The Journal of Animal & Plant Sciences, 25(5): 2015, Page: 1297-1302
ISSN: 1018-7081

COMPARATIVE ANALYSIS OF PROTEIN PROFILE OF VACCINE STRAIN AND LOCAL ISOLATES OF AVIAN INFECTIOUS BURSAL DISEASE VIRUS BY WESTERN BLOT

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ABSTRACT

Infectious bursal disease (IBD), commonly known as "Gumboro Disease is an infectious viral disease of chickens inducing severe immunosuppression. In Pakistan, imported vaccine is used to prevent the disease but there have been outbreaks even in vaccinated flocks. This indicates that there is variation among the locally prevalent viral strains and commercially available vaccine strain. In present study, twenty-five local strains of IBD virus (IBDV) were isolated, purified and their structural polypeptides were studied by polyacrylamide gel electrophoresis (PAGE). Then their protein profiles were compared with commercial vaccine D-78 strain. Distinct banding patterns were observed in local isolates on basis of which the viral isolates were placed in three groups. Group-1 contained polypeptides of molecular weights 95Kd, 60Kd, 56Kd, 45Kd, 31Kd, 21Kd and 14Kd. In group-2, bands of 56Kd and 32Kd were missing whereas in group-3 the bands of 56Kd and 31Kd were missing while comparing with group-1. It was observed that 60Kd molecular weight protein band was common in all the three groups but it was absent in the vaccine D-78 strain. Vaccine D-78 strain appeared to have the banding pattern as 95Kd, 53Kd, 46Kd, 40Kd, 32Kd and 27Kd. Western blot analysis of the proteins were also conducted using antibodies raised against vaccine strain D-78. It was observed that the antibodies bind against all the protein bands of vaccine D-78 but did not bind with all the polypeptides of locally prevalent field viruses. This confirms that antigenic heterogenicity exists among the indigenous strains and the imported vaccine strains of IBDV.

Key words: Infectious bursal disease virus, Protein profile, SDS-PAGE, Western blot, Antigenic heterogenicity, Pakistan.

INTRODUCTION

Gumboro, the Infectious Bursal Disease (IBD) is caused by an acute, extremely transmittable disease that consequently ends up in death and immunological disorder in immature chickens. Since its discovery in Gumboro, Delaware, the disease has caused heavy economic losses on poultry trade all over the world (Kegne and Chanie, 2014).

The etiologic agent of IBD is a double stranded RNA virus belonging to the family Birnaviridae (Jordan, 1990). IBD virus (IBDV) is single cased, non-enveloped virus that contains a bi-segmented, double-stranded RNA genome. It can be cultured in different cell lines however the mammalian cell lines are best for culturing the virus for vaccine production (Mannan *et al*., 2009). The virus is composed of five major proteins. VP1 (95 Kilo Dalton (Kd)) molecular weight is the RNA dependent RNA polymerase protein of the virus (Kibenge and Dhama, 1997), VP2 (40Kd) forms the antigenic region, responsible for the induction of neutralizing antibodies and for serotype, VP3 (32 Kd) is a group specific antigen that is recognized by non neutralizing antibodies, some of which cross react with both serotypes, VP4 (28Kd) is a non structural polypeptide involved in auto processing of the polyprotein and VP5 has a regulatory function and play a key role in virus release and dissemination (Havarstein *et al.*, 1990).

The IBDV exist as two antigenically distinct serotypes commonly referred as serotype 1 and serotype 2. Most isolates of chicken origin have been classified as serotype 2 that cause disease symptoms in chickens under ten weeks of age. Symptoms do not appear in older chickens. Antibodies against serotype 2 are common in turkeys but sometimes found in chickens and ducks as well (Isaac *et al.,* 2002). Because of prevalence of different strains of the virus, it has become more difficult to control IBD (Jordan, 1990).The variant strains provoke infection in the presence of even high antibody titers. Such strains do not cause clear symptoms, but they do cause immune-suppression (Jackwood *et al.,* 2005).

Chickens that are 3-6 weeks age are the most vulnerable to IBD. The IBDV mutate rapidly and is resistant to high temperature and chemicals and can live in feces, bedding, contaminated feed and water for about four months. Transfer of virus is mainly through fecal oral course. Spread through aerosol is not considerable. Transmission of IBDV through embryos or semen has not been observed (Jackwood *et al.,* 2005).

Incubation phase of the virus is about 3 days after which it is shed following infection which last up to two weeks. The disease is highly transmittable, can

spread through the movement of poultry products, tools, feed supply bags, transportation vehicles and workers. Movement of the virus between wild birds and chickens is possibly due to consumption of contaminated water, or contact of respiratory or conjunctiva membranes to polluted poultry dust (Okoyo and Uzoukwu, 2005).

In Pakistan, IBD was first recorded at the Government Poultry Farm, Peshawar, in 1987. Since then it has caused heavy economic losses by reduced egg production as well as by mortality of chickens due to lack of immunity against diseases. Despite concerted efforts to induce improved vaccination schedules, there has been outbreak of the disease in the vaccinated flocks in different countries of the world including Pakistan. The IBDV isolates from these outbreaks have shown to differ antigenically and pathogenically from the standard IBDV. They have been designated as variants of the original strain (Sharma *et al.,* 2009). This is due to the reason that IBDV has a potential for antigenic heterogenicity. The present study focuses on the isolation and purification of the locally prevalent IBDV strains from different poultry farms as well as comparison of their structural polypeptides with that of the commercial vaccine D-78 strain by using the techniques of SDS-PAGE and western blot.

MATERIALS AND METHODS

Sampling: Twenty five samples of bursa of fabricious were collected from IBD infected flocks from different poultry farms located in district Sahiwal. The infected chickens suspected to be IBDV positive were collected and slaughtered. Six samples were collected from layers and 19 samples were obtained from broilers. All the samples were given specific identity code and were refrigerated till further processing (Table 1)

Preparation of inoculum: IBDV infected bursa of fabricious were finely divided in sterile GKN medium (glucose (1g), KCl (0.2g), NaCl (8g), phenol red 1% (2ml), H_2O (900ml)). The suspension was then washed again with GKN and minced with sterile scalpel. The homogenate was frozen and thawed three times to release viruses from the cells. Then it was centrifuged at 1500rpm for 15 minutes at 10^{o}C. The supernatant was collected and pellet was discarded. Finally supernatant was filtered and the filtrate was stored at -20^{o}C.

The above filtrate was then purified on a sucrose gradient by ultra-centrifugation at 63,000 x g for 5 hours at 4^{o}C. The virus band was collected using syringe. The collected material was dialyzed against TNE (Tris. NaCl.EDTA) buffer for overnight at 4 ^{o}C using molecular porous membrane tubing. The dialyzed material was recovered and centrifuged at 50,000 x g for 5 hours at 4^{o}C. The pellet was suspended in sterile distilled water and was stored at -20^{o}C. The IBDV inoculums were confirmed to be free of contamination for other poultry viruses by electron microscopy.

Chicken antisera to IBD virus: Antisera against IBDV was raised in three specific pathogen free chickens by infecting chicken intra ocularly using 0.5 ml of commercially imported inactivated vaccine D-78. All chickens were bled after 14 days to separate the antisera. The sera were separated by centrifugation at 400 g for 15 min and stored at -20°C.

SDS-PAGE of IBDV proteins: The proteins of IBDV vaccine D-78 and locally prevalent field isolates of IBDV were analyzed on 3.5% stacking gel and 12.5% separating gel using the discontinuous SDS-PAGE system. For this purpose 40 µl of each of the purified virus sample was mixed with 20µl of sample buffer (1M TrisHCl, SDS 20%, Beta mercaptoethanol, glycerol and H_2O) and heated for 3 minutes in boiling water bath. Then after adding the loading dye Bromophenol blue the samples were loaded in the gel and were run at a constant voltage of 220V and current of 60mA. When the dye reached the bottom, the plates were disassembled and gel was stained with comassie blue stain for 6 hours. Then the gel was placed overnight in destaining solution (Methanol 450 ml, Glacial acetic acid 100ml, H_2O up to 1L)

Transfer of IBDV proteins to nitrocellulose membrane: The IBDV proteins separated by SDS-PAGE were transferred to nitrocellulose membrane (Immunobiolin-NC, pore size 0.45µm Sigma chemicals, USA) using the semi-dry electrophoresis transfer method. For this purpose the semi-dry transblot unit (Semi-phorHoefer scientific instruments, USA) was used. The membrane was then dried completely. After that it was rewetted with distilled water.

Western blot analysis of protein profiles: First of all the non-specific adsorption sites on the nitrocellulose membrane were blocked with fish gelatin solution for one hour. After that the blot was washed twice with Tween-20 solution. The membrane was placed in specific antibodies (1:20 dilution) rose against IBDV vaccine D-78, for 6 hours. Then it was washed with Tween-20 solution and then alkaline phosphatase labeled chicken IgG conjugate (1: 500) was added. Afterwards the membrane was washed three times. Nitro blue Tetrazolium (NBT) and Bromochloroindolyl phosphate (BCIP) were used as substrate for alkaline phosphatase enzyme. The blot was developed at room temperature for 30 minutes. The reaction was stopped with 20mM EDTA solution made in Phosphate buffer saline.

RESULTS

SDS-PAGE of IBDV proteins: For SDS-PAGE low range protein markers of molecular weights, 220Kd, 94Kd, 67Kd, 60Kd, 43Kd, 35Kd, 30Kd, 20Kd and 14Kd were used as standard to compare the molecular weights of the polypeptides of viruses. The SDS-PAGE analysis of vaccine strain D-78 showed that it contains proteins of molecular weights 95Kd (VP1), 53Kd (precursors of VP2**,** VP3 and VP4), 46Kd (VPX), 40Kd (VP2), 32KD (VP3) and 27Kd (VP4). The protein with molecular weight 40Kd was found to be the major component of the vaccine strain D-78 virus.

The molecular weights of the proteins of field isolates of IBDV were also analyzed through SDS-PAGE. The banding pattern of the field isolates revealed that there are three types of field isolates of IBDV that were placed in three groups. Group-1 presented the banding pattern of molecular weights 95Kd, 60Kd, 56Kd, 45Kd, 31Kd, 21Kd and 14Kd. Group-2 comprised of 95Kd, 60Kd, 45Kd, 21Kd and 14Kd bands. While Group-3 appeared to have 95Kd, 60Kd, 21Kd and 14Kd bands. The 60Kd molecular weight protein was found to be the major component of field isolates of IBDV which was absent in the vaccine (Figure 1).

Western blot analysis of protein profiles: Western blot was conducted using the antibodies which were raised in chickens vaccinated with D-78 commercial vaccine. In the blot, the banding pattern of 95Kd, 53Kd, 46Kd, 40Kd, 32Kd and 27Kd proteins was obtained, which was of D-78. While the bands present in the field strains were absent in the vaccine. Moreover, the major protein of 60Kd present in field isolates was not hybridized (Figure 2).

Table 1. Site of sample collection, type, age and size of the flock from which the samples were collected

S. No.	Name of Poultry farm	Flock-ID	Type, age and size of flock
1	Alam farm, Sahiwal	B-1	Broiler, 4 weeks, 1300
2	Alam farm, Sahiwal	B -2	Broiler, 6 weeks, 4000
3	Sajad farm, Sahiwal	B -3	Broilers, 2 weeks, 300
4	Nadeem farm, Sahiwal	L-4	Layers, 4 weeks, 2200
5	Nawaz farm, Sahiwal	B-5	Broilers, 18 days, 2000
6	Majeed farm, Sahiwal	F-6	Layers, 5 weeks, 2500
7	Asif Farm, Sahiwal	F-7	Broilers, 2 weeks, 2500
8	Asif Farm, Sahiwal	F-8	Layers, 6 weeks, 3000
9	Asif Farm, Sahiwal	F-9	Broilers, 3 weeks, 2000
10	Asif Farm, Sahiwal	F-10	Layers, 3 weeks, 2000
11	Saleem farm, Ranala	F-11	Broilers, 3 weeks, 3000
12	Saghir farm, Ranala	F-12	Broilers, 5 weeks, 5000
13	Saleem farm, Ranala	F-13	Layers, 4 weeks, 2500
14	Saghir farm, Ranala	F-14	Broilers, 3 weeks, 400
15	Qadeer farm, Okara	F-15	Broilers, 19 days, 2000
16	Qadeer farm, Okara	F-16	Layers, 6 weeks, 2000
17	Qadeer farm, Okara	F-17	Broilers, 6 weeks, 2500
18	Qadeer farm, Okara	F-18	Broilers, 4 weeks, 1500
19	Ramazan farm, Okara	F-19	Broilers, 3 weeks, 3000
20	Shabir farm, Okara	F-20	Broilers, 4 weeks, 2500
21	Saleem farm, Ranala	F-21	Broilers, 4 weeks, 2000
22	Saghir farm, Ranala	F-22	Broilers, 3 weeks, 1500
23	Tahir farm, Ranala	F-23	Broilers, 6 weeks, 2500
24	Yaqoob farm, Chichawatni	F-24	Broilers, 5 weeks, 1000
25	Akram farm, Chichawatni	F-25	Broilers, 5 weeks, 3000

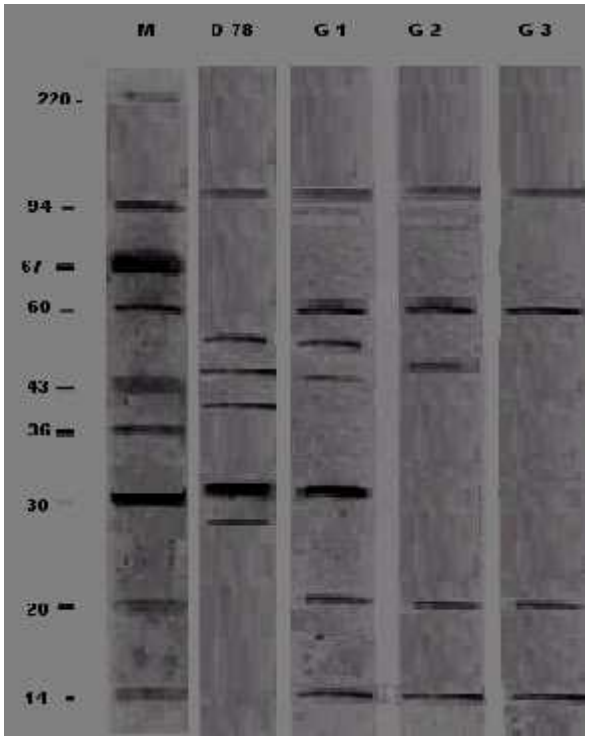

Figure 1: SDS-PAGE pattern of proteins of vaccine strain D-78 and the three groups G1, G2 and G3 of field strains of IBDV. M is protein molecular weight marker in Kilo Dalton (Kd).

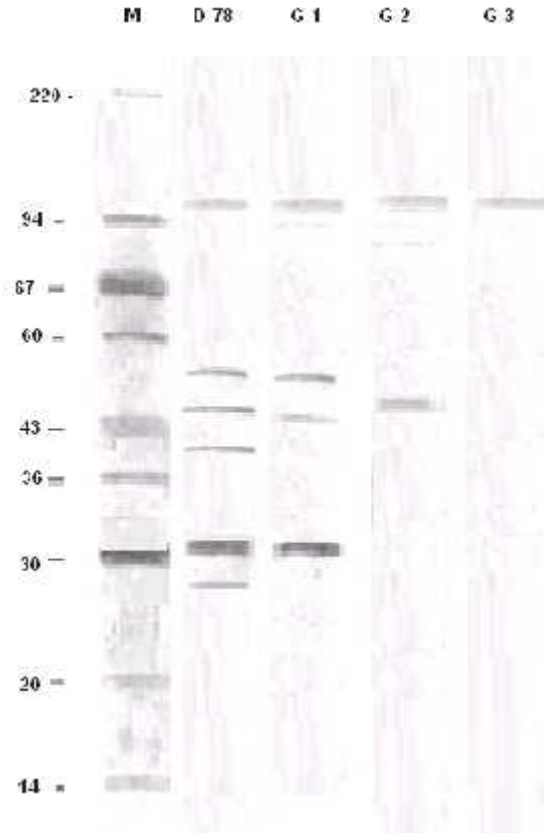

Figure 2: Western Blot of protein profiles of Vaccine strain D-78 and the three groups G1, G2 and G3 of field strains of IBDV. M is protein molecular weight marker in Kilo Dalton (Kd).

DISCUSSION

Infectious bursal disease can be prevented only by vaccination. Therefore, regular vaccination plan is followed by the farm holders. Live attenuated vaccine as well as the killed vaccine is being employed in commercial farming in Pakistan as well as other countries. The objective of present study was to identify the reasons why the vaccinated flocks of chickens are getting IBDV infection. For this purpose local strains of IBDV were isolated from infected samples of bursa of Fabricious. Bursa of Fabricious is a good source of IBDV because it provides a good microenvironment for the replication of IBDV (Tsai and Saif, 1992). Moreover, it was observed that mostly the birds positive for IBDV were of the age between two to three weeks, with bursa covered with gelatinous yellow transudate and was edematous. This is due to the reason that the disease occurs in early age and is of short duration and after complete destruction of lymphocytes the virus leaves the tissue.

The overall idea revealed by analyzing the results of SDS-PAGE banding pattern of viral isolates and Western blot with antibodies raised against vaccine D-78 Strain is that the precursor proteins of virus can also be virulent. In case of the field isolates, 60Kd precursor protein was found to be the major component in the three groups that could be the cause of virulence. A protein of almost similar molecular weight was earlier detected in infectious bursal disease virus field isolates of Pakistan. Additional proteins were also found in the three groups but absent in the vaccine. The 14Kd protein was also reported earlier (Anjum *et al.*, 1997). One of the reasons for the variation in protein profile is differences in the cleavage sites of the precursor proteins, especially when different host systems are used to grow the virus (Muller and Bechet, 2002). Moreover, the type and amount of cellular proteases made available by B-cells or fibroblast during the maturation of the virion also plays a role in the efficient processing of viral precursors.

From present study it appears that 60Kd molecular weight protein that gave a major band in all the isolates have vital role in the virulence of IBDV. Since this protein was absent in vaccine strain, the flocks are not completely immunized against all the viral proteins. Thus, causing outbreak of the disease despite intensive vaccination program followed far and wide in the country. Knoblich *et al.*, 2000 has also reported that the emergence of diverse strains of IBDV has complicated the protection against IBD infection. Also the ability of vaccine virus to protect against variant challenge is associated with strain of challenge and vaccine viruses.

Correspondingly, Hsieh *et al.* (2010) and Rojs *et al.* (2011) separately reported that the variant isolates differ pathologically and serologically from classical IBDV strains and they contain different neutralizing epitopes which causes vaccination failures (Hsieh *et al.*, 2010; Rojs *et al.*, 2011). As a result, vaccines prepared from native strains have been observed to provide better protection due to more antigenic relatedness Therefore, it is recommended to produce indigenous vaccines using the locally prevalent strains of IBDV in Pakistan.

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