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Research Article

# Protection and Efficacy of Cell Culture Propagated Montanide Adjuvant Based Inactivated Vaccine Against Hydropericardium Syndrome in Poultry

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## Abstract

The present study was designed to propagate field isolate of Fowl adenovirus-4 in chicken embryo liver (CEL) cell culture for development of efficient cell culture based inactivated vaccine. A pathogenic field isolate of fowl adenovirus-4 was propagated in chicken embryo liver cell culture. The liver homogenate virus ($10^{3.0}TCID_{50}$/ml) and cell culture propagated FAV-4 ($10^{3.0}TCID_{50}$/ml) was used for preparing water based and oil based inactivated vaccines. The post-vaccination antibody response to all vaccines was tested by Enzyme Linked Immunosorbant Assay (till 3 week post vaccination) and the chickens were subjected to challenge protection studies. The groups injected with oil base (Montanide) and water based conventional liver homogenate vaccines showed an average S/P (Sample/positive) value of 0.341 and 0.323 respectively, the groups given P-1 cell culture passaged oil (Montanide) based and water based vaccines showed an average S/P ratio 0.989 and 0.800 respectively. Cell culture based montanide adjuvanted vaccine showed highest antibody response (S/P) among all groups. Cell culture passaged vaccinated groups survived and provided 100% protection against challenge. Liver homogenate based vaccines provided 80% protection. As a whole the cell culture passaged vaccines qualified the known standards of safety, sterility and potency.

***Keywords:*** *Fowl adenovirus-4, Hydropericardium Syndrome, Chicken embryo liver cell culture, Inactivated vaccine*

# Kümes Hayvanlarında Hidroperikardium Sendromuna Karşı Hücre Kültüründe Üretilmiş Montanide Adjuvant Bazlı İnaktive Edilmiş Aşının Koruyucu Etkisi

## Özet

Bu çalışma tavuk embriyo karaciğer (CEL) hücre kültüründe Fowl adenovirus-4'ün saha izolatını üreterek etkili hücre kültürü temelli inaktive edilmiş aşı geliştirmek amacıyla tasarlanmıştır. Fowl adenovirus-4'ün patojenik saha izolatı tavuk embriyo karaciğer hücre kültüründe üretildi. Karaciğer homojenat virus ($10^{3.0}TCID_{50}$/ml) ve hücre kültüründe üretilmiş FAV-4 ($10^{3.0}TCID_{50}$/ml) su bazlı ve yağ bazlı inaktive edilmiş aşılar hazırlamak amacıyla kullanıldı. Tüm aşılara karşı aşılama sonrası antikor cevabın oluşup oluşmadığı Enzyme Linked Immunosorbant Assay (aşılama sonrası 3 haftaya kadar) ile test edildi ve tavuklar etkene karşı koruma çalışmasında kullanıldı. Yağ bazlı (Montanide) ve su bazlı konvansiyonel karaciğer homojenat aşılar enjekte edilen gruplar sırasıyla 0.341 ve 0.323 ortalama S/P (Örnek/Pozitif) değeri gösterdi. P-1 hücre kültürü pasajlanmış yağ bazlı (Montanide) ve su bazlı aşılar enjekte edilen gruplar sırasıyla 0.989 ve 0.800 ortalama S/P değeri gösterdi. Montanide adjuvantlı hücre kültürü temelli aşı tüm gruplar içerisinde en yüksek antikor cevabı (S/P) gösterdi. Hücre kültürü pasajlanmış aşılı gruplar hayatta kaldılar ve etkene karşı %100 koruma gösterdiler. Karaciğer homojenat bazlı aşılar %80 koruma sağladı. Sonuç olarak, hücre kültürü pasajlı aşılar bilinen güvenlik, sterilite ve potansiyel standartlarını sağlamıştır.

***Anahtar sözcükler:*** *Fowl adenovirus-4, Hidroperikardium sendromu, Tavuk embriyo karaciğer hücre kültürü, İnaktive aşı*

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# INTRODUCTION

Hydrpericardium syndrome (HPS) is re-emerging disease caused by Fowl adenovirus serotype-4 resulting in huge economic losses to poultry industry in Pakistan since 1987. In affected chickens disease is characterized by accumulation of transparent straw colored watery fluid in the pericardium and swollen discolored fragile liver [1].

HPS has been controlled using autogeneous formalin inactivated vaccine prepared from infected liver homogenate [2]. Different vaccine formulations are being used in the field but none of them provides prompt, effective and long-lasting immune response against the natural outbreaks of HPS. Immunization of broiler chicks with a single dose of inactivated liver homogenate provides protection against hydropericardium syndrome although double shot of inactivated liver organ vaccine may be much more efficacious in breeders [3]. Infected liver homogenate based vaccines result in spreading of HPS and outbreaks even occur after vaccination. So extensive use of infected liver homogenate based vaccine should also be minimized by attempting to propagate the virus on eggs and cell cultures for the production of inactivated vaccines [4]. Adjuvants have been effective in enhancing antibody response of inactivated vaccines. Montanide ISA 70 adjuvant has been efficacious in different animal models [5]. Despite lot of work on HPS, literature regarding motanide adjuvant based cell culture passaged FAV-4 inactivated vaccines is scanty.

The present study was designed to develop an efficacious cell culture based inactivated vaccine against HPS.

# MATERIAL and METHODS

### *Source of Virus*

A field isolate Pak/NARC-3317/2008 of FAV-4 recovered from clinically affected birds with HPS was used as vaccine virus in this study. This virus was earlier stored in the repository of National Reference Lab for poultry Diseases (NRLPD), National Agricultural Research centre, Islamabad. The virus was confirmed through PCR using standard procedure [6]. Amplification of a 730 bp variable part of the hexon gene was done by PCR to confirm the presence of viral DNA. The PCR products were analyzed on 1% agarose gels containing 0.1% ethidium bromide along with 1 Kb DNA ladder. PCR product was visualized by placing the gel in Gel documentation system.

### *Propagation of FAV-4 in Cell Culture*
### *Chicken Embryo Liver (CEL) Cell Culture*

Primary chicken embryo liver cells (CEL) were prepared in 25 $cm^2$ cell culture flasks using the standard protocol [7]. CEL monolayer was first infected with 0.2 ml of FAV-4 virus suspension and incubated up to 72 h or till >75% CPE was noticed. The flasks were freeze thawed thrice and virus was clarified after centrifugation at 1.500 rpm at 4ºC for 10 min. This virus propagated in CEL cell culture was saved at -70ºC till used. The preparation was labeled as FAV-4-CEL cell culture.

### *Vaccine Preparation*

Using liver homogenate FAV-4 and CEL cell culture propagated FAV-4 two types of vaccines with different combinations were prepared following the standard protocols of vaccine production [7]. The stock virus was inactivated for 48 h using formaldehyde to attain final concentration of 0.02%. To test viral inactivation the material was propagated in chicken embryo liver cell culture and tested for presence of FAV-4 by PCR. The inactivated virus stocks were saved at 4ºC till further used. This virus was used for preparing inactivated water based and oil based vaccines.

For preparing oil based liver homogenate inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie.

***A***

| | |
|---|---|
| FAV-4 liver homogenate ($10^5$ $TCID_{50}$/ml) | 3 ml |
| Montanide ISA-70 | 7 ml |

For preparing water based liver homogenate inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

***B***

| | |
|---|---|
| FAV-4 liver homogenate ($10^5$ $TCID_{50}$/ml) | 3 ml |
| Adjuvant (10% Aluminium hydroxide) | 0.1 ml |
| Water | 6.9 ml |

For preparing cell culture propagated oil based inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

***C***

| | |
|---|---|
| FAV-4/CEL/P1 stock ($10^5$ $TCID_{50}$/ml) | 3 ml |
| Montanide-70 | 7 ml |

***D***

For preparing cell culture propagated water based inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

| | |
|---|---|
| FAV-4/CEL/P1 stock ($10^5$ $TCID_{50}$/ml) | 3 ml |
| Adjuvant (10% Aluminium hydroxide) | 0.1ml |
| Water | 6.9 ml |

### *Vaccination of Birds*

100 day old broiler chicks were reared at the animal house of National Reference Laboratory for poultry

Diseases at NARC. For this purpose chicks were divided into five groups and kept in chicken isolators *(Table 1)*. In groups A and B a dose 0.2 ml per bird of inactivated liver vaccines were given subcutaneously at 8$^{th}$ day of their age in oil base and water base combination respectively. In group C and D birds were immunized with 0.2 ml of CEL cell culture propagated FAV-4. Group E was injected with Phosphate buffered saline (PBS).

### *Blood Sampling*

Blood samples were collected weekly from birds of all groups including control up to 3 week post-vaccination. Sera were separated by centrifuging at 1.500 rpm for ten minutes and further tested for immune response by ELISA using protocol given below.

### *Challenge Studies*

A field isolate of FAV-4 (Pak/NARC-3317/2008) was used for challenge studies. For this purpose birds in vaccinated and control groups were challenged three week post vaccination using $10^5$ $TCID_{50}$ of field isolate. Clinical observations were recorded and postmortem was done upon death of any bird during the experiment. After two weeks of challenge all surviving birds were necropsied and checked for HPS lesions.

### *Indirect ELISA*

Humoral immune response against FAV-4 was assessed by antibody detection using indirect ELISA in each group. ELISA was standardized by introducing some modifications in the procedure earlier reported in literature [8]. ELISA plate (96 well flat bottom polystyrene microtiter plate) was coated with 1:10 dilution of CEL cell culture propagated FAV-4 antigen in carbonate bicarbonate buffer (pH-9.6). 50 µl of diluted antigen was used for coating plates. After incubation of 90 min at 25°C plate was washed three times with PBS -Tween 20. Blocking of plate was done by addition of 100 µl of blocking solution (5% BSA) in each well. Plate was incubated for 1 h at 25°C and washed thrice with PBS-Tween-20 solution. Test samples were diluted (1.17) in dilution buffer (2% BSA) and 50 µl of diluted test sample was added in each well including positive (FAV-4 antiserum raised in chicken) a and negative control (Serum from Uninfected control chicken). Plate was incubated for 1 h at 25°C. After washing Plate 50 µl of 1:500 dilution of Horseraddish peroxidase conjugate was added and incubated for 40 min. 50 µl of Substrate (OPD 30% in phosphate citrate buffer pH 5.0 and 70% $H_2O_2$) was added in each well and incubated for 15 min. Reaction was stopped by adding 50 µl of 1M $H_2SO_4$ in each well. The absorbance values (OD) were read with ELISA reader at 492 nm. OD values from individual samples were used to calculate Mean sample-to-positive (S/P) ratio.

### *Ethical Committee Report*

The study was approved by the Intuitional Ethical Committee of the Animal Sciences Institute, NARC, thorough letter No. 01521/ASI/NARC.

## RESULTS

The virus was confirmed as Fowl adenovirus-4 through PCR (730 bp) *(Fig. 1)*. The virus inoculum from positive known FAV-4 serotype upon chicken embryo liver cells propagation showed cytopathic effects (CPE) after 48 h. CPE were characterized by rounding and clumping of cells *(Fig. 2)*. This was referred as passage 1 of FAV-4 and was used for cell culture based vaccine preparation. Confirmation of cell culture propagation of FAV-4 was done by PCR *(Fig. 3)*.

HPS specific antibody response was not detected at first week post vaccination by ELISA. HPS specific Antibody response of chicks in groups A, B, C, D, and E was detected positive by ELISA during second week post vaccination. Antibody response by ELISA was interpreted by finding the cut off value between negative and positive samples. Cut off value (0.116) was estimated as mean of known negative S/P ratio (0.026) plus two standard deviation [9].

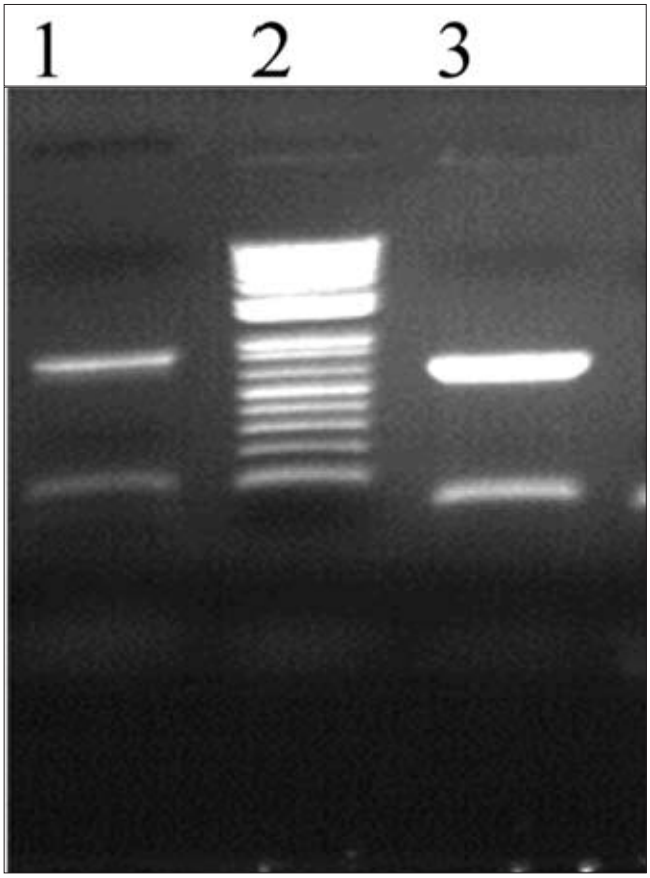


**Fig 1.** PCR amplification of hexon gene from field samples (Lane-1 FAV-4 positive sample 7 30bp hexon gene, Lane-2 Marker 1-kb plus, Lane-3 positive control)

**Şekil 1.** Saha örneklerinden hekzon geninin PCR amplifikasyonu (1. sıra FAV-4 pozitif örnek 7 30 bp hekzon geni, 2. sıra Markır 1-kb artı, 3. sıra pozitif kontrol)

**Fig 2.** CPE of P-1 CEL cell culture
**Şekil 2.** P-1 CEL hücre kültürünün sitopatik etkisi

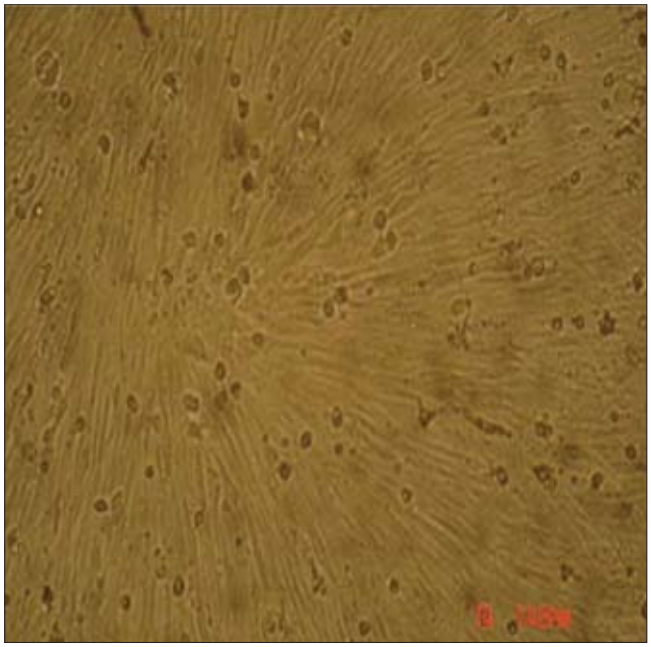

**Fig 3.** Uninfected Control monolayer
**Şekil 3.** Enfekte olmamış control monolayer

Samples giving OD higher than cut off values were considered positive and found protective.

The ELISA antibody response in groups C and D (Cell culture based montanide adjuvanted and water based vaccines) indicated mean S/P value of 0.665 and 0.496, respectively. These values were significantly higher ($P<0.05$) than those in groups A and B (mean S/P 0.287,C2, mean S/P 0.276) and were above cut off value. A significant

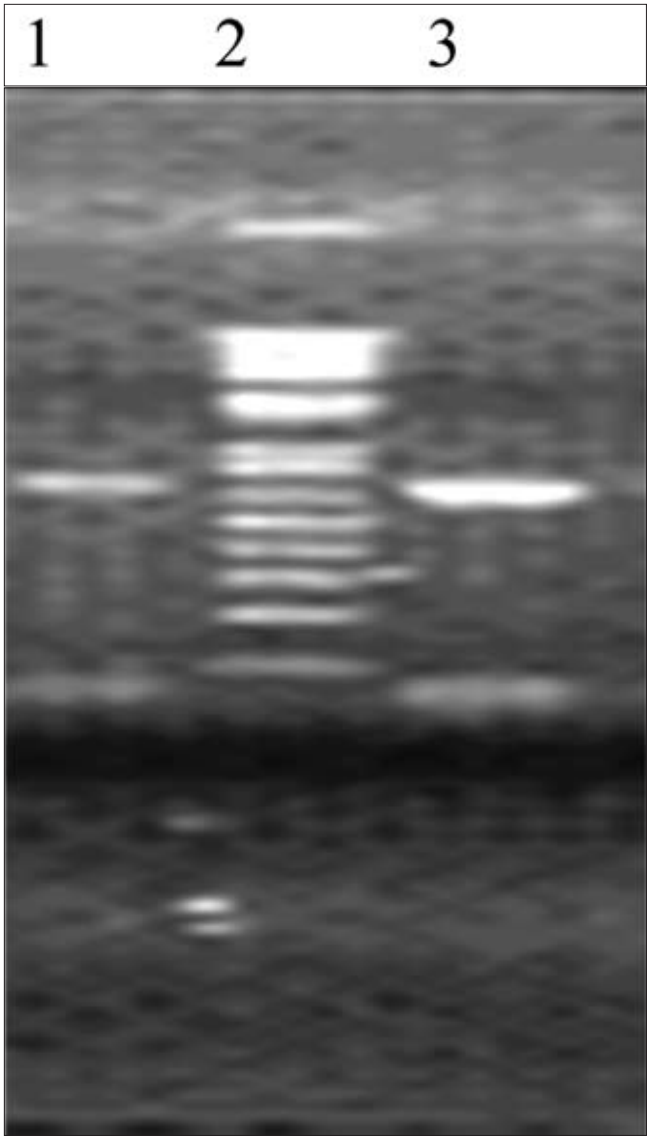


**Fig 4.** PCR data regarding FAV-4 grown in Chicken embryo liver cell culture **(**Lane-1 730 bp hexon FAV-4 P-1 CEL, Lane-2 Marker 1-kb plus, Lane-3 FAV-4 positive control)
**Şekil 4.** Tavuk embriyo karaciğer hücre kültüründe üretilmiş FAV-4 ilişkin PCR (1. Sıra 730 bp hekzon FAV-4 P-1 CEL, 2. sıra Markır 1-kb artı, 3. sıra FAV-4 pozitif kontrol)

increase ($P<0.05$) in ELISA S/P was recorded at 24$^{th}$ day PV in all groups *(Table 1)*.

Cell culture vaccine based on Montanide adjuvant showed highest antibody response (S/P 0.989). The chicks in unvaccinated control group did not indicate any seroconversion against HPS FAV-4 virus.

A challenge protection study was done for evaluation of the efficacy of cell culture based adjuvanted vaccine in comparison with liver homogenate based vaccines. Birds immunized with cell culture based adjuvanted vaccine having highest S/P (0.989) by ELISA showed 100% protection against hydropericardium syndrome. Birds in groups A and B immunized with liver homogenate vaccine

***Table 1.*** *Comparitive antibody response of inactivated oil based and water based vaccines by ELISA in terms of S/P values*
***Tablo 1.*** *ELISA ile belirleneni inaktive edilmiş yağ bazlı ve su bazlı aşıların karşılaştırmalı antikor cevapları (S/P değerine göre)*

| ID | Vaccine Type | 1 Week Post Vaccination Mean S/P | 2 Week Post Vaccination Mean S/P | 3 Week Post Vaccination Mean S/P |
|---|---|---|---|---|
| A | Tissue homogenate(Liver) Vaccine (Montanide ISA 70 based) | 0.053 | 0.287 | 0.341 |
| B | Tissue homogenate (Liver) Vaccine (Water based) | 0.082 | 0.276 | 0.323 |
| C | CEL Culture Propagated P-1 (Montanide ISA 70 based) | 0.043 | 0.665 | 0.989 |
| D | CEL Culture Propagated P-1 (Water based) | 0.032 | 0.496 | 0.800 |
| E | PBS (Control) | 0.050 | 0.053 | 0.054 |

(commercially used in Pakistan) showed 80% protection. Birds in group E (negative control) showed 90% mortality. Dead birds showed typical signs of HPS (Watery fluid around heart).

## DISCUSSION

Hydropericardium Syndrome (HPS) is re-emerging disease of broilers (3-6 weeks old) and breeders (6-20 weeks old) resulting in high mortality [10]. Formalin-inactivated liver organ vaccines are the only available source of vaccines against HPS in Pakistan which have been unable to control/eradicate disease [4]. For the development of an efficacious vaccine against HPS chicken embryo liver cell culture was used for continuous passages in this study. Protective efficacy of an oil adjuvanted cell culture adapted vaccine has been found superior to the liver homogenate vaccine [11].

In the present study CEL cell culture based montanide and water adjuvanted vaccines (C and D) were tested for efficacy in chicken. The results revealed that virus neutralizing antibody response rose at day 16 after the administration of vaccines. At 24$^{th}$ day post vaccination there was an increase in the antibody response. Cell culture montanide adjuvanted vaccine (C) at 16$^{th}$ and 24$^{th}$ day post vaccination showed significantly higher level ($P<0.05$) of antibody response (S/P) in birds as compared to cell culture water based vaccinated group (D).

In the present study the efficacy of cell culture based inactivated adjuvanted vaccines was tested and compared with liver homogenate vaccines (Commercially used in Pakistan) of HPS. The results revealed that in Group C (cell culture based montanide adjuvanted vaccine) and D (cell culture based water adjuvanted vaccine) the antibody response at week 2 post vaccination was significantly higher ($P<0.05$) than groups A and B (Tissue homogenate based inactivated vaccines). In general, the antibody response in Montanide adjuvanted cell culture based vaccine-inoculated birds (C) was significantly higher throughout the period of the experiment in comparison to the liver homogenate vaccine-administered groups (A and B) and cell culture based water adjuvanted vaccinated group (D). These observations suggested the superiority of Inactivated Montanide adjuvanted cell culture based vaccine over the commercial liver homogenate vaccines.

During earlier days of investigation many attempts were made for the control of hydropericardium syndrome in broilers by using formalin inactivated liver homogenate vaccines and there have been a lot of contrary findings regarding the efficacy of such liver homogenate vaccines [12,13]. The results of the present study suggested that chicken embryo liver cell culture based inactivated vaccines performed best in experimental conditions as compared to liver homogenate vaccine. Our results are in close agreement with already reported work [4].

The results of challenge protection study revealed that cell culture propagated montanide adjuvant and water based inactivated vaccines having higher ELISA S/P values gave maximum protection of 100% to chicks which is comparable with a recent study [5] who attained 94% protection of birds using Montanide adjuvanted egg adapted FAV-4 vaccine. The liver homogenate vaccine-inoculated groups A and B showed 80% protection.

The objective of the present study was to develop cell culture based efficacious vaccine against Hydropericardium syndrome in poultry. Cell culture based inactivated montanide adjuvanted vaccine performed best in experimental conditions and provided 100% protection. It is therefore recommended that Commercial tissue homogenate based vaccine should be replaced with cell culture based inactivated vaccines.

## REFERENCES

**1. Ahmad I, Afzal M, Malik MZ, Hanif W:** Studies on the disease pattern and etiology of hydropericardium syndrome (Angara disease) in broiler chickens in Pakistan. *Pak J Agric Res,* 10, 195-199, 1989.

**2. Chishti MA, Afzal M, Cheema AH:** Preliminary studies of the development of vaccine against the hydropericardium syndrome. *Rev Sci Tech Off Int Epiz,* 8, 797-801, 1989.

**3. Hussain I, Munir R, Ahmad R:** Evaluation and comparison of hydropericardium syndrome vaccines in broiler chicks. *Pak Vet J,* 19, 88-90, 1999.

**4. Khan AA, Sabri AN, Mansoor MK, Hussain I:** Hydropericardium syndrome in Pakistan: A review. *World's Poul Sci J,* 8, 647-654, 2005. DOI: 10.1079/WPS200576

**5. Aslam A, Hussain I, Mahmood MS, Khan A, Akhtar M:** Development and evaluation of montanide adjvanted vaccine for protection of chicken against inclusion body hepatitis hydropericadndrium syndrome. *Pak J Z,* 44, 109-116, 2012.

**6. Ganesh K, Suryanarayana VV, Raghavan R:** Detection of fowl adenovirus associated with hydropericardium hepatitis syndrome by a polymerase chain reaction. *Vet Res Commun,* 26, 73-80, 2002. DOI: 10.1023/A:1013361906791

**7. Naeem K, Niazi T, Malik SA, Cheema AH:** Immunosuppressive potential and pathogenecity of an avian adenovirus isolate involved in hydropericardium syndrome in broilers. *Avian Dis,* 39, 723-728, 1995.

**8. Balamurugan VJ, Katari M, Tiwari AK, Verma KC, Toroghi R, Jadhao SJ:** Development of sandwich ELISA for the detection of fowl adenovirus 4 associated with hydropericardium syndrome in experimentally infected chicken. *Acta Virol,* 45, 95-100, 2001.

**9. Mansoor MK, Hussain I, Arshad M, Muhammad G:** Preparation and evaluation of chicken embryo-adapted fowl adenovirus serotype 4 vaccine in broiler chickens. *Trop Anim Health Prod,* 43, 331-338, 2010. DOI: 10.1007/s11250-010-9694-z

**10. Chandra R, Shukla SK, Kumar M, Garg SK:** Electron microscopic demonstration of an adenovirus in the hepatocytes of birds experimentally infected with hydropericardium syndrome. *Vet Rec,* 140, 70-71, 1997. DOI: 10.1136/vr.140.3.70-b

**11. Icochea E, Alba M, Fiory L, Ramirez A:** Efficacia de tres vacunas inactivades contra la hepatitis a corpusculos de inclusion y syndrome de hidropericardio en el Peru. *Revue Sci Tech Off Inter Epiz,* 115, 1025-1030, 2001.

**12. Anjum AD:** Experimental transmission of hydropericardium syndrome and protection against it in commercial broiler chickens. *Avian Pathol,* 19, 655-660, 1990. DOI: 10.1080/03079459008418721

**13. Hussain I, Munir R, Ahmad R:** Evaluation and comparison of hydropericardium syndrome vaccines in broiler chicks. *Pak Vet J,* 19, 88-90, 1999.