DEVELOPMENT OF SANDWICH ELISA FOR THE DETECTION OF FOWL ADENOVIRUS 4 ASSOCIATED WITH HYDROPERICARDIUM SYNDROME IN EXPERIMENTALLY INFECTED CHICKEN

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Summary. – A sandwich ELISA was standardized to detect fowl adenovirus (FAV) group I antigen in various tissues, namely liver, spleen, bursa, thymus and kidneys of chicks experimentally infected with fowl adenovirus 4 (FAV-4) isolated from cases of inclusion body hepatitis-hydropericardium syndrome (IBH-HPS). The assay was found to be more sensitive and more specific in comparison to an agar gel immunodiffusion (AGID) test, as it could detect FAV antigen below the titer of 20,000 $TCID_{50}$/ml and below 1.14 μg in 5% (w/v) suspensions of liver tissue. In 2-week-old experimentally infected chicks, the antigens were detectable by ELISA in liver from 3 to 15 days, in thymus from 3 to 7 days, and in kidneys, bursa and spleen from 3 to 10 days post infection (p.i.). Maximum antigen concentration in terms of ELISA absorbance values was detected in liver and kidneys, which could be used as tissues of choice for virus isolation or detection of viral antigens from IBH-HPS cases.

Key words: fowl adenovirus 4; hydropericardium syndrome; sandwich ELISA

Introduction

IBH-HPS is an emerging disease of poultry caused by FAV-4, a non-enveloped virus belonging to the *Adenovirus C* species of the *Aviadenovirus* genus of the *Adenoviridae* family. It is characteristic by hydropericardium and hepatitis with intranuclear inclusion bodies in hepatocytes (Jaffery, 1988; Ahmad *et al.*, 1989; Anjum *et al.*, 1989). The disease causes heavy economic losses due to high mortality (10–70%) in 3–6-week-old broiler chicks. The disease has been reported from many parts of Asia including India (Jaffery, 1988; Gowda and Satyanarayana, 1994; Abe *et al.*, 1998). Isolation of FAV-4 from many poultry farms of the country experiencing IBH-HPS has been reported (Oberoi *et al.*, 1996). The disease has been experimentally reproduced in 1–3-week-old broiler chicks by inoculating FAV-4 propagated in chick embryo liver (CEL) cell cultures (Kataria *et al.*, 1997). In India, supportive tentative diagnosis of IBH-HPS is based on gross and histopathological changes in the affected chicken (Gowda and Satyanarayana, 1994), but confirmatory diagnosis is routinely done by virus isolation or detection of the virus in infected tissue by electron microscopy (Kataria *et al.*, 1996; Chandra *et al.*, 1997). As these methods are cumbersome, time consuming and costly, development of cost-effective, rapid and sensitive diagnostic test is essential or a need of the hour. Since presence of FAV-4 antibodies has been detected even in healthy chicken (Yates *et al.*, 1976), detection of FAV-4 antigens in suspected tissues is more relevant in the diagnosis of infection than quantification of FAV-4 antibodies in serum.

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Abbreviations: AGID = agar gel immunodiffusion, CEL = chicken embryo liver; CPE = cytopathic effect; FAV = fowl adenovirus; FAV-1, FAV-4, FAV-8 = fowl adenovirus 1, 4, 8; IBDV = infectious bursal disease virus; IBH-HPS = inclusion body hepatitis-hydropericardium syndrome, p.i. = post infection; NDV = Newcastle disease virus; BSA = bovine serum albumin

Therefore, for the diagnosis of the disease, it is necessary to detect the virus or viral antigens in tissues of infected chicks by sensitive techniques. ELISA, first described by Engvall and Perlmann (1971), is a rapid, inexpensive, and highly sensitive serological test that has been applied to the diagnosis of many diseases.

Hence, this paper deals with the development of a sandwich ELISA for detection of FAV-4 antigens in various tissues of chicks experimentally infected with the virus.

Materials and Methods

Viruses. Two field isolates of FAV-4, namely AD: IBH-157/95 and AD: IBH 317/95 were propagated in CEL cell cultures prepared from 14-16-day-old chicken embryos using Medium 199 with 15% of newborn calf serum. The viruses were titrated in the same type of cultures on the basis of cytopathic effect (CPE). EDS 76 virus (*Adenoviridae* family), CELO virus (FAV-1) (species *FAV-A*, genus *Aviadenovirus*) and infectious bursal disease virus (IBDV) serotype I (species *Infectious bursal disease virus*, genus *Avibirnavirus*, family Birnaviridae) were also propagated in CEL cell cultures, while Newcastle disease virus (NDV, F and LaSota strains) was propagated in embryonated chicken eggs.

Preparation of semipurified viral antigen. Cell cultures infected with the AD: IBH157/95 isolate showing CPE in more than 80% of cells were harvested and partially purified by the method of Capua *et al.* (1995) with slight modification. The harvest (500 ml) was freeze thawed thrice, homogenized with half volume of chloroform and centrifuged at 6,000 rpm for 30 mins. The obtained supernatant was centrifuged at 100,000 x g for 3 hrs at 4°C and the viral pellet was resuspended in 0.5 ml of TE buffer (100 mmol/l Tris-HCl and 10 mmol/l EDTA, pH 8.0). The viral suspension was centrifuged at 100,000 x g for 2 hrs over 3 ml of 30% (w/v) sucrose cushion and the resulting viral pellet was resuspended in 0.5 ml of TE buffer. This semipurified viral antigen was used for raising hyperimmune sera.

Hyperimmune serum against the AD: IBH 157/95 isolate of FAV-4 was prepared in 4-month-old chickens and adult guinea pigs as described by Saiffudin and Wilks (1990) with some modifications. The semipurified viral antigen of protein concentration of 790 µg/ml was mixed with an equal volume of Freund's complete adjuvant and 1ml aliquots were injected at two different sites intradermally to three healthy chickens free of FAV-4 antibodies and to four guinea pigs. After 2 and 4 weeks, the second and third booster were administered intramuscularly to the animals. Ten days after the last booster, maximum blood was collected from the immunized animals, the serum was separated, adsorbed with liver powder prepared from healthy chicken by the method of Sambrook *et al.* (1989), and stored in aliquots at -20°C until used.

Experimental infection of chickens. For standardization of ELISA, ten 5-week-old healthy chickens free of FAV antibodies were inoculated intraperitoneally with 5 x 10^{-4} $TCID_{50}$ (0.5 ml) of the AD: IBH 157/95 isolate propagated in CEL cell cultures and observed daily for mortality. Various tissues namely liver, spleen, bursa, thymus and kidneys were collected from the dead chickens showing gross lesions of IBH-HPS.

A hundred-day-old unvaccinated broiler chicks procured from a poultry farm were raised in isolation in a shed belonging to the Division of Avian Diseases. At the age of 2 weeks, all the chicks were screened for FAV antibodies by an AGID test and were found negative.

Forty chicks of this group were infected orally with 3 x 10^4 $TCID_{50}$ (0.3 ml) of the AD: IBH 157/95 isolate propagated in CEL cell cultures and remaining chicks were kept as control. Three chicks each from infected and control groups were sacrificed at days 3, 5, 7, 10, 15, 21, and 28 p.i. and samples of liver, spleen, bursa, thymus, and kidneys were collected and kept at -20°C until tested.

Preparation of tissue samples. Samples of liver, spleen, thymus, bursa, and kidneys collected from healthy chicks (negative control), dead chicks showing gross lesions of HPS (positive control) and experimentally infected chicks at various days p.i. were triturated with PBS to prepare 10% (w/v) suspensions. These were centrifuged at 3,000 rpm for 20 mins and the obtained supernatants were tested by ELISA.

ELISA. A sandwich ELISA, based on the procedure of Saifuddin and Wilks (1990) with minor modifications, was employed. An antigen was prepared from the AD: IBH 157/95 isolate of FAV-4 according to Saifuddin and Wilks (1990). The assay was carried out in 96-well, flat-bottom, polystyrene microtiter plates (Nunc, Denmark) in total volume of 50 µl. A hundred µl of blocking solution was used. The reactions were carried out at room temperature (20–25°C) to avoid non-specific absorbance. A bicarbonate buffer pH 9.6 and PBS containing 0.05% Tween-20 (PBS-Tween 20) were used for diluting the coating guinea pig FAV-4 antibody and for washing the plates, respectively. PBS Tween-20 containing 2% bovine serum albumin (BSA) was used for diluting test samples, chicken FAV-4 antibodies and chicken HRPO conjugate (Sigma), whereas 5% BSA was used for blocking the plates. The substrate solution consisted from 30% of a solution of ortho-phenylenediamine in a phosphate-citrate buffer pH 5.0 and 70% of 0.012% H_2O_2. Absorbance at 492 nm (A_{492}) was measured using a Micro Scan computerized spectrophotometer (ECL, India). The highest dilutions of the guinea pig and chicken hyperimmune sera giving with the semi purified viral antigen an A_{492} value of 1.0 were employed in checker board titration.

Sensitivity of ELISA was evaluated by determining minimum viral antigen detected in infected CEL cell culture supernatants, positive liver samples and the semipurified viral antigen. The supernatants of CEL cell cultures infected with the isolates AD: IBH 157/95 (titer of 2 x 10^6 $TCID_{50}$/ml) and AD: IBH 317/95 (titer of 2 x 10^5 $TCID_{50}$/ml), and the semipurified viral antigen (protein concentration of 2.28 mg/ml) were used concentrated and in dilutions of 1:25, 1:50, 1:80, 1:100, 1:200, and 1:400. The non-infected CEL cell culture supernatants at the same dilutions were used as controls. The infected and non-infected liver suspensions serially diluted from 1% to 10% were also employed.

Results and Discussion

Dilutions of 1:4,000 and 1:3,000 of the hyperimmune guinea pig and chicken sera, respectively, were found to give

with the 1:50 dilution of the semipurified viral antigen an A_{492} value of 1.03. The sera from a non-infected guinea pig and chicken gave an A_{492} value of 0.23 at the same dilutions. The A_{492} values of healthy chicken tissues, namely liver spleen, bursa, thymus, and kidney varied from 0.18 to 0.27 with a mean ± SD of 0.23 ± 0.03. The negative control without any antigen gave values from 0.19 to 0.21. The mean values for the unabsorbed and adsorbed hyperimmune sera reacted with the antigen from healthy chicken liver were 0.45 and 0.23, respectively. This indicated that the adsorption of the sera with the healthy liver tissue reduced the non-specific reaction, which probably contributed to the high sensitivity of this assay (Dawson *et al.*, 1980). Saiffudin and Wilks (1990) have reported that adsorption of a gamma globulin preparation with a liver and kidney powder from SPF chicks reduced the A_{492} value of the reaction with healthy tissues to 0.15. The higher A_{492} value of the reaction with healthy tissues in our study might be due to presence of other proteins in the serum in addition to FAV-4-specific antibodies. As the cut off point between ELISA-positive and ELISA-negative A_{492} values was taken the mean value of the negative controls (antigens from non-infected tissues) plus the threefold of SD, i.e. 0.32. Accordingly, samples giving A_{492} values higher than the cut off point were considered positive. We interpreted the results of the ELISA by comparison of positive and negative reference samples. Shafren and Tannock (1988) and Saiffudin and Wilks (1990) employed a similar approach in the ELISAs developed for avian encephalomyelitis virus and FAV, respectively.

The developed ELISA was found to be specific as it reacted only with viruses belonging to FAV. The semipurified preparation of FAV-4 antigen gave an A_{492} value of 1.6, whereas the cell culture supernatant containing the FAV-4 isolate AD: IBH 157/95 gave an A_{492} value of 1.39. FAV-1 in undiluted infected cell culture supernatant and allantoic fluid gave A_{492} values of 0.62 and 0.54, respectively. EDS-76 virus reacted partially and gave an A_{492} value of 0.45 as it shared some antigens with the FAV group I (McFerran, 1978). The reason for the higher value for FAV-4 and lower values for FAV-1 is that the hyperimmune serum was raised against FAV-4. This finding is contradictory to that of Saifuddin and Wilks (1990). In the latter study, IBDV serotype I and NDV did not cross-react with an anti FAV-4 serum in ELISA (A_{492} values of 0.3 and 0.24–0.31, respectively).

In our study, SD values of homologous and heterologous viruses reacting in ELISA ranged from 0.016 to 0.03 (Fig. 1). These findings further support the specificity of the ELISA developed.

Undiluted cell culture supernatants containing either the FAV-4 isolates AD: IBH 157/95 or AD: IBH 317/95 or no FAV-4 gave A_{492} values of 1.41, 1.15 and 0.35, respectively. The 1:100 dilution of AD: IBH 157/95 and the 1:80 dilution of AD: IBH 317/95 gave corrected values of 0.17 and 0.15,

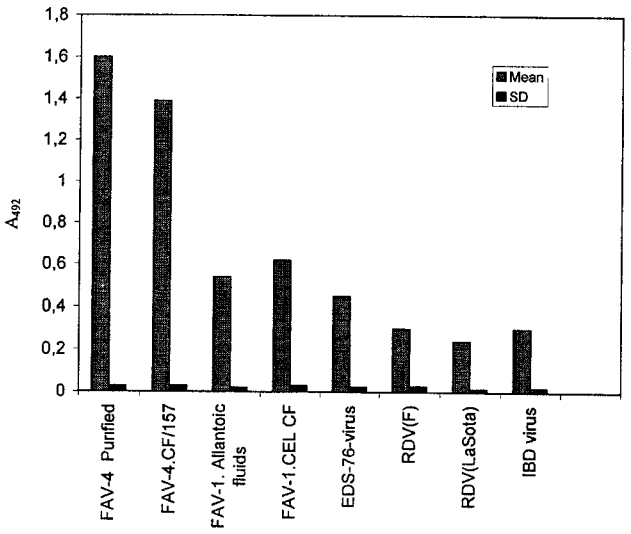

Fig. 1
Reactivity of hyperimmune sera with various fowl adenoviruses and other avian viruses in ELISA

respectively. The corresponding titers of these two isolates were ~20,000 $TCID_{50}$/ml and ~12,500 $TCID_{50}$/ml, respectively. The 1:100 dilution of the semipurified viral antigen gave a corrected value of 0.20 corresponding to protein concentration of ~1.14 μg/ml. The cell culture preparation at the same dilution, however, failed to react in an AGID test. This indicates a lower sensitivity of AGID test compared to the ELISA developed.

Fig. 2 shows the results of an experiment analyzing the sensitivity of the ELISA developed. The mean A_{492} value of a positive liver suspension was calculated for each concentration. The 10% suspension gave a value of 1.16, which gradually declined with a decrease in the percentage of the suspension. The viral antigens could be detected in 4% and more concentrated suspensions. However, the

Table 1. Reaction of FAV-4-positive and FAV-4-negative tissues in ELISA

	A_{492} values (mean ± SD)	
Tissues	Negative tissues	Positive tissues (corrected values)
Liver	0.264 ± 0 0135	0.87 ± 0.183
Spleen	0.184 ± 0 0185	0.322 ± 0 046
Bursa	0 25 ± 0 0167	0.376 ± 0 055
Thymus	0 224 ± 0 023	0 296 ± 0.056
Kidney	0.274 ± 0 010	0.453 ± 0.029

Corrected values were obtained by subtracting mean A_{492} values of negative controls

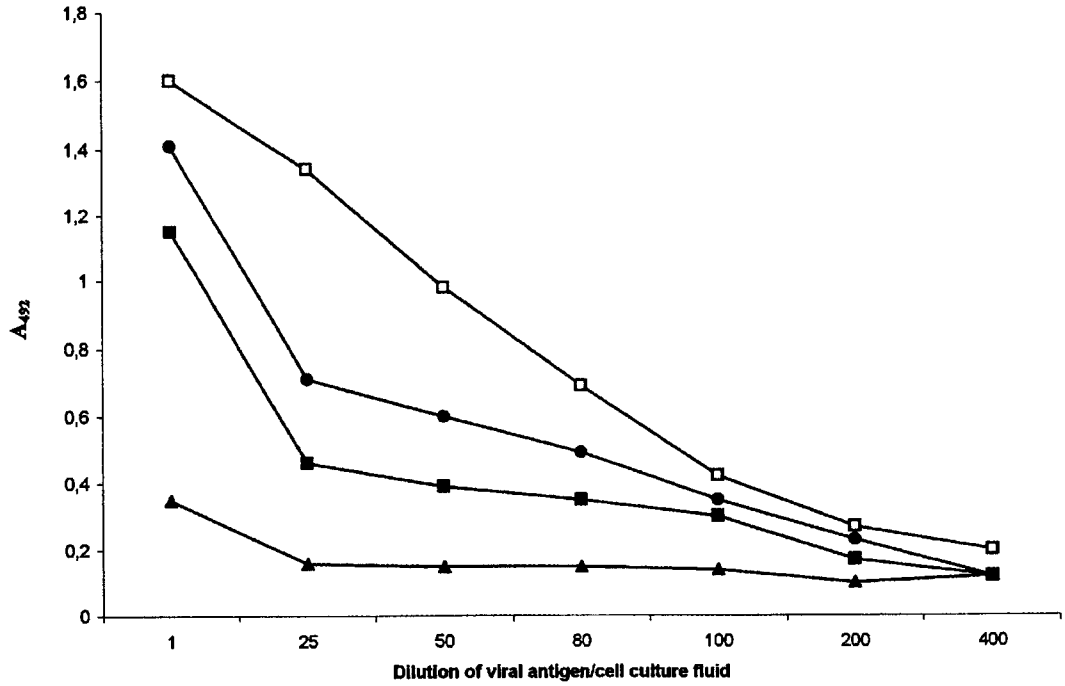

Fig. 2
Detection limit of FAV-4 antigen by ELISA
Semipurified FAV-4 antigen (□), FAV-4 AD: IBH157/95 isolate culture fluid (●), FAV-4 AD: IBH317/95 isolate culture fluid (■), uninfected cell culture fluid (▲).

uncorrected value of 0.37 gave a marginal corrected value of 0.05 in the case of the 4% suspension, while the 5% suspension gave clear-cut positive reaction. As compared to AGID test, the latter could detect FAV antigen in liver suspension of 7% and above.

The reactivity of positive and negative tissue samples in the ELISA gave variable values (Table 1). Detection of viral antigens by the ELISA was attempted in various tissue samples of chicks experimentally infected with FAV-4. It is evident from results presented in Tables 2 and 3 that a high concentration of viral antigens was detected in liver by the ELISA from days 3 to 15 p.i. with a peak value of 0.73 at day 5. The viral antigens were detected in bursa, spleen and kidneys from days 3 to 10 with a peak at days 5, 5 and 7, respectively. In thymus, the viral antigens were detected from days 3 to 7 with a peak of 0.21 at day 3. Of the tissues tested, liver gave the highest values followed by kidneys and bursa. Saiffudin and Wilks (1991), while studying the pathogenesis of acute viral hepatitis-IBH following oral administration of FAV-8 to 2-day-old SPF chickens, have detected the viral antigens in liver and bursa from days 2 to 9 p.i. with a peak at days 6 and 5, respectively, and in thymus, spleen and kidneys from days 3 to 6, 4 to 7, and 5 to 6, respectively, with a peak at days 4, 6 and 6, respectively. All control groups were found negative for FAV group I antigen in various tissues. Saiffudin and Wilks (1992) have demonstrated the viral antigens in bursa from days 2 to 9 p.i. and in spleen from days 3 to 7 with a peak at day 5 by ELISA. In thymus, the viral antigens were detected from days 3 to5 p.i. with a peak of 0.28 at day 4.

The levels of the viral antigens in terms of ELISA absorbance values obtained in our study are in agreement with those reported by Saiffudin and Wilks (1991,1992) except for the kidney tissues. In our study, kidney samples

Table 3. Detection of viral antigen in different tissues at various intervals by ELISA following experimental infection with FAV-4

Tissues	Days of viral antigen detection (day of peak)	Peak A_{492} values
Liver	3-15 (5)	0.73
Spleen	3-10 (5)	0 30
Bursa	3-10 (5)	0.32
Thymus	3-7 (3)	0.21
Kidney	3-10 (7)	0.43

Corrected A_{492} values were used (see Table 1).

Table 2. Detection of viral antigen in different tissues at various intervals by ELISA following experimental infection of chickens with FAV-4

Tissue	A_{492} values (range) Days p.i.						
	3	5	7	10	15	21	28
Liver	0.30-0.64*	0.40-1.09	0.25-043	0.26-0.31	0.08-0.28	<0.05	<0.06
	(3)**	(3)	(3)	(3)	(2)	(–)	(–)
Spleen	0.06-0.27	0.24-0 38	0.10-0. 28	0.20-0.34	<0.08	<0.04	<0.02
	(2)	(3)	(3)	(3)	(–)	(–)	(–)
Bursa	0.05-0.34	0.21-0.40	0.08-0.28	0.08-0.13	<0.06	<0.05	<0.06
	(2)	(3)	(2)	(2)	(–)	(–)	(–)
Thymus	0.16-0.26	0.18-0.26	0.14-0.20	<0.08	<0.03	<0.03	<0.01
	(3)	(3)	(3)	(–)	(–)	(–)	(–)
Kidney	0.07-0.23	0.08-0.32	0.40-0 46	0.05-0.10	<0 01	<0.02	<0.03
	(2)	(2)	(3)	(1)	(–)	(–)	(–)

*Corrected A_{492} values were used (see Table 1). Values over 0.09 were considered significantly positive.
**Values in parenthesis indicate No. of positive samples of 3 samples tested.
(–) = all 3 samples were negative.

gave a peak of 0.43 at day 7 p.i. compared to 0.09 at day 6 p.i. for FAV-8 as reported by Saiffudin and Wilks (1991) This difference might be due to the fact that FAV-4 has been proved experimentally to cause extensive damage to the kidneys in addition to hydropericardium and hepatitis (Kataria, 1997; Anjum *et al.*, 1989; Cheema *et al.*, 1989), whereas FAV-8 causes only hepatitis in experimentally infected chicks.

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Note of Editor-in-Chief. Terms "fowl adenovirus(es) (FAV)" and "FAV group I" used in this article are not compatible with the presently valid virus taxonomy (Van Regenmortel MHV, Fauquet CM, Bishop DHL: *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego-San Francisco-New York, 2000).

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