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Research Article

Protein Profile of Normal and Hydropericardium Infected Poultry Liver Using Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis and Western Blotting

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ABSTRACT

Preparation of autogenous vaccine against Hydropericardium syndrome (HPS) and subsequent inoculation to chickens is common practice in Pakistan. However, due to lack of quality control testing and evaluation procedures, the accurate quantity of virus in a given clinical sample of liver always remains questionable. Therefore, the present study has been conducted to differentiate the protein profile of normal and HPS infected liver lysate with respect to the availability of immunogenic proteins using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting technique. Using commercially available vaccine against HPS, hyperimmune serum was raised in immunologically naïve broiler birds (3–weeks old, n = 60) and subjected to agar gel precipitation test (AGPT) for screening HPS positive serum samples. Of the total examined (n=30), only 5 birds were found positive. The commercially available HPS autogenous vaccines, liver from HPS positive birds and normal liver samples were subjected to protein extraction and measurement (Ganesh K et al, 2002). SDS–PAGE analysis revealed 15–structural polypeptide bands in both HPS infected poultry liver and autogenious vaccine (Ottomans Parma). Contrary to this, a 10–structural polypeptide bands were observed in normal liver. The polypeptides of HPS autogenious vaccine (Ottomans Parma) and both normal and HPS infected livers, separated on 9% gel were transferred onto nitrocellulose membrane (0.22 µm) by wet system known as Western blotting as performed by Rajesh Kumar and Rajesh Chandra (2004). The western blot analysis of proteins of hydropericardium virus infected liver and HPS autogenious vaccine separated on 9% gel showed one immunogenic protein molecular weight between 20–15 kDa. The difference is used for identification and differentiation between clinically diseased and healthy birds as well as autogenious vaccine.

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INTRODUCTION

Hydropericardium was first recognized in broiler–rearing area of Angara Goth Karachi in 1987 Muneer et al 1989. The disease was thought to be a multifactorial syndrome initially, which might have occurred either due to bacterial infections, poisoning with disinfectant, insecticides (McCune et al., 1962) or toxic fat (Sanger et al., 1958). Nevertheless, the Koch's postulates, from liver homogenate of the infected birds, have been proved by a number of researchers (Khawaja et al., 1988; Cheema et al., 1989; Muneer et al., 1989). Now, Fowl adenovirus serotype–4 (FAdV–4) has been considered the cause of HPS in birds (Afzal., 1990., Rabbani et al., 1998; Hess et al., 1999).

To protect the broiler flock from HPS, since 1989, autogenous vaccine prepared from infected liver suspension has been used (Chisti et al., 1989; Afzal and Ahmed, 1990; Anjum, 1990). However, the protection provided by this vaccine is not long lasting and disease has been reported even in vaccinated birds (Afzal and Ahmad., 1990). Though the disease can be diagnosed from gross lesions and histopathological changes in the liver, various serological tests such as agar gel immunodiffusion (AGID), counter immuno–electrophoresis (CIE) (Parimal Roy et al., 2001), indirect haemaglutination and ELISA (Chandra et al., 2000) has been found useful.

Without known quality control procedures, many commercial labs in Pakistan are manufacturing autogenious liver homogenate vaccine. As the quantity of the virus is not being measured, some of the livers used in making the vaccine may not either have sufficient concentration of virus or no virus at all. It is, therefore, necessary that a method may be developed to evaluate the quality of the manufactured vaccine. The present study has been conducted to differentiate the protein profile of normal and HPS infected liver lysate with respect to the availability of immunogenic proteins using SDS–PAGE and western blotting technique,which will be used for identification and differentiation between clinically diseased and healthy birds as well as autogenious vaccine.

MATERIAL & METHODS

Based upon clinical history and necropsy lesions suspected of HPS, Infected liver samples (n = 30) were collected aseptically from poultry farms in and around district Lahore. The normal liver samples were collected from clinically healthy birds at UVAS Lahore. The samples were transported at 4^0C and stored

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at −20^{0}C until used. The 3−types of autogenious vaccines and one cell cultured vaccine against HPS virus used in the analysis were purchased from local market at Lahore Pakistan.

Preparation of Antibodies against HPS Virus

Sixty chicks (3−week old) were used to raise antibodies against HPS virus as per method described previously (Kumar et al. (1997) The birds were divided into four groups based on four type of commercially available HPS vaccine.

The birds were bled on 24th day; serum was collected and stored at −20 °C until used.

The author raised the Sera in different groups to check the body response to different commercially available HPS Vaccine in the form of antibody titter. Moreover it was also performed to save the time. if one vaccine don't give best response, then the antibodies of another can be used.

The oil based cell culture HPS vaccine (Rhone Merieux, (USA)) was used as an antigen in AGPT(Kumar et al. (1997) because it give us clear precipitation line in AGPT during optimization for screening antibodies against HPS virus. The positive sera were stored at −20 °C.

Preparation of Infected /Normal Liver Sample for SDS−Page

To make component peptides for use in SDS−PAGE, the positive liver samples (tested by AGPT) were homogenized and subjected to sonication by giving 10 shots at the interval of five minutes each using sonicator (EILA Japan). Liver samples were processed by various methods but the bands obtained from chloroform treatment were clearly visible and well separated, so chloroform treatment method was preferred. Chloroform was added to the supernatant (2:1) , shake slowly for 15 minutes manually and then subjected to sonication again. The sonicated samples were placed in ultra centrifuge machine (Merck Japan) for 15 minutes at the speed of 4,000rpm.. After centrifugation, the upper most layers was taken in a sterile microfuge tube and subjected to ammonium sulphate precipitation (Jayanta K. Pal et al (2004). Likewise, the normal liver and HPS autogenous vaccines were processed. A stock solution containing BSA (100mg/ml) of was prepared in normal saline which was further diluted to prepare concentrations of 1, 20, 40, 60, and 80 88.mg/ml.

Using bovine serum albumin as standard, the UV spectrophotometer (at 280 nm wavelength)(yes standard) was used to calculate protein concentrations in diseased, healthy and vaccine samples(Kumar R, Chandra R,2004),

Characterization of the HPS Virus Using SDS−PAGE Method

Working solution for loading gel was prepared (Maiti NK and Sarkar P, 1997) Jayanta K. Pal et al (2004) Unstained protein markers (Fermentas USA) were used. Samples polypeptides were separated using a distinctive gel of 10% acrylamide composition to analyze the entire profile of supernatants A 30 mL of gel−mixture was prepared by mixing already prepared monomer stock (30%) (9 mL), resolving gel buffer (31.6%) (7.5 mL), 10% SDS (300 µL), ammonium persulphate 10% (150 µL) and TEMED (12 µL) and water (12 mL),. While the stacking gel (6%) was prepared to a total quantity of 10 mL. The electrophoresis tank was filled with 6L of tank buffer (Electrophoresis buffer 10%) and the casting stand (containing gel) was placed vertically in electrophoresis tank. Finally, 30 µL of denatured protein samples were loaded in each well and the gel was run at voltage 4.54V/cm. The power was turned off when dye border reached near the gel bottom. Gel was removed from the cassette and placed in a staining dish containing deionized water. After a quick rinse, the water was poured off and stain was added. Coomassive blue dye (0.1%) in 50% methanol and protein bands in polyacrylamide gel was detected in 10% glacial acetic acid. Gel was agitated for 3−4 hours, stained in acetic acid and methanol to remove the extra dye.

Identification of Immunogenic Proteins using Western Blotting Technique

The Bio−Ice cooling unit (BIO−USA) was filled with water stored it in freezer at −85 °C until used. The transfer buffer (5%) was chilled to 4 °C (to improve heat dissipation.)The Nitrocellulose membrane (0.22 µm pore size) and the filter paper were cut to the dimensions of the gel(containing the fractionated protein).The gel sandwich was prepared as described by Kumar R, Chandra R.(2004) The cassette containing sandwich was placed in module(Bio−red USA) in such a way that the gel was at cathode side and nitrocellulose membrane on anode side. The frozen Bio−Ice cooling unit was added, Placed in tank (Bio−red USA) and the tank was completely filled with transfer buffer(5%).The blot was run at 100 volts for one hour. Upon completion of the run, the blotting sandwich was disassembled and the membrane was removed for development.

A duplicate of western blot was stained for visualization of the bands. The blot was stained using working stain (2%) to visualize protein bands on nitrocellulose. The blots were differentiated in running tap water. Later on the stain was completely removed by washing in tap water. The unstained western blot was incubated overnight in blocking buffer (3%)at room temperature to block non specific sites on membrane. The blotting paper was washed 3 times in washing buffer (0.5%) for 10 minutes each to remove unbound blocking agent. Filter paper (Whatman 3MM) and blotting paper, both were cutted into equal size using a clean blade and gloves. The filter paper was saturated with serum diluted in blocking buffer(3%)(1:4) and then assembled with blotting membrane having protein bands and were sealed by clumping between two glass plates .The sandwich was incubated at room temperature for 45 minutes. The filter paper was removed and blotting membrane was washed three times by shaking in 100 ml/wash, in PBS−Tween 20 solution(washing buffer) for 5 minutes each at room temperature. The horseradish peroxidase conjugated anti−chicken IgG was diluted in 10 ml blocking solution(3%) (1:50).The filter paper was saturated with diluted horseradish peroxidase conjugated anti−chicken IgG and were sealed by clumping between two glass plates .The sandwich was incubated at room temperature for 45 minutes. The filter paper was removed and blotting membrane was washed three times by shaking in 100 ml/wash , in washing buffer for 5 minutes each, at room temperature.

0.05 gm of noble agar(Merck's)was completely dissolved in 50 ml deionized water by gentle heating.4 ml of substrate was mixed with 4 ml of melted agar and poured slowly on blotting membrane to prevent bubble formation. Also the filter paper was fully saturated in 5 ml substrate solution and placed on blotting membrane. Results were recorded within 15 minutes.

RESULTS

Sixty broilers were divided into four groups viz A, B, C and D (n = 15 each).Three different liver homogenates vaccines (A, B, C) and one oil based cell culture vaccine (D) (Rhone Merieux Georgia) was used for raising hyper immune serum against HPS virus. The birds were kept for screening of antibodies against HPS virus before vaccination. The birds were immunized according to the schedule given in Table−1 at the dose rate of 0.5 ml/bird at 1st day of vaccination 0.75 ml/bird on 7th day and 1 ml/bird on 14th day. Serum was collected at 9th day after 2nd boosting. All serum samples were then subjected to Agar gel

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precipitation test (AGPT). Serum raised in response to oil based cell culture HPS vaccine given clear precipitation line

Hyper immune sera was raised using four types of vaccine against HPS Virus in broiler (n=60) .Positive sera was screened out by using AGPT. Serum raised in response to oil based cell culture HPS vaccine given clear precipitation line

The 20 % homogenates of HPS infected livers (collected from different poultry farms) were reacted with hyperimmune serum raised against oil based cell culture HPS vaccine (using AGPT). Precipitation lines of identity were observed in three liver samples. The positive samples were stored at −4°C till further used.

Absorbance of the sample by spectrophotometer was used to determine the concentration of protein samples from the calibration curve (Table 2). A standard curve was created by plotting concentration of the standard versus regression analysis of A280, as shown in Figure1

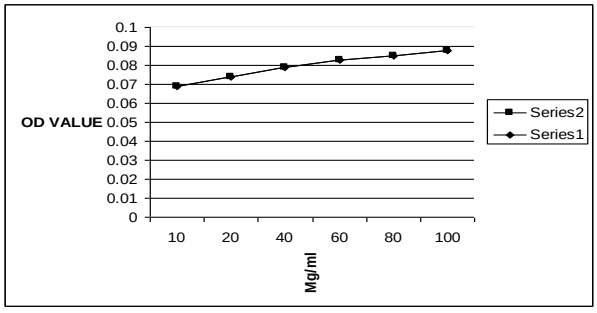

Figure 1: Standard Curve of Known Protein Sample

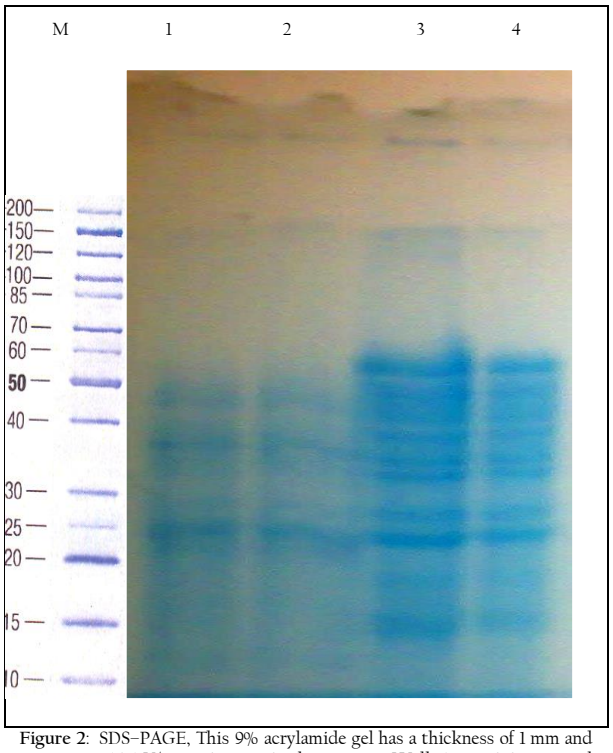

Figure 2: SDS–PAGE, This 9% acrylamide gel has a thickness of 1 mm and was run at 4.54 V/cm ,using maxi gel apparatus. Wells 1,containing normal liver and well 2 & 3 containing HPS autogenous vaccine 1 & 2. Wells 4, containing infected liver. All the wells were loaded with 30 mg/mL of total proteins.

Table–1: Total protein concentrations of samples

	Infected liver	Normal liver	Vaccine (A,B,C)
Conc. mg/mL	30	30	30
Optical density	0.078	0.078	0.078

The polypeptides of normal and HPS infected livers were resolved by SDS–PAGE using discontinuous buffer system. In 9% acrylamide gel, normal liver and HPS autogenious vaccine–1 yielded 10 polypeptides while HPS infected liver and HPS autogenous vaccine–2, yielded 15 polypeptide bands (Table 3) which were visualized on Coomassie brilliant blue R 250 staining (Figure–2).

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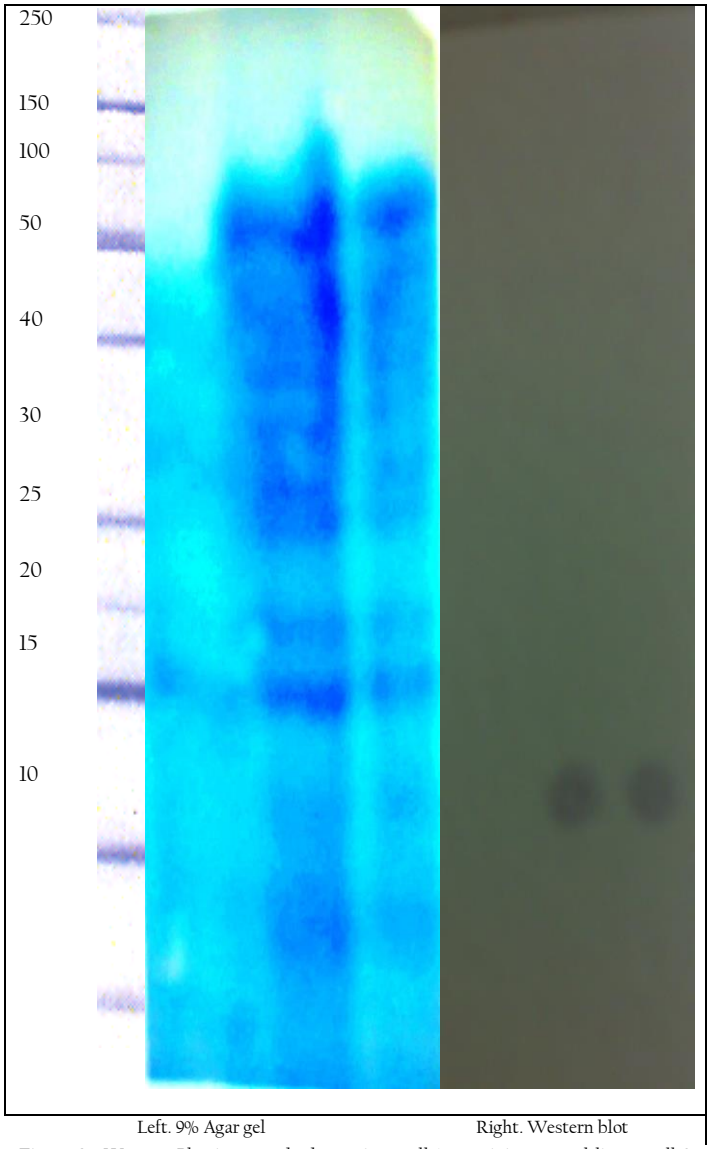

Figure 3: Wastern Blotting, standerd proteins, well 1 containing normal liver., well 2 containing HPS autogenous vaccine, well 3 infected liver

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Table 2: Molecular weights of structural polypeptides as determined by SDS-PAGE

Structural polypeptides	Molecular weight (kDa)			
	Normal liver	HPS auto vaccine –1	HPS auto vaccine –2	HPS infected liver
I	115	115	115	115
II	100	100	100	100
III	85	85	85	85
IV	70	70	70	70
V	60	60	60	60
VI	–	–	55	55
VII	50	50	50	50
VIII	–	–	45	45
IX	35	35	35	35
X	32	32	32	32
XI	–	–	30	30
XII	–	–	25	25
XIII	20	20	20	20
XIV	–	–	13	13
XV	15	15	15	15

1, containing normal liver and well 2 & 3 containing HPS autogenous vaccine 1 & 2. Wells 4, containing infected liver. All the wells were loaded with 30 mg/mL of total proteins.

The polypeptides of HPS autogenious vaccine, normal and HPS infected livers, separated on 9% gel were transferred onto nitrocellulose membrane (0.22 µm) by Western blotting technique(Kumar R, Chandra R.(2004).An immunogenic band was detected in well containing HPS virus infected liver homogenate and HPS autogenous vaccine (ottoman Parma) in the form of color spot. The position of spot was compared with stained western blot (duplicate). It was found to be between 20–15 kDa both in HPS infected and HPS autogenious vaccines (Figure–4)

DISCUSSION

In the present study, the infected liver and HPS autogenous vaccine revealed 15–polypeptides and one immunogenic band which is different from previous observations made by number of researchers. For example, Maiti NK and Sarkar P, 1997 has verified 11 polypeptides, ranging in molecular weights from 100 to 9 kDa in FAV serotype 1. Nazerian et al. (2002) reported 11 structural polypeptides in FAV serotype 2 that ranged in molecular weight from 97 to 14 kDa. Li et al, reported at least 14 polypeptides in chicken embryo lethal orphan (CELO) virus (fowl adenovirus type 1). Rajesh and Rajesh (2004) observed a total of 12 polypeptides ranging in molecular weight between 13.8 and 110.0 kDa and also seven immunogenic polypeptides ranging in molecular weight between 15.8 and 110.0 kDa.

Contrary to this, normal liver revealed 10–polypeptides with a difference of 5–polypeptide & one immunogenic band from diseased or infected liver.

The present study revealed that some HPS autogenious vaccine may not contain even single immunogenic proteins and thus, may not be able to provoke an effective immune response. The local manufacturer should take into consideration the fact and appropriate measure should be adopted such as described here; evaluation of vaccine using SDS PAGE and western blotting along with normal and diseased clinical samples (liver).

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