mean antibody titers of groups A and B were (38, 16) and (310, 204), respectively, which differ significantly from each other but titers of both groups were significantly lower than those of group C (404, 806). Mean antibody titers of group D and E at 3-4 weeks post-immunization was 4, 4 and 4, 4, respectively, which were considered baseline.

Cell-mediated immunity

The response of chickens to PHA-P injection measured at 48 and 72 h post-PHA-P injection are presented in Table I. In this study, chickens of group C exhibited significantly higher swelling than all of the other groups in response to PHA-P injection, indicating that lymphoproliferation ability was highest in the chickens vaccinated with Montanide-adjuvanted IBH-HPS vaccine. Group B exhibited significantly higher lymphoproliferation than Group A. The control groups D and E also exhibited normal T cell activation in response to PHA-P injection, which was comparable to the swelling exhibited by group C.

Table I.- Skin thickness in mm (Mean±SE) in PHA-P-induced, delayed type hypersensitivity (DTH) reaction in different groups at 48 and 72 hours

Group	48 h	72 h
A	0.70 ± 0.02 ^c	0.68 ± 0.04 ^c
B	0.92 ± 0.04 ^b	1.16 ± 0.01 ^b
C	1.44 ± 0.05 ^a	1.50 ± 0.02 ^a
D	0.56 ± 0.03 ^c	0.75 ± 0.04 ^c
E	0.64 ± 0.01 ^c	1.21 ± 0.02 ^b

^{a-c}Values with different superscripts within a column vary significantly at P<0.05

Group A, vaccinated with formalin-inactivated IBH-HPS vaccine; Group B, vaccinated with commercial oil-based IBH-HPS vaccine; Group C, Montanide-adjuvanted IBH-HPS vaccine; Group D, unvaccinated, challenged, positive control; Group E, unvaccinated, unchallenged, negative control.

Mean body weight gain

The mean body weights of the chicks in the various groups are presented in Table II. The chicks in groups A, B, and D had lower mean body weight than those in the other groups. Along with reduced weight gain, the chickens of Groups A, B, and D

showed mild dullness and depression with a reduced feed intake.

Table II.- Body weight in grams (Mean±SE) at weekly intervals in experimental groups after challenge.

Group	0 week	1 week	2 weeks	3 weeks	4 weeks
A	273 ± 32 ^c	375 ± 33 ^c	550 ± 14.5 ^b	800 ± 15 ^c	1250 ± 31 ^a
B	258 ± 27.8 ^c	450 ± 34 ^c	620 ± 26.4 ^b	850 ± 38 ^c	1470 ± 19 ^a
C	265 ± 31.5 ^c	640 ± 24 ^b	800 ± 29 ^b	1350 ± 32 ^a	1770 ± 21.5 ^a
D	268 ± 28.6 ^c	375 ± 19.5 ^c	550 ± 25 ^b	860 ± 12.5 ^b	1150 ± 27 ^a
E	262 ± 23.6 ^c	575 ± 22 ^b	680 ± 24 ^b	1350 ± 18.2 ^a	1580 ± 33 ^a

*these broiler chickens were vaccinated at 15 days of age

^{a-c} values with different superscripts within a column vary significantly, P<0.05

Group A: vaccinated with formalin-inactivated IBH-HPS vaccine; Group B: vaccinated with commercial oil-based IBH-HPS vaccine; Group C: Montanide-adjuvanted IBH-HPS vaccine; Group D: unvaccinated, challenged positive control; Group E: unvaccinated, unchallenged negative control.

Mean organ to body weight ratios

The mean ratios of organ to body weight of chicken are presented in the Table III. In Groups A and D, there was significant reduction in the mean ratio of the BF or spleen to body weight, but significant higher mean ratio of liver to body weight compared to other groups. Grossly, chicks from Groups A and B had discolored and swollen livers. Enlargement of the liver and progressive atrophy of the BF and thymus were present in Groups A and D. Chicks in Group E (unvaccinated, unchallenged) showed no signs of hydropericardium syndrome.

Protection against challenge

To evaluate the protective efficacy of immunization against IBH-HPSV challenge, all vaccinated and positive control chickens were challenged with 10^{4.8} EID₅₀ of IBH-HPSV, at 21 days of age. As shown in Table IV, the *Montanide-adjuvanted* vaccine in Group C conferred 95% protection, comparable to Group B (80%) and Group A (70%), against clinical signs and mortality, whereas protection was minimal in Group D, the unvaccinated control. After challenge, 65 percent of

Table III.- Organ to body weight ratio (Mean±SE).

Organ	Group	1 week	2 weeks	3 weeks	4 weeks
Bursa	A	0.15 ± 0.00 ^c	0.13 ± 0.01 ^c	0.14 ± 0.00 ^c	0.17 ± 0.02 ^c
	B	0.19 ± 0.02 ^b	0.16 ± 0.01 ^b	0.18 ± 0.01 ^b	0.19 ± 0.00 ^b
	C	0.20 ± 0.01 ^b	0.22 ± 0.01 ^b	0.24 ± 0.01 ^a	0.25 ± 0.00 ^a
	D	0.11 ± 0.00 ^c	0.10 ± 0.00 ^c	0.13 ± 0.02 ^c	0.15 ± 0.01 ^c
	E	0.29 ± 0.03 ^a	0.30 ± 0.00 ^a	0.27 ± 0.00 ^a	0.29 ± 0.02 ^a
Spleen	A	0.12 ± 0.01 ^c	0.13 ± 0.00 ^b	0.12 ± 0.00 ^c	0.13 ± 0.00 ^b
	B	0.13 ± 0.02 ^b	0.13 ± 0.00 ^b	0.14 ± 0.00 ^b	0.15 ± 0.02 ^a
	C	0.15 ± 0.01 ^a	0.15 ± 0.00 ^a	0.16 ± 0.00 ^a	0.15 ± 0.01 ^a
	D	0.11 ± 0.04 ^c	0.12 ± 0.01 ^c	0.10 ± 0.00 ^c	0.12 ± 0.01 ^c
	E	0.16 ± 0.00 ^a	0.15 ± 0.02 ^a	0.16 ± 0.02 ^a	0.17 ± 0.01 ^a
Liver	A	6.00 ± 0.03 ^a	6.10 ± 0.09 ^a	5.75 ± 0.06 ^b	5.08 ± 0.08 ^b
	B	5.75 ± 0.02 ^b	5.75 ± 0.02 ^b	4.95 ± 0.06 ^c	5.00 ± 0.05 ^b
	C	5.00 ± 0.04 ^b	5.15 ± 0.04 ^b	4.75 ± 0.09 ^c	5.11 ± 0.02 ^b
	D	6.30 ± 0.05 ^a	6.50 ± 0.07 ^a	6.00 ± 0.05 ^a	5.75 ± 0.09 ^b
	E	4.85 ± 0.06 ^c	4.45 ± 0.08 ^c	5.00 ± 0.04 ^b	4.75 ± 0.03 ^c

^{a-c} values with different superscripts within a column vary significantly, P<0.05

Group A, vaccinated with formalin-inactivated IBH-HPS vaccine; Group B, vaccinated with commercial oil-based IBH-HPS vaccine; Group C, Montanide-adjuvanted IBH-HPS vaccine; Group D, unvaccinated, challenged, positive control; Group E, unvaccinated, unchallenged, negative control.

Table IV.- Protection level of different vaccines after challenge.

Group	No. of chicks	Total mortality	% mortality	% Protection
A	20	6	30	70
B	20	4	20	80
C	20	1	5	95
D	20	13	65	35
E	20	--	--	N.A.

* Chicks were challenged at 7 days post-vaccination

Group A, vaccinated with formalin-inactivated IBH-HPS vaccine; Group B, vaccinated with commercial oil-based IBH-HPS vaccine; Group C, Montanide-adjuvanted IBH-HPS vaccine; Group D, unvaccinated, challenged, positive control; Group E, unvaccinated, unchallenged, negative control.

the chickens in the unvaccinated control group died due to IBH-HPS. There was a considerable amount of clear straw-colored fluid accumulated in the pericardial sac of these chickens, which showed clinical signs and/or died due to the disease. Livers of these chickens were discolored, pale yellow, swollen, and friable, and the kidneys were swollen. The chickens surviving the challenge were slaughtered and postmortem (PM) examination was performed. These birds also exhibited typical PM lesions of IBH-HPS.

DISCUSSION

Inclusion body hepatitis-hydropericardium syndrome (IBH-HPS), is an emerging disease of

poultry, and has recently been detected in some countries of Asia and America, particularly in broiler birds aged 3-6 weeks (Chandra *et al.*, 2000). The chickens vaccinated with formalin-inactivated IBH-HPS vaccines are not fully protected against IBH-HPSV challenge; there was a rise in antibody (IHA) titer in the 1st week post-immunization that peaked in the 2nd week (GMT 64) followed by decline (GMT 38, 16) in the 3rd and 4th weeks post-immunization. These results are in accordance with those of previous workers (Afzal and Ahmad, 1990; Ahmad *et al.*, 1990). There was a low but consistent IHA titer in pre-vaccination (GMT 2-8) and Groups D and E (GMT 4, 2, respectively). An IHA titer of less than 1:8 is considered negative (Afzal and Ahmad, 1990). When broiler chickens were

immunized with commercial oil-based IBH-HPS vaccine, the IHA titer at the 3rd week was significantly increased (GMT 310), with a decreasing trend at the 4th week (GMT 204). The *Montanide-adjuvanted* IBH-HPS vaccine, on the other hand, showed an increasing weekly trend in the IHA titer, which remained consistently high at the 3rd and 4th weeks (GMT 404, 806, respectively). The results of the present study are thus in line with Hussain *et al.* (1996).

The repository adjuvant holds the antigen at the site of deposition, delays its absorption, and subsequently released antigen behaves as a secondary stimulus to the sensitizing action of the earlier-released antigen (Frost and Lance, 1973). A depot theory of adjuvant action has been proposed for water-in-oil emulsions (Freund *et al.*, 1937). Inactivated antigens require an oily formulation for the production of efficacious vaccines (Dupuis *et al.*, 2006). Montanide™ ISA 206 and ISA 70 also allow the manufacture of water in oil in water (W/O/W) and water in oil (W/O) comprised of a high grade injectable mineral oil specifically developed to not trigger a cellular immune response (Cox *et al.*, 2003). These adjuvants can be selected to avoid the commonly seen side effects that are associated with other mineral oil emulsions, such as incomplete Freund's adjuvant, which may produce inflammatory reactions, granulomas, and ulcers at the injection site (Hafeez, 2011). As a result, 70%, 80%, and 95% chickens were protected against challenge with IBH-HPSV in Groups A, B, and C, respectively, whereas in Group D there was 35% only, who survived. Immunization with formalin-inactivated IBH-HPS vaccine provided good protection against challenge, but was not long-lasting, requiring administration of a booster. Formalin-inactivated vaccines are prepared by mincing the livers of chickens from flocks that have been infected with adenovirus and induced to develop clinical IBH-HPS syndrome. Neither the severity of induced infection nor the flock history and laboratory conditions have been uniform, resulting in an inconsistent titer of the virus among and/or between the different batches of vaccine. The immune response provoked by these vaccines is not always consistent and predictable. By using these liver homogenates there is a greater risk of

secondary bacterial infection as well (Khan *et al.*, 2005). Oil-adjuvanted vaccines, however, confer good, long-lasting protection against challenge (Sahidullaha *et al.*, 2008).

When lectin PHA-P is injected intradermally into the chickens, the response primarily involves stimulation of T cell division with minimal effect on B cells (Tizard, 1994; Naeem *et al.*, 1995a). Therefore, lymphoproliferation in response to PHA-P is considered a good *in vivo* measure of T lymphocyte function. In this study, lymphoproliferative ability was significantly higher in the chickens vaccinated with Montanide-adjuvanted IBH-HPS vaccine (Group C), followed by the chickens vaccinated with commercial oil-based vaccine (Group B), and compared with other groups. The avian adenovirus has a predilection for lymphoid tissues, which can result in immunosuppression (Naeem *et al.*, 1995b) and the birds were further immunocompromised owing to concurrent infections, with the involvement of lymphoid organs (Pettit and Carlson, 1972). The mean body weight gain was significantly lower ($P < 0.05$) in the chickens of Groups A and D, followed by Group B, as compared with group C and E. These findings agree with the report of Shivachandra *et al.* (2003). There was significant reduction in the ratio of the BF or spleen to body weight in Groups A and B, followed by Groups C and E, and there was significantly greater reduction in the ratio of the BF or spleen to body weight in Group D (challenged, unvaccinated). There was significantly higher mean ratio of liver to body weight in Groups A and D, followed by Groups B and E, and there was lower mean ratio of liver to body weight in Group C. The gross findings of pale enlarged liver, and atrophy of BF and spleen were similar to those reported by Jaffery (1988).

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REFERENCES

- ABE, T, NAKAMURA, K, TOJO, H., MASE, M., SHIBAHARA, T, YAMAGUCHI S. AND YUASA, N., 1998. Histology, immuno-histochemistry and ultra-

- structure of hydropericardium syndrome in adult broiler breeders and broiler chicks. *Avian Dis.*, **42**:606-12.
- AFZAL, M. AND AHMAD, I., 1990. Efficacy of an inactivated vaccine against hydropericardium syndrome in broiler. *Vet. Rec.*, **126**:59-60.
- AHMAD, I., MALIK, M.I, IQBAL, K., AHMAD, K. AND NAZ, S., 1990. Efficacy of formalinized liver-organ vaccine against Angara disease in broilers. *Vet. Archiv.*, **60**: 131-8.
- ASRANI, R.K., GUPTA, B.K., SHARMA, S.K., SINGH, S.P. AND KATOCH, R, C., 1997. Hydropericardium hepatopathy syndrome in Asian poultry. *Vet. Rec.*, **141**:271-273.
- AUCOUTURIER, J. AND GANNE, V., 2000. Assessment of oil adjuvants in Newcastle disease vaccine. In: *Proceedings of the 20th World Poultry Congress, Montreal, Canada, August 20-25*.
- BALAMURUGAN, V. AND KATARIA, J.M., 2006. Economically important non-oncogenic immunosuppressive viral diseases of chickens-current status. *Vet Res Communi.*, **30**: 541-566.
- BEARD, C.W., 1970. Demonstration of type specific antibody in mammalian and avian sera by immunodiffusion. *Bull. Wld. Hlth. Org.*, **42**: 779-785.
- BELLOC, C., DUPUIS, L., DEVILLE, S., AUCOUTURIER, J AND LAVAL, A., 2008. Evaluation of safety and immune response induced by several adjuvants included in *Pasteurella multocida* vaccines in chickens. *Rev. Med. Vet.*, **159**: 371-375.
- BENKO, M., HARRACH, B., BOTH, G.W., RUSSELL, W.C., ADAIR, B.M., ADAM, E., DE JONG, J.C., HESS, M., JOHNSON, M., KAJON, A., KIDD, A.H., LEHMKUHL, H.D., LI, Q.G., MAUTNER, V., PRING, A., KERBLUM, P. AND WADELL, G., 2005. Family Adenoviridae. In: *Virus taxonomy. Eighth report of the international committee on taxonomy of viruses* (eds. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball). Elsevier, New York, pp. 213-228.
- BURGH, M.J., 1977. A simple method of recording and analyzing serological data. *Avian Dis.*, **22**:362-365.
- CHANDRA, R., SHUKLA, S.K. AND KUMAR, R., 2000. The hydropericardium syndrome and inclusion body hepatitis in domestic fowl. *Trop. Anim. Hlth. Prod.*, **32**:99-111.
- COX, S.J., AGGARWAL, N., STATHAM, R.J AND BARNETT, P.V., 2003. Longevity of anti- body and cytokine responses following vaccination with high potency emergency FMD vaccines. *Vaccine*, **21**, 1336-1347.
- DUPUIS, L., ASCARATEIL, S., AUCOUTURIER, J. AND GANNE, V., 2006. Seppic vaccine adjuvants for poultry. *Ann. N. Y. Acad. Sci.*, **1081**: 202-205.
- FREUND, J., CASALS, J. AND HOSMER, E.P., 1937. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc. Soc. exp. Biol. Med.*, **37**: 509-513.
- FROST, P. AND LANCE, E.M., 1978. On the mechanism of action of adjuvant. *Immunology*, **35**:63-68.
- HAFEEZ, H.M., 2011. Avian adenovirus infection with special attention to IBH-HPS & EDS. *Pakistan Vet. J.*, **31**: 85-92.
- HESS, M., RAUE, R. AND PRUSAS, C., 1999. Epidemiological studies on fowl adenoviruses isolated from cases infectious hydropericardium. *Avian Dis.*, **28**: 433-9.
- HUSSAIN, I., ANJUM, A.A. AND KHAN, I.H., 1996. An oil emulsion vaccine against Hydropericardium syndrome in broiler chickens. *Pakistan Vet. J.*, **16**: 125-127.
- HUSSAIN, I., MUNIR, R., AKTHAR, M. AND AHMAD, R., 1999. Evaluation and comparison of Hydropericardium syndrome vaccine in broiler chicks. *Pakistan Vet. J.*, **19**: 88-90.
- JAFFER, M.S., 1988. A treatise on Angara disease (hydropericardium-pulmonary oedema- hepatonephritis syndrome. *J. Pak. Vet. med. Assoc.*, **34**: 1-33.
- KHAN, A.A., SABRI, A.N., MANSOOR, M.K. AND HUSSAIN, I., 2005. Hydropericardium syndrome in Pakistan: a review. *World Poul. Sci. J.*, **61**: 647-654.
- KUMAR, R. AND CHANDRA, R., 2004. Studies on structural and immunogenic polypeptides of hydropericardium syndrome virus by SDS-PAGE and western blotting. *Comparative Immunol. Microbiol. Inf. Dis.*, **27**, 155-161
- KUMAR, R., CHANDRA, R., SKUKLA, S.K., AGRAWAL, D.K. AND KUMAR, M., 1997. Hydropericardium syndrome in India: a preliminary study on causative agent and control of the disease by inactivated autogenous vaccine. *Trop. Anim. Hlth. Prod.*, **29**:158-164.
- LAWRENCE, G.W., SAUL, A., GIDDY, A.J., KEMP, R. AND PYE, D., 1997. Phase I trial in humans of an oil-based adjuvant SEPPIC MONTANIDE ISA 720. *Vaccine*, **15**, 176-178.
- MAHMOOD, M.S., SIDDIQUE, M., HUSSAIN, I., KHAN, A. AND MANSOOR, M.K., 2006. Protection capability of recombinant plasmid DNA vaccine containing VP2 gene of very virulent infectious bursal disease virus in chickens adjuvanted with CpG oligodeoxynucleotide. *Vaccine*, **24**: 4838-4846.
- MANSOOR, M.K., HUSSAIN, I., ARSHAD, M., MUHAMMAD, G., HUSSAIN, M.H AND MEHMOOD, M.S., 2009. Molecular characterization of Fowl adenovirus 4 (FAV0-4) isolate associated with fowl hydropericardium-hepatitis syndrome in Pakistan, *Pakistan J. Zool.*, **41**: 269-276.
- MANZOOR, S. AND HUSSAIN, I., 2003. Reverse passive haemagglutination (RPHA) test for detection and quantification of hydropericardium syndrome virus (HPSV). *Pakistan J. Life Soc. Sci.*, **1**:141-143.

- MCFERRAN, J.B AND ADAIR, B.M., 1977. Avian adenovirus-A review. *Avian Dis.*, **6**; 189-217.
- MCFERRAN, J.B., 1997. Group 1 adenovirus infections, In: *Diseases of poultry* (eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif). 10th ed. Ames: Iowa State University Press, pp. 608-20.
- MONREAL, G., 1996. History and development of research about avian adenoviruses. *Proc. Intl. Symp. Adeno Reo viruses Inf. Poul. Germany*, pp. 4-9.
- NAEEM, K., RABBANI, M., HUSSAIN, M. AND CHEEMA, A.H., 1995b. Development of cell culture vaccine against Hydropericardium syndrome in poultry. *Pakistan Vet. J.*, **15**: 150-151.
- NAEEM, K., NIAZI, T., MALIK, S.A. AND CHEEMA, A.H., 1995b. Immunosuppressive potential and pathogenicity of an adenovirus isolate involved in hydropericardium syndrome in broilers. *Avian Dis.*, **39**: 723-728.
- PETTIT, J.R. AND CARLSON, H.C., 1972. Inclusion body hepatitis in broiler chickens. *Avian Dis.*, **16**: 858-863.
- RAHMAN, S., ASHFAQ, M., ANJUM, A.D. AND SINDHU, T.A., 1989. Indirect haemagglutination test for detecting Angara disease agent antibody. *1st Int. Conf. Trade Show Poul. Prod. Karachi*, pp. 73-74.
- REED, L.J. AND MUENCH, H., 1938. A simple method of estimating fifty percent end point. *Am. J. Hyg.*, **27**:493-494.
- SAHIDULLAHA, RAHMA, S.U., SHAN, M.K., RABBANI, M., NASEEM, S., KHAN, S.H AND MUHAMMD, K., 2008. Development of standard protocols for preparation and evaluation of liver homogenate vaccines against hydropericardium syndrome virus in poultry. *Pakistan. Vet. J.*, **28**: 163-166.
- SHANE, M.S., 1996. Hydropericardium-hepatitis syndrome, a current world situation. *Zootec. Int.*, **18**:20-27.
- SHIVACHANDRA, S.B., SAH, R.L., SINGH, S.D., KATARIA, J.M. AND MANIMARAN, K., 2003. Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAV-4) associated with hydropericardium syndrome. *Vet. Res. Commun.*, **27**: 39-51.
- SINGH, A., GREWAL, G.S., MAITI, N.K AND OBEROI, M.S., 2006. Effect of fowl adenovirus-1 (IBH isolate) on humoral and cellular immune competency of broiler chicks. *Comp. Immunol. Microbiol. Infect. Dis.*, **29**, 315-321.
- STEEL, R.G.D. AND TORRIE, J.H., 1984. *Principles and procedures of statistics*. McGraw Hill Book Co, New York, USA.
- TIZARD, I., 1994. *Immunology: an introduction*. 4th ed. NY: Saunders, New York.

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