Isolation and molecular identification of infectious bursal disease (IBD) virus from commercial poultry: Effects of field isolate on cell mediated immune response and serum biochemical parameters in broilers

*Zain ul Abidin1, Aisha Khatoon2, Tariq Mahmood Butt1, Sajjad Hussain1, Ayesha Kanwal1, Sajjad Ali1 and Asma Aziz1

1. Veterinary Research Institute, Zarrar Shaheed Road Lahore Cantt-13 Pakistan.
2. Department of Pathology, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad, Pakistan.

Abstract:
Belonging to genus Aviibirnavirus and family Birnaviridae infectious bursal disease virus (IBDV) is a double stranded RNA virus and it causes an acute highly infectious disease in poultry resulting in watery diarrhea, anorexia, high morbidity and mortality and hemorrhagic lesions on breast and leg muscles leading to down grading of poultry meat. The present study was designed to isolate and molecularly identify the causative agent (IBDV) from a clinically suspected flock of infectious bursal disease and to check the effects of isolated virus on cell mediated immune response and serum biochemical parameters in broilers along with reference strain (IBDV-2512). Bursae were collected and subjected to trituration and supernatent when inoculated in 9-day old embryonated chicken eggs resulted in the death of all the embryos during first three blind passages. Every triturate produced a clear and distinct line of precipitation with IBDV-known antisera in agar gel precipitation test (AGPT). Serum samples collected at the time of occurrence of disease presented a low anti-IBDV titer which was between 1:2 and 1:8 as elucidated by indirect haemagglutination inhibition (IHA) test while serum samples of same flock collected 14 days after first sampling presented a drastic increase in anti-IBDV antibodies that was between 1:64 and 1:512. Reverse transcriptase polymerase chain reaction (RT-PCR) revealed a product of approximately 743bp of VP2 gene of IBDV for all three suspected samples along with the reference strain. Broiler birds of 3 weeks of age when injected with field isolated resulted in decreased lymphoproliferative response as elucidated by tuberculin test and serum biochemical parameters were also altered in field isolated injected birds and these alterations were more or less similar to that of birds injected with reference strain (IBDV-2512) suggesting high pathogenicity of isolated virus.

Key Words: Infectious bursal disease; molecular identification; commercial poultry; cell mediated immune response; serum biochemical parameters; broilers.

Introduction
Infectious bursal disease (IBD) is a life threatening problem in the poultry industry, adversely affecting its economy through high mortality potential in a flock. IBD is an acute highly infectious disease caused by a double stranded RNA virus belonging to the genus Aviibirnavirus, family Birnaviridae (Bidin et al., 2001). This disease results in watery diarrhea, anorexia, high mortality, hemorrhagic lesions on breast and leg muscles leading to rejection of meat from market (Hamoud et al., 2007) and immunosuppression leading to secondary bacterial infections and most importantly the adverse effects of mycotoxins present in feed (Abidin et al., 2011).

Different serological tests are being performed for the diagnosis and confirmation of IBD like indirect haemagglutination test (IHA), agar gel precipitation test (AGPT) and enzyme linked immune-sorbent assay (Okwor et al., 2011). These tests mostly involve the principle of antigen-antibody reaction while identification of the agent (IBDV) using real time-polymerase chain reaction (RT-PCR) is a molecular technique that allows a much quicker identification of IBDV (Wu et al., 1992). Presence of this disease causes alterations in different hematological and serum biochemical parameters in poultry (Zeryehun et al., 2012). Apart from many other alterations, the most important stress to birds caused by the virus is immunosuppression. The predilection site for virus replication is bursa where its peak titer can be detected between 3-5 days after IBDV infection (Kim et al., 2000). This disease causes suppression of both humoral and cell mediated immune responses in birds. The virus also causes a transient inhibition of proliferative responses of T-cells against mitogens in vitro (Sharma et al., 2000).
Keeping in view the above discussion the present study was designed to isolate and molecularly identify the IBD virus from field and to check the extent of alterations induced by field isolate regarding different biochemical parameters and cell-mediated immune response when compared with IBDV reference strain 2512.

**Materials and Methods**

**Collection of samples**

An outbreak of IBD reported at Lahore in broiler farm of 70,000 birds at 4th week of age. The mortality was up to 40% and morbidity was 100% at 4th day of outbreak. The farm was visited and observed for clinical signs, morbidity and mortality. Watery diarrhea, anorexia, vent picking and lameness were observed and necropsy examination showed increase in the size of bursae with clear nephritis (Figure 1 and 2). Total 20 bursae were collected for IBD virus isolation and molecular identification along with 24 serum samples. The same farm was followed and 24 serum samples were again collected 14 days post outbreak (convalescence). The serum samples were subjected to IHA test for determination of anti-IBD-IHA-antibody titers.

**Isolation of virus**

Fifteen bursae were divided into 3 groups containing 5 bursae each and processed for virus isolation while remaining five bursae were preserved for molecular identification. Bursae of each group were triturated using phosphate buffered saline (PBS) and 0.1ml of triturate was injected in 16 embryonated chickens eggs (9 day of age) via chorio-allantoic membrane (CAM) route while 4 embryos were taken as control. Candling was performed on daily basis and any mortality up to 48 hrs was discarded, however; dead embryos after 48 hrs were used for further processing. Embryos along with CAM were collected, triturated and again inoculated in 9 day old chicken embryonated eggs (2nd passage). Similarly three passages were given to each isolate presenting the respective groups.

**Agar gel precipitation test (AGPT)**

The triturate collected after each passage was subjected to agar gel precipitation test (AGPT) by the method of Okwor et al., (2011) with few modifications to check the antigen-antibody response specific for IBD virus. Briefly described, 6 wells were made (with a central well surrounded by 5 wells in a circle) using sterilized well cutter in an immunodiffusion plate containing 1% Nobel agar prepared in PBS having a PH of 7.2. The triturated sample was added in the surrounding wells with known IBD hyperimmune serum in the central well. The plate was incubated at 37ºC and observed under diffused light at 24, 48 and 72 hrs interval for the presence of line of precipitation between central and any surrounding wells.

**Indirect haemagglutination (IHA) test**

Serum antibody titer for IBDV was determined using indirect haemagglutination (IHA) test according to the method of Hussain et al., (2003). Briefly described a quantity of 5 ml of human blood group “O” with EDTA (anticoagulant) was centrifuged at 1500 rpm for 5 minutes and plasma along with buffy coat was separated. Using PBS, red blood cells (RBCs) were washed thrice. The washed RBCs were sensitized with antigen (IBDV) by adding 2 ml of PBS, 2 ml of antigen and 1 ml of washed RBCs in a tube and placing it in incubator at 37ºC for 45 minutes. The treated RBCs were again washed thrice and used in test (IHA). Two fold serial dilution of test samples (in PBS) were made in titration plate and equal quantity of sterilized RBCs were added in each well and plates were incubated at 37ºC for 30 minutes.

**Molecular identification using RT-PCR**

Three bursae from field samples and one bursa from bird after experimental induction of disease by injecting IBDV strain 2512 (positive control) were subjected to RT-PCR based diagnosis of the disease. RNA was isolated using TRIzol method. In brief, the bursal tissue samples were homogenized in TRI reagent (Cat # PR-118, Molecular Research Centre (MRC), Inc.) followed by RNA extraction with chloroform and precipitation with isopropanol. After giving it one wash with 70% ethanol, RNA was dissolved in RNase-free water and stored at -20ºC.

RT-PCR was performed following the method explained by Lone et al., (2009) using the primer pair; FJL1 5’ GCCCAAGGTCTACACCAT 3’ and RJL2 5’ CCCGGATTATGTCTTTTGA 3’ that amplified a product of approximately 743bp from the VP2 gene of IBDV (Jackwood and Sommer, 1997). The amplified gene fragment was electrophoresed on 1% agarose gel and its size was confirmed by comparing with 1kb DNA ladder (O’GeneRuler 1kb DNA ladder ready-to-use).
**Induction of disease**

Thirty specific-pathogen-free (SPF) broiler birds of 3-week age were selected and divided in 3 groups containing 10 birds each. Group A was taken as control; Group B was given IBDV reference strain 2512 (Charles River Laboratories Inc.) at the rate of 0.1ml of EID50 (virus titer 10^{4.08}/100µl) via eye-droppings and Group C was given IBDV field isolate at the rate of 0.1ml of EID50 (virus titer 10^{5.66}/100µl) via eye-droppings. These birds were given basal diet (ad-lib) with aflatoxin levels less than 1ng/g and proper managemental conditions were provided. The work was carried out at VRI, Lahore Pakistan and guidelines of ethical use of animals (Dua, 2004) were strictly followed.

**Lymphoproliferative response to avian tuberculin**

This test was performed following the method of Khatoon et al., (2013) to check the cell-mediated immune response. Briefly described, five birds from each group were randomly selected and avian tuberculin (VRI, Lahore Pakistan) was injected in the inter-digital space between 3rd and 4th digit of right foot at the rate of 0.2ml/bird at day 3 post-infection (p.i). Similarly, PBS was injected (0.2ml/bird) between 3rd and 4th digit of left foot as control using a constant tension micrometer. The thickness of intra-digital space was measured at 0, 24, 48 and 72 hours time intervals to evaluate the cell-mediated response. The thickness was expressed as tuberculin mediated minus PBS-injected (control) swelling (millimeters) at each time point in all three groups.

**Serum biochemical parameters**

Serum samples of five birds from each group were collected at 3, 4, 5 and 6 day post-infection (p.i) and were used for determination of albumin, total proteins, alanine aminotransferase, ALP, AST, triglycerides, cholesterol, urea, creatinine, calcium, sodium, chloride and potassium using commercially available kits of Fluitest (Germany) and samples were analyzed by spectrophotometer (Uvikon 930, Kontron, Germany).

**Statistical analysis**

Data thus obtained was subjected to statistical analysis using one-way analysis of variance (one-way ANOVA) test (P≤0.05).

**Results**

**Isolation of virus**

During 1st passage 4 embryos out of 16 injected embryonated eggs were found dead during 48 hours which were discarded (considered as accidental deaths). After 48 hours all the embryos were dead. There were distinct hemorrhages at the lower abdomen and hind legs (Figure 3A and B) while chorio-alantoic membranes had pinpoint hemorrhages (Figure 4). Dwarfing of embryos was also observed with subcutaneous oedema. 2nd and 3rd passage revealed no mortality during 48 hours while all embryos were found dead after 48 hours and embryos showed more severe lesions on hind legs and lower abdomen. Dwarfing of the embryos was more pronounced during these passages.

**Agar-gel precipitation test (AGPT)**

The triturates of bursae of all three groups showed a clear line of precipitation between 48 and 72 hours in agar-gel precipitation while all the passaged triturates showed much clear and prominent line of precipitation between 24 and 48 hours of AGPT.

**Indirect haemagglutination test (IHA)**

Sero-prevalence of anti-IBD antibodies of both periods following IHA have been shown in Table 1. Samples collected at the time of outbreak had titers between 1:2 to 1:8 with 2 samples having titer of 1:2; 13 with 1:4 and 10 with 1:8 while drastic increase in titer was observed in samples collected 14 days post outbreak (convalescence) was observed and these titers were between 1:64 and 1:512 (4 samples with titer of 1:64; 12 with 1:128; 6 with 1:256 and 3 with 1:512).

**Molecular identification using RT-PCR**

RT-PCR analysis of three field samples (isolates) along with IBDV strain 2512 (positive control) produced a PCR product of approximately 743bp (Figure 5).

**Lymphoproliferative response to avian tuberculin**
Lymphoproliferative response to avian tuberculin of all three groups has been mentioned in Table 2. At 24 and 48 hours post-tuberculin injection, the responses of chicks of both tested groups (B and C) were significantly lower as compared to control while lymphoproliferative responses of both groups (B and C) were non-significantly different from control at 72 hours post-tuberculin injection.

**Serum biochemical parameters**

Serum biochemical parameters of albumin, total proteins, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), triglyceride and cholesterol have been given in Table 3 while that of urea, creatinine, calcium, sodium, chloride and potassium have been mentioned in Table 4.

**Albumin and Total proteins:** Serum albumin and total protein values of group B and C were non-significantly different from control at day 3 and 4 (p.i) while at day 5 and 6 (p.i) albumin and total protein values of group B and C were significantly lower as compared to control group.

**Alanine aminotransferase:** Alanine aminotransferase values of group B was significantly higher than control at day 3, 4 and 5 while that of group C values though were non-significantly different from control yet closer to values of group B. However; at day 6 values of both group (B and C) were significantly higher than control.

**Alkaline Phosphatase:** Serum alkaline phosphatase values of group B and C were non-significantly different from control at day 3 and 4 (p.i) while these values were significantly higher for group B and C when compared with control at day 5 and 6 (p.i).

**Aspartate Aminotransferase:** Serum Aspartate aminotransferase values of group B were significantly higher than control during all days while these values in group C were higher than control at day 5 and 6 while at day 3 and 4 (p.i) these values though were non-significantly different from control yet closer to AST values of group B.

**Triglycerides:** Triglyceride values of group B were significantly lower as compared to control during all days of sampling while triglyceride values of group C during all days though were non-significantly different from control yet closer to values of group B.

**Cholesterol:** Cholesterol values of group B and C were non-significantly different from control at day 3 and 4 (p.i) while at day 5 and 6 (p.i) values of group B and C were significantly lower as compared to control.

**Urea:** Urea concentration of group B and C were non-significantly different from control at day 3 (p.i) while at day 4, 5 and 6 (p.i) these values were significantly higher than control in group B and C.

**Creatinine:** Creatinine values of group B and C were non-significantly different from control at day 3 and 4 (p.i) while at day 5 and 6 (p.i) creatinine values of group B and C were significantly higher as compared to control.

**Calcium:** Calcium levels of group B and C were non-significantly different from control at day 3 and 4 (p.i) while at day 5 and 6 (p.i) serum calcium levels of both groups (B and C) were significantly lower as compared to control.

**Sodium:** Sodium levels of group B were significantly lower as compared to control during all days while group C had significantly lower sodium levels as compared to control at day 6 (p.i) only. However; at day 3, 4 and 5 (p.i) values of group though were non-significantly different from control yet closer to the values of group B.

**Chloride:** Serum chloride levels of group B and C were significantly higher as compared to control during all days of sampling (day 3, 4, 5 and 6 post-infection).

**Potassium:** Serum potassium levels of group B were significantly higher than control at day 5 and 6 (p.i) while that of group C, the values were non-significantly different from control during all days of sampling (3, 4, 5 and 6). Despite of non-significant values of group C as compared to control these values were closer to values of group B.
### Table 1: Anti-IBD antibody titers of 25 samples collected at the time of outbreaks and 25 samples collected 14 days post outbreak (convalescence) using indirect haemagglutination test (IHA)

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Number of samples</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
<th>1:128</th>
<th>1:256</th>
<th>1:512</th>
<th>1:1028</th>
</tr>
</thead>
<tbody>
<tr>
<td>At time of outbreak</td>
<td>25</td>
<td>2</td>
<td>13</td>
<td>10</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>14 days after outbreak (convalescence)</td>
<td>25</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>4</td>
<td>12</td>
<td>6</td>
<td>3</td>
<td>-----</td>
<td>------</td>
<td>-------</td>
</tr>
</tbody>
</table>

**Each dilution showing number of positive samples during specific time interval**

### Table 2: Lymphoproliferative response to avian tuberculin of all three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>24 h post Injection</th>
<th>48 h post Injection</th>
<th>72 h post Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Control)</td>
<td>0.21±0.04 a</td>
<td>0.16±0.09 b</td>
<td>0.07±0.01 a</td>
</tr>
<tr>
<td>B (IBD strain 2512)</td>
<td>0.13±0.03 b</td>
<td>0.08±0.06c</td>
<td>0.12±0.04 a</td>
</tr>
<tr>
<td>C (Field Isolate)</td>
<td>0.14±0.01 bc</td>
<td>0.09±0.04 c</td>
<td>0.13±0.05 a</td>
</tr>
</tbody>
</table>

**Groups: (Group A= Control; Group B= IBDV reference strain; Group C= Field Isolate)
**

### Table 3: Serum concentrations (albumin, total protein, alanine aminotransferase, alkaline phosphatase, Aspartate aminotransferase, Triglycerides and Cholesterol) of all three groups at day 3, 4, 5 and 6 post-infection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin g/dl</td>
<td>A</td>
<td>1.71±0.11 a</td>
<td>1.69±0.44 ab</td>
<td>1.68±1.21 a</td>
<td>1.73±5.74 a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.59±0.42 a</td>
<td>1.51±0.87 b</td>
<td>1.32±1.73 b</td>
<td>1.09±4.66 b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.66±0.39 a</td>
<td>1.52±0.91 ab</td>
<td>1.38±2.74 b</td>
<td>1.14±4.88 bc</td>
</tr>
<tr>
<td>Total Protein g/dl</td>
<td>A</td>
<td>4.98±0.62 a</td>
<td>5.11±0.49 c</td>
<td>5.47±1.11 a</td>
<td>5.51±5.09 b</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.11±0.74 ab</td>
<td>3.99±0.71 c</td>
<td>3.23±3.01 b</td>
<td>3.11±5.89 c</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4.47±0.43 a</td>
<td>4.09±0.98 c</td>
<td>3.74±1.09 bc</td>
<td>3.37±2.93 c</td>
</tr>
<tr>
<td>Alanine Aminotransferase (ALT) u/L</td>
<td>A</td>
<td>3.48±0.32 a</td>
<td>3.51±0.28 ab</td>
<td>3.60±2.44 a</td>
<td>3.81±3.67 c</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.79±0.55 b</td>
<td>6.67±0.77 c</td>
<td>8.84±2.83 b</td>
<td>11.81±3.13 b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4.48±0.19 a</td>
<td>5.11±0.93 a</td>
<td>7.13±1.44 ab</td>
<td>10.35±3.79 bc</td>
</tr>
<tr>
<td>Alkaline Phosphatase (ALP) u/L</td>
<td>A</td>
<td>184.10±0.22 a</td>
<td>192.71±0.63 ab</td>
<td>194.55±1.93 a</td>
<td>202.41±2.42 c</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>199.34±0.38 ab</td>
<td>212.12±0.88 a</td>
<td>249.53±1.72 bc</td>
<td>258.61±3.72 ab</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>190.11±0.41 a</td>
<td>199.71±0.73 ab</td>
<td>217.41±2.01 ab</td>
<td>233.49±5.06 b</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (AST) u/L</td>
<td>A</td>
<td>177.12±0.66 a</td>
<td>180.11±0.44 a</td>
<td>185.23±1.74 a</td>
<td>186.49±2.95 b</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>209.12±0.34 bc</td>
<td>272.27±0.99 b</td>
<td>313.59±2.99 bc</td>
<td>401.11±5.49 b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>197.93±0.19 a</td>
<td>227.09±0.53 a</td>
<td>282.11±3.09 bc</td>
<td>307.71±2.84 ab</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>A</td>
<td>40.11±0.29 a</td>
<td>41.22±0.49 ab</td>
<td>39.99±2.54 a</td>
<td>43.01±3.07 a</td>
</tr>
</tbody>
</table>
**Groups:** (Group A = Control; Group B = IBDV reference strain; Group C = Field Isolate)

***Values in each column followed by different small letters are statistically different (P ≤ 0.05).***

Table 4: Serum concentrations (urea; creatinine, calcium, sodium, chloride and potassium) of all three groups at day 3, 4, 5 and 6 post-infection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea mg/dl</td>
<td>A</td>
<td>3.39±0.19a</td>
<td>3.42±0.17b</td>
<td>3.66±1.49a</td>
<td>3.62±1.22a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.81±0.27b</td>
<td>4.78±0.29a</td>
<td>5.14±1.26b</td>
<td>6.88±2.01b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.48±0.17a</td>
<td>4.02±0.83a</td>
<td>4.96±0.83bc</td>
<td>5.47±3.53b</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>A</td>
<td>0.69±0.87b</td>
<td>0.66±1.53a</td>
<td>0.68±0.69b</td>
<td>0.71±1.11a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.83±0.66bc</td>
<td>0.92±1.49a</td>
<td>0.98±0.47a</td>
<td>1.13±5.93b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.74±0.73bc</td>
<td>0.78±0.93a</td>
<td>0.88±0.86a</td>
<td>0.96±6.01bc</td>
</tr>
<tr>
<td>Calcium mmol/L</td>
<td>A</td>
<td>1.63±0.51a</td>
<td>1.68±0.81b</td>
<td>1.71±0.48a</td>
<td>1.80±2.13b</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1.60±0.84b</td>
<td>1.51±0.47a</td>
<td>1.31±0.69bc</td>
<td>0.88±3.79a</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.66±0.45bc</td>
<td>1.62±1.06a</td>
<td>1.48±0.63a</td>
<td>1.17±2.99a</td>
</tr>
<tr>
<td>Sodium mmol/L</td>
<td>A</td>
<td>128.11±2.22a</td>
<td>126.22±1.87bc</td>
<td>129.76±4.44a</td>
<td>132.54±3.36b</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>125.45±0.94b</td>
<td>111.09±3.82b</td>
<td>98.22±3.21b</td>
<td>71.11±1.92b</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>125.88±2.87ab</td>
<td>121.24±4.69bc</td>
<td>106.44±2.01bc</td>
<td>84.84±1.41bc</td>
</tr>
<tr>
<td>Cholesterol mg/dl</td>
<td>A</td>
<td>138.11±0.76a</td>
<td>143.24±0.91b</td>
<td>142.47±2.19a</td>
<td>149.71±2.58bc</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>134.14±0.48bc</td>
<td>120.34±0.72ab</td>
<td>111.14±1.63bc</td>
<td>93.85±3.11a</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>135.11±0.37bc</td>
<td>131.44±0.47bc</td>
<td>128.97±3.16c</td>
<td>105.22±5.49a</td>
</tr>
</tbody>
</table>

**Groups:** (Group A = Control; Group B = IBDV reference strain; Group C = Field Isolate)

***Values in each column followed by different small letters are statistically different (P ≤ 0.05).***

**Table 4: Serum concentrations (urea; creatinine, calcium, sodium, chloride and potassium) of all three groups at day 3, 4, 5 and 6 post-infection**
Figure1: Enlarged bursa along with nephritis in IBD suspected broilers collected from the farm
Figure 2: Enlarged bursa removed from the IBD suspected bird

Figure 3A: Hemorrhagic lesions on the chicken embryo injected with the triturate of bursae
Fig 3B: Hemorrhagic lesions on hind legs and body of the embryo injected with triturate of bursae

Figure 4: Chorio-allointoic membrane isolated from triturate inoculated embryonated chicken eggs
Discussion

Broiler birds were suspected for IBD on the basis of high mortality (40%) and clinical signs. Similarly mortality was also observed by Nunoya et al., (1992) in Japan and Van den Berg et al., (1991) in Belgium due to very virulent pathotype of IBDV while Chowdhury et al., (1996) reported 20-30% mortality due to it in Bangladesh. Chorio-alantoic (CAM) route was followed for the inoculation of triturate in the embryonated eggs as this part is more sensitive for virus replication than the allantoic sac. Normal incubation period for IBDV is 48 hours and all the inoculated embryos were found dead (100% mortality) after this time period in this study and this 100% mortality remained consistent during all three blind passages of the triturate. Pin-point hemorrhages were found on hind legs and CAM during all passages with dwarfing of embryo. Similarly Yamaguchi et al., (1996) and Ahmad et al., (2005) reported severe hemorrhagic lesions on CAM and surface of embryo and 100% mortality due to virulent IBDV during early passages. The triturate of bursae along with triturates obtained from every passage were found positive for agar gel precipitation test (AGPT) as a clear and distinct line of precipitation was found between wells containing sample and hyper-immune serum specific for IBDV. The basic concept behind AGPT is actually antigen-antibody interaction and presence of clear precipitation confirmed the suspected samples to be positive for IBDV. Similarly diagnosis of IBDV following AGPT was also performed by Hussain et al., (2003) and Okwor et al., (2011).

Reference strain along with three samples produced a PCR product of approximately 743 bp. The primers used were of VP2 gene and positivity of samples along with reference strain was suggestive of the fact that the test samples were positive for IBDV. The reason behind performing RT-PCR along with AGPT and IHA was that in these tests sometimes a negative tissue might give non-specific agglutination producing false negative results but RT-PCR is more sensitive assay as compared to AGPT and IHA and molecular identification using VP2 gene primers confirmed the presence of IBDV in samples. Molecular identification using RT-PCR was also reported by Lone et al., (2009) who performed RT-PCR for identification of 743bp segment of VP2 gene of IBDV in Pakistani isolates.

Low levels of antibodies against IBDV were detected at the time of occurrence of clinical signs which is suggestive of IBDV challenge in the field and it can be assumed that one shot of IBDV vaccine could not conferred the protective immunity. Generally speaking a titer of 1:64 is considered sufficient to protect and provide the birds specific immunity against IBDV (Lukert and Saif, 1997), however; this titer was very low in all samples at the time of appearance of disease and all samples had a titer between 1:2 to 1:8. Normally vaccination for IBDV is done between 5 to 10 days of age in broilers and atleast 10-14 days are needed to maintain minimal protective titer and during this period birds are most susceptible to IBDV infection. Low titer in present study might be co-related to many factors and one such factor is vaccination failure. Vaccine type, condition, handling, storage and transportation along with condition of birds and route of administration of vaccine is very important regarding proper vaccination programme. Another factor is emergence of new viral strains might result in the failure of classical vaccines (Hussain et al., 2003). Developing countries also have a problem of poor quality vaccines which might result in the
poor protection of birds against the diseases (Vui et al., 2002). Antibody titers 14 days after the first sampling (convalescence) showed drastic increase in the antibody titer against IBDV which were between 1:64 to 1:256 suggestive of the fact that surviving birds had combated IBDV infection and the immune system of birds was quite good leading to production of antibodies against viral particles and these antibodies would prevent the occurrence of future infection. Similarly Hussain et al., (2003) reported a low and high titers in broilers during 0-3 and 3-6 weeks of age respectively.

Oral infection with IBDV results in the replication of virus in macrophages and lymphocytes present in the gut and furthermore it infects bursa within 16 hours which is the predilection site for the growth of IBDV where it further replicates before viraemia develop. From blood virus goes to different visceral organs like spleen, liver, thymus and kidney. Within 2-3 days clinical signs of IBD appear in birds (Lima et al., 2005). In our study the clinical signs specific for IBD also appeared within 3 days post oral infection of IBDV and birds became dull, anorexic and passed whitish feces.

The alterations in the levels of antibody production have implications for several immunological responses in situ. Among them are the responses to mitogens such as phyto-haemagglutinin-P (PHA-P) and avian tuberculin which are taken as a responsible measurement of T-lymphocytes function (Qureshi et al., 1997; Khatoon et al., 2013). Decreased lymphoproliferative response to avian tuberculin was observed in birds infected with reference strain (IBD 2512) as well as field isolate suggesting that the isolated virus had equal or nearly equal capability of causing immunosuppression or more specifically decreased cell mediated immune response in birds. A decreased lymphoproliferative response to avian tuberculin was also observed by Khatoon et al., (2013) in ochratoxin A infected white Leghorn cockerels.

Albumin and total protein values of groups infected with reference strain and field isolate were drastically reduced at day 5 and 6 post-infection (p.i) while ALT, AST and ALP values were adversely increased in both treatment groups mostly at day 5 and 6 (p.i). The decreased albumin and total protein values and increased ALT, AST and ALP values in serum were also reported by Ley et al., (1983); Nunoya et al., (1992) and Zeryhun et al., (2012) due to vIBDV while Afaleq (1998) reported a decrease in total protein and Panigraphy et al., (1986) reported a decreased albumin values in serum of birds infected with IBDV. Reduction in total protein and albumin is attributed by liver damage which is a major source of plasma protein. It is also observed that reduced functional ability of liver also leads to hypoalbuminaemia and decreased protein levels in serum might also result due to proteinuria due to kidney damage. This proteinanemia might also result from the anorexic state of birds due to IBDV. Increase in ALT values occurs due to its release from hepatocytes as a result of necrosis or rupturing of hepatocytes causing irreversible damage (Abidin et al., 2013). Increased levels of AST, ALT and ALP in IBD infected groups is an indication of pathological changes in kidney and liver which are common features of IBDV infection (Henry et al., 1980; Tan et al., 2004). These damages might occur due to aplastic bone marrow following IBDV infection leading to hypoxic state (Nunoya et al., 1992).

Increased cholesterol values in serum might be associated with liver damage as most of the metabolism of cholesterol occurs in liver. In this study decreased levels of cholesterol as well as triglycerides were observed in IBDV infected groups which might be associated with anorexia along with watery diarrhea leading to reduced availability and absorption of fatty acids. Similarly Zeryhun et al., (2012) reported a decrease in levels of cholesterol in IBDV infected birds however; contrary to our study, Panigraphy et al., (1986) reported a higher concentration of cholesterol in the serum of IBDV infected birds as compared to control.

Increased urea and creatinine values were observed in this study in the serum samples of IBDV infected birds. Zeryhun et al., (2012) also reported an increase in uric acid and creatinine values of IBDV infected birds while Panigraphy et al., (1986) reported a decrease in the uric acid values of the chicks infected with IBDV. Increase in urea and creatinine values in serum could be attributed the kidney damages (Abidin et al., 2013) which can be observed in the infectious bursal disease.

IBDV infected birds showed a decrease in the calcium, sodium and potassium levels while an increase in chloride levels in the serum as compared to control. Decreased calcium levels might be due to reduced absorption of dietary calcium in the intestine possibly due to diarrhea and anorexia. Stress due to IBDV induced dehydration and anorexia might also lead to secretion of corticosteroid leading to decreased calcium in serum. The possible reasons for decreased sodium levels are dehydration, anorexia and decreased water intake leading to excessive water loss from the body causing a reduction in serum sodium levels (Rupley, 1997). Similarly increased chloride and decreased potassium levels could be possibly associated with diarrhea specifically seen in IBDV infected birds. Decreased potassium levels (hypokalemia) could also be attributed by kidney damage (Finco, 1997). Similar to our study Zeryhun et al., (2012) also reported a decrease in calcium, sodium and potassium levels while increase in chloride levels while Afaleq (1998) reported a decrease in calcium levels in IBDV infected birds.
Conclusion

The outbreak was of infectious bursal disease as indicated by death of embryos after inoculation in eggs, positive AGPT, increase in titer at convalescence period and most importantly samples positive for 743bp segment of VP2 gene of IBDV. The isolated virus drastically affected and altered the activity of T-lymphocytes as indicated by avian tuberculin assay and serum biochemical parameters more or less similar to those birds infected with the reference IBDV strain 2512.

References


Dua K. (2004): Veterinary ethics and jurisprudence, Kalyani Publishers, New Delhi, India


Okworo EC, Eze DC, Okinkwo KE, Ibu JO. (2011): Comparative evaluation of agar gel precipitation test (AGPT) and indirect haemagglutination test (IHA) for the detection of antibodies against infectious bursal disease (IBD) virus in village chickens. African Journal of Biotechnology, 10: 16024-16027


