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RESEARCH ARTICLE

Comparative Pathogenicity of Liver Homogenate and Cell Culture Propagated Hydropericardium Syndrome Virus in Broiler Birds

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ABSTRACT

Comparative pathogenicity of liver homogenate and cell culture propagated agents of hydropericardium syndrome was studied in broiler birds. In Experiment I, 25-day-old while in experiment II, broiler birds at different ages were inoculated through different routes. In Experiment I, liver homogenate caused 64% mortality through intramuscular route and 33.33% mortality through oral route. The cell culture propagated HPS virus caused 60 and 13.33% mortality in broiler birds through intramuscular and oral routes, respectively. In Experiment II, none of the day-old-chicks died when challenged with liver homogenate and cell culture propagated HPS virus through S/C and oral route. The liver homogenate and cell culture propagated HPS virus caused higher mortality in different age groups of broiler birds through s/c route compared to oral route. The values of hemoglobin (Hb) and packed cell volume (PCV) showed highly significant ($P<0.05$) reduction indicating anemia. The values of Hb and PCV of the broiler birds inoculated with infectious liver homogenate were significantly lower as compared to birds inoculated with cell culture propagated HPS virus. The results indicated that the liver homogenate is more pathogenic than cell culture propagated HPS virus. These changes may be due to adoptability of the original FAdVs (fowl adenovirus) after continued passages in the culture of chicken embryo liver cells. Importance of this study in vaccine production is also discussed.

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INTRODUCTION

Hydropericardium syndrome (HPS) has caused huge economic losses to poultry industry in Pakistan since 1987 when it was first reported at Angara Goth. It is causing heavy economic losses to the poultry farmers; more than Rs. 80 million/annum (Khalid, 2003). The causative agent of hydropericardium syndrome is a DNA non-enveloped icosahedral fowl adenovirus (FAV) serotype 4, belonging to the Adenovirus genus of the family Adenoviridae, which can be propagated in chicken embryo liver and kidney primary cell cultures.

Viruses belonging to Aviadenovirus genus are much diverged and could be classified into 5 (A to E) species and also into 12 serotypes. Adenoviral infections are widely prevalent among birds including poultry and wild species. Most of these viruses do not produce any clinical signs as the primary pathogens and their role as the opportunistic factors is indicated. However, some fowl adenoviruses (FAdV) directly affect health and could cause disease conditions and symptoms such as inclusion body hepatitis, hydropericardium syndrome, respiratory disease, decreased egg production, diarrhea, poor growth, and reduced feed conversion (Blicharz *et al*., 2011).

HPS is responsible for high mortality (20 to 80%) in broiler birds starting at 3rd week of age and peaks for 4-8 days, while morbidity is low (Saif, 2001). The transmission of disease occurs vertically and laterally by the oral-fecal route. The disease has been brought under control by the use of formalin-inactivated vaccines, prepared from infected liver homogenate, and of inactivated cell culture vaccines. The vaccines are effective in the face of natural outbreaks or experimental challenge and significantly reduce the mortality. To develop vaccines, knowledge about the pathogenicity of virus is very essential (Balamurugan and Kataria, 2004).

The liver homogenate prepared from naturally infected liver of the broiler birds can reproduce the disease. The disease was reproduced from liver homogenate by a number of workers (Khawaja *et al*., 1988; Muneer *et al*., 1989; Mansoor *et al*., 2011). According to recent report, formalin-inactivated liver organ vaccines failed to protect the Pakistan broiler industry from this destructive disease of economic importance. Liver organ vaccine showed significantly low protection as compared to chicken embryo adapted fowl adenovirus serotype 4 vaccine in broilers. Protection level was estimated on the basis of clinical signs, gross lesions in the liver and heart, histopathological lesions in the liver, and mortality (Mansoor *et al*., 2011). However the fowl adenovirus strain of HPS are different from non HPS strains, as Masaji *et al*. (2010), characterized HPS short fiber protein of different strains from Japan, India and Pakistan and reported that FAdV-4 strains from HPS (HPS–FAdV-4) fell into a different cluster from FAdV-4 strains not derived from HPS. Fiber genes of FAdV-4 strains of HPS and conventional FAdV-4 isolates were compared for pathogenicity to chickens and results showed that HPS strains were pathogenic while conventional strains caused no mortality and clinical signs.

Despite lot of work on HPS, literature regarding the comparative pathogenicity and pathogenesis of HPS virus obtained from different sources is scanty, therefore, the present project was designed to study and compare the pathogenicity of HPS virus from infected liver homogenate and cell culture propagated. Purpose of present study was to investigate the pathogenicity of fowl adenovirus serotype 4 causing hydropericardium in different age groups of broiler birds through natural and parenteral routes, to study comparative pathogenicity of inoculum prepared directly from infected liver and cell culture propagated virus and to study various hematological values such as Hb and PCV in infected birds and it's comparison with normal birds. Ahmad and Hassan (2004) reported that formalinized aqua based liver organ vaccines for hydropericardium syndrome are effective for control of the disease, while on other hand contaminated vaccines are considered to be responsible for its massive spread throughout the country therefore it is needed to improve the vaccine production by adopting safer methods. This study can help to standardize the protocols for development and evaluation of avian HPS virus vaccines from infected liver homogenate and cell culture for their comparative immunogenicity and protection.

MATERIALS AND METHODS

Tissue processing and source of virus

Seed stock virus in the form of infected livers was obtained from a commercial production unit. Infected livers were homogenized using electric tissue homogenizer (Nissei, AM-11, Japan) at 5000 rpm for 5 minutes to prepare 20% (w/v) suspension in PBS. Tissue suspension was clarified by removing coarse debris by staining through cheesecloth followed by centrifugation at 7000 rpm for 20 minutes at 4°C (H-200 NR Kokusan Corporation, Japan). Clarified supernatant was gently decanted into sterile container. Antibiotics (Benzyl Penicillin (Na-Salt) 2500 I.U., Streptomycin sulphate 1mg, Polymxin B sulphate 500 I.U. and Fungizone (Amphotericin B) 4µg per ml) were added to supernatant, kept at 37°C for one hour and then stored at -20°C. The propagated virus was titrated to calculate the tissue culture infective dose 50 ($TCID_{50}$) $10^{-3.55}$ prior to the infection using Reed and Muench method (Reed and Muench, 1938).

Experiment-I: Virus challenge

One hundred, day old broiler chicks were purchased from a commercial hatchery. The birds were vaccinated against Newcastle disease and IBD. At the age of 25-days birds were divided into 5 groups (A-E). Birds in group A and B were inoculated with different dilutions (1ml) of liver homogenate through I/M and oral route while birds in group C and D were inoculated with different dilutions (1ml) of infected chicken embryo liver cell culture supernatant through I/M and oral route. The group E was kept as uninfected control (Table 1).

Hematology

The blood sample (3 ml) of experimental birds was collected in a disposable syringe containing EDTA (@1mg/ml) prior to infection as representative of healthy chickens and then on 2nd or 3rd day of post infection from the surviving birds. Hb estimation and PCV were determined following the protocol of Benjamin (1986).

Necropsy and Mortality

The experimentally infected birds as well as control birds were kept under observation to record the morbidity and mortality in each group. The necropsy of the dead birds was performed to record the lesion and confirmation of HPS.

Experiment-II: Virus Challenge

One hundred twenty five, day old broiler chicks were divided into five groups (A-E) and vaccinated as per schedule of experiment I. In each group two birds were given cell culture virus and two birds were given liver homogenate virus through S/C and oral routes, while one bird was kept as control (Table 2). Birds in group A were inoculated on day one of their age, group B at 7 days of age, group C on 14 days of age, and group D on 21 days of age and group E on 28 days of age (Table 2). Blood samples were collected prior to the infection and then on 2nd or 3rd day post infection from the surviving birds. Parameters observed were same as mentioned in experiment I.

RESULTS AND DISCUSSION

Hydro-pericardium syndrome is an infectious disease primarily of broiler birds. It has emerged as a severe hazard to poultry producers and it causes high morbidity and variable mortality (Shane, 1996; Mansoor *et al*., 2011). Mortality ranges from 20-75% in Pakistan (Cheema *et al*., 1989) and 30-80% in India (Kumar *et al*., 1997).

Nakamura *et al*. (1999 and 2000) inoculated (I/M) undiluted infectious liver homogenate in day old chicks as

well as in 4-week-old broilers and recorded 100% mortality within two to five days post inoculation. However, mortality rate dropped to 20 and 0 % in groups inoculated with diluted virus (10^{-1} and 10^{-2} LD_{50}), respectively. The mortality was not recorded in the present study in one-day old broiler chicks either infected through S/C or oral route using 20% w/v of infectious liver homogenate (Table 2). The resistance of these birds to infectious material may be due to the presence of maternal antibodies as these birds were purchased from a commercial hatchery rearing parent flocks in nearby area. However, these antibodies could not be detected by AGPT conducted (data not shown), nor the disease history was reported in the parent flocks by the owner on inquiry.

In the present study the day-old and 4-week-old broiler birds were inoculated through different routes with cell culture propagated virus (Table 2). None of the birds from day-old chicks died of HPS whether infected through s/c or oral route. The mortality rates of 80% and 40% were recorded in 4-week-old broiler birds infected through S/C and oral routes, respectively (Table 2). Nakamura *et al*. (1999) recorded mortality rates of 40, 25 and 0% in 5-week-old broiler birds infected through i/m route with 10^{-7}, 10^{-5} and 10^{-3} $TCID_{50}$ of HPS virus, respectively. Dahiya *et al*. (2002) reported 83 and 50% mortality in 4-week-old broiler birds inoculated subcutaneously and orally, respectively with 10^{-4} $TCID_{50}$ virus. Although the cell culture propagated virus has the ability to induce infection and mortality in broiler birds above 20 days of age. However, the mortality rate varies depending on the dose of infectious virus in the inoculums and the route of inoculation. Ahmad and Burgess (1998) observed that viral isolation in cell culture has several limitations. It is therefore not possible in most of cases to titrate the infectious virus from tissue of infected birds. The development of CPE is slow and visual appraisal of end point titers is often equivocal. Therefore the cell culture based vaccines for FAdV are not very successful

The maximum mortality rate of 40% recorded in two-week-old chicks inoculated with10^{0} cell culture propagated virus through s/c route, while none of the birds died when virus was introduced by oral route (Table 2). Mazaheri *et al*. (1998) reported 100% mortality 7th day post inoculation in day-old broiler chicks infected orally with10^{-5} plaque forming units, while it was 80% in 2-week-old broiler birds infected intramuscularly with10^{-8} plaque forming unit and 46% mortality in 1-week-old chickens infected orally with same dilution of cell culture propagated virus.

In Experiment I of the present study, the 25-day-old broiler birds were inoculated intramuscularly with different dilutions of Liver homogenate and the overall mortality was recorded as 64% (Table 1), these results are in accordance with the results of Abdul-Aziz and Hassan (1995), who also recorded the 100% mortality due to liver homogenate inoculated through intramuscular route. The 60% mortality was recorded in the 25-day-old broiler birds inoculated orally with same liver homogenate virus. This suggests that liver homogenate of HPS infected broiler birds causes high mortality in 25-day-old broiler birds through parenteral routes than oral route. But Abe *et al*. (1998) reported 20.2 and 26.6% mortality in 16-day-old and 25-day-old chickens, respectively, naturally infected with HPS. The findings of the present experiment show that by increasing the dilution of liver homogenate the mortality decreases. The highest mortality was recorded in 4-week-old and 1-week-old broiler chickens, inoculated subcutaneously with 10^{0} cell culture propagated HPS virus (Table 2). The most successful route of infection is subcutaneous inoculation irrespective of the age of the birds (Naeem *et al*., 2001). Postmortem examination showed most predominant gross lesion of accumulation of up to 15 ml of clear, watery or jelly like fluid (Table 1 and Table 2) in the pericardial sac with misshapen and flabby heart in birds that died of experimental infection in experiment I and II. Pathogenicity was determined by giving score according to accumulation of fluid in pericardial sac. Other lesions comprised of hemorrhages in the hear musculature and other organs, congestion and edema in lungs, swollen pale friable enlarged liver and pale kidneys with prominent tubules. The bursa of Fabricious was also swollen in experimentally induced HPS cases. Presentation of almost same lesions has been reported by other workers (Ganesh and Raghavan, 2000; Ganesh *et al*., 2002; Mansoor *et al*., 2011).

In the present study the liver homogenate and cell culture propagated HPS virus caused 80% mortality in 1-week-old broiler chickens through s/c route. But 60 and 0% mortality was recorded through oral route by the same concentration of liver homogenate and cell culture propagated HPS virus, respectively (Table 2). Our results also demonstrated 100% mortality in 3-week-old broiler birds inoculated subcutaneously with 10^{0} liver homogenate and 60% through oral route. But cell culture propagated HPS virus caused 80 and 40% mortality through s/c and oral routes, respectively (Table 2). In corroboration, 100% mortality in 3-week-old broiler birds inoculated with 10-5 Plaque forming unit of cell culture propagated virus intramuscularly was observed by Mazaheri *et al*. (1998) Nakamura *et al*. (2000) also recorded 20% mortality in 3-week-old birds infected with 0.1 ml of 10^{-7} PFU of GIAAV virus intramuscularly. Khawaja *et al*. (1988) reported 100% mortality through intrapericardial route, 80% by s/c and 40% by oral route.

The results indicated that the birds at an age of 3 weeks and older than 3 weeks are more prone to HPS infection regardless of the route of inoculation. However, the virus pathogenicity is much higher by s/c and i/m routes as compared with oral infection. Lower pathogenicity of HPS for broiler birds by oral route might be due to inactivation/degradation of virus by gastrointestinal tract secretions as the presence and importance of the antiviral substances in alimentary tract with respect to protection of the chicken against the viscerotropic pathotype of NDV has been reported (Janet and Robert, 1975).

The mean Hb and PCV values of healthy and infected birds were statistically analyzed to compare the pathogenicity of liver homogenate and cell culture propagated HPS virus (Table 3 and Table 4). The partial nested design was applied to analyze the hematological values pre and post infection. In experiment I the Mean Hb value of the healthy birds was 13.21 ± 0.1146 that decreased significantly ($P<0.05$) to 8.51 ± 1.51. Similarly the mean PCV value of the healthy broiler birds reported

Table 1: Mortality record of the 25 day old broiler birds inoculated with liver homogenate and cell culture propagated HPS virus (n=100)

Group	Virus	Route	Dilution	Mortality After Days						Total Chicks Died	Mortality %	Post mortem lesion Hydropericardium
				1st	2nd	3rd	4th	5th	6th			
A	Liver Homogenate	I/M	10^{-1}	2	1	2	-	-	-	5	100	++++
			10^{-2}	-	1	2	1	-	-	4	80	++++
			10^{-3}	-	-	-	1	1	1	3	60	++++
			10^{-4}	-	-	-	1	1	-	2	40	+++
			10^{-5}	-	-	-	-	-	2	2	40	+++
B	Liver Homogenate	Orally	10^{-1}	-	-	-	2	1	-	3	60	++++
			10^{-2}	-	-	-	-	2	-	2	40	+++
			10^{-3}	-	-	-	-	-	-	0	0	-
C	Cell Culture Virus	I/M	10^{0}	-	1	1	2	-	-	4	80	++++
			10^{-1}	-	-	2	-	1	1	4	80	++++
			10^{-2}	-	-	1	-	1	-	2	40	+++
			10^{-3}	-	-	-	1	1	-	2	40	+++
D	Cell Culture Virus	Orally	10^{0}	-	-	-	-	-	2	2	40	++++
			10^{-1}	-	-	-	-	-	-	0	0	-
			10^{-2}	-	-	-	-	-	-	0	0	-
E	Control											-

++++ = Pericardial fluid > 8ml, +++ = Pericardial fluid > 5ml, ++ = Pericardial fluid > 3ml

Table 2: Mortality record of the different age groups of broiler birds inoculated with liver homogenate and cell culture propagated HPS virus (n=125).

Group	Age	Virus	Route	No of Chicks	*Mortality After Days						Mortality %	Post mortem lesion Hydropericardium
					1st	2nd	3rd	4th	5th	6th		
A	Day old	Liver Homogenate	S/C	A1=5							0	-
			Orally	A2=5							0	-
		Cell Culture Virus	S/C	A3=5							0	-
			Orally	A4=5							0	-
		Control		A5=5								
B	One week	Liver Homogenate	S/C	B1=5					2	2	80	+++
			Orally	B2=5					1	2	60	+++
		Cell Culture Virus	S/C	B3=5				1	1	2	80	+++
			Orally	B4=5				0	0	0	0	-
		Control		B5=5								
C	Two week	Liver Homogenate	S/C	C1=5					1	2	60	++++
			Orally	C2=5						2	40	+++
		Cell Culture Virus	S/C	C3=5					1	1	40	+++
			Orally	C4=5					0	0	0	-
		Control		C5=5								
D	Three week	Liver Homogenate	S/C	D1=5					2	3	100	++++
			Orally	D2=5					1	2	60	+++
		Cell Culture Virus	S/C	D3=5					2	2	80	++++
			Orally	D4=5						2	40	++
		Control		D5=5								
E	Four week	Liver Homogenate	S/C	E1=5					2	2	80	++++
			Orally	E2=5					1	2	60	++++
		Cell Culture Virus	S/C	E3=5					1	3	80	+++
			Orally	E4=5						2	40	++
		Control		E5=5								-

++++ = Pericardial fluid > 8ml, +++ = Pericardial fluid > 5ml, ++ = Pericardial fluid > 3ml; * Mortality not earlier than 4th day of post inoculation

was 34.10±0.27 that significantly decreased ($P<0.05$) to 22.43±0.36 (Table 4). These findings are in line with Niazi *et al*. (1989), Gowda and Satyanarayana (1994) and Asrani (1997) who recorded marked anemia among HPS affected birds. Vairamuthu *et al*. (2004) also reported a great reduction in the hematological values in the naturally HPS infected broiler chickens. Results showed drop in the hematological values of infected birds, which is supported by finding of other researchers who reported that HPS affected birds showed severe anemia with significant reductions in all hematological parameters except the mean corpuscular volume. The decrease in hematological values and severe anemia may be due to low cell production or concurrent infection such as with chicken anemia virus (Chandra *et al*., 2000).

The results of the present study showed that liver homogenate virus caused high mortality and severely decreased hematological values than the cell culture propagated HPS virus inoculated through intramuscular, subcutaneous and oral routes. This shows that the liver homogenate virus is more pathogenic than cell culture propagated virus. It is thought that the virus pathogenicity

Table 3: Comparison of mean hematological values (Mean±SD) of HPS infected and healthy broiler birds in Experiment- I

Group	Route	Sub groups	Hemoglobin (g/dl)		Packed Cell Volume (%)	
			Healthy Birds	Infected Birds	Healthy Birds	Infected Birds
A	Intramuscular	A1	13.20±0.05	7.08±0.08	38.50±0.25	24.34±0.06
		A2	13.23±0.03	7.18±0.03	38.74±0.45	24.32±0.01
		A3	13.17±0.15	8.03±0.06	37.96±0.63	26.27±0.08
		A4	13.19±0.05	8.20±0.05	37.31±0.67	25.37±0.18
		A5	13.21±0.02	8.23±0.08	38.96±0.07	25.93±0.55
B	Oral	B1	13.22±0.03	7.36±0.07	37.38±0.33	24.38±0.05
		B2	13.15±0.13	7.60±0.10	38.62±0.05	24.33±0.06
		B3	13.22±0.03	8.20±0.26	38.70±0.41	25.18±0.47
C	Intramuscular	C1	13.25±0.03	8.03±0.06	37.78±0.69	25.93±0.55
		C2	13.08±0.08	8.22±0.02	39.12±0.33	25.50±0.18
		C3	13.23±0.03	8.11±0.03	36.81±1.58	25.17±0.49
		C4	13.20±0.01	8.26±0.03	37.89±0.29	25.06±0.67
D	Oral	D1	13.14±0.13	8.23±0.02	37.58±0.06	26.17±0.16
		D2	13.32±0.30	9.83±0.30	36.35±0.22	32.64±2.13
		D3	13.13±0.12	10.16±0.10	38.71±0.18	32.56±4.39
E	Control	Mean	13.26±0.03	13.26±0.03	37.65±0.69	37.65±0.69
	Total	Mean ± SD	13.21±0.11	8.51±1.51	38.04±0.89	27.21±4.21

Table 4: Comparison of mean hemoglobin and packed cell volume values (Mean±SD) of HPS virus infected and infected Broiler Birds in Experiment-II

Group	Age	Virus	Route	Hemoglobin (g/dl)		Packed Cell Volume (%)	
				Healthy Birds	Infected Birds	Healthy Birds	Infected Birds
A	Day old	Liver	S/C	12.26±0.25	10.05±0.08	38.62±0.05	35.27±0.38
		Homogenate	Orally	11.00±0.50	9.91±0.38	37.31±0.67	34.10±0.27
		Cell Culture	S/C	11.73±0.25	10.16±0.28	39.12±0.32	34.07±0.91
		Virus	Orally	12.33±0.28	9.83±0.28	37.85±0.33	35.84±0.23
B	One week	Liver	S/C	13.16±0.14	8.10±0.10	37.53±0.34	24.67±0.54
		Homogenate	Orally	13.28±0.20	8.53±0.45	37.30±0.78	25.74±0.22
		Cell Culture	S/C	13.25±0.05	8.66±0.76	36.33±1.36	25.98±0.35
		Virus	Orally	13.20±0.26	11.76±0.40	38.85±0.16	36.85±1.51
C	Two week	Liver	S/C	13.23±0.25	8.25±0.25	38.85±0.24	25.17±0.48
		Homogenate	Orally	13.23±0.02	8.20±0.26	38.43±0.56	25.49±0.21
		Cell Culture	S/C	13.23±0.25	10.33±0.28	38.43±0.18	31.12±0.25
		Virus	Orally	13.25±0.25	10.93±0.60	37.98±0.45	30.47±0.81
D	Three week	Liver	S/C	13.15±0.13	7.25±0.25	39.19±0.73	24.57±0.14
		Homogenate	Orally	13.16±0.15	8.23±0.25	38.08±0.87	26.03±0.39
		Cell Culture	S/C	13.36±0.23	8.33±0.28	37.49±1.25	24.35±0.05
		Virus	Orally	13.41±0.52	8.33±0.15	37.39±1.59	25.78±0.35
E	Four week	Liver	S/C	12.83±0.47	7.40±0.36	35.28±1.18	21.29±0.80
		Homogenate	Orally	13.30±0.26	7.66±0.28	34.56±1.01	22.07±1.08
		Cell Culture	S/C	13.58±0.38	7.45±0.18	33.63±0.52	23.89±0.36
		Virus	Orally	13.50±0.20	8.16±0.28	34.10±0.27	22.43±0.36

is decreased due to passages in cell culture or due to adaptation to the cell culture; furthermore chances of contamination are very less in cell culture method as compared to virus isolated from liver homogenate. However the isolation of the aviadenoviruses using chicken embryo liver (CEL) cell culture and chicken embryo fibroblast cell culture with further identification and determination of the pathogenicity seems to be very important, since the pathogenicity of the isolates within the same serotype can be widely differ. The cross neutralization tests and /or molecular biological tools are necessary to serotype the isolated virus and to determine a new serotype (Hafez, 2011).

This study may help to develop cell culture based vaccine for HPS which can be updated with regular isolation of virus from the field. Contaminated vaccines are considered to be responsible for its massive spread throughout the country therefore it is needed to improve the vaccine production by adopting safer methods.

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