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Isolation of infectious bursal disease virus from cell culture and bursal tissue and confirmation by RT-PCR and electron microscopy

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Abstract
In this study, isolation of the infectious bursal disease virus was carried out from two different sources viz. virus adapted in cell culture and bursal tissue obtained from field outbreaks. The cell culture adapted UA/BZ/2 strain of the virus was propagated in chicken embryo fibroblast upto 8th passage and 1000 ml of the viral suspension was prepared. The bursal tissue from infected chickens obtained from the field outbreak cases of Infectious Bursal Disease in Hisar, Haryana was triturated and 10% suspension in phosphate buffered saline was prepared. Both the suspensions were then subjected to centrifugatiion and ultracentrifugation to obtain the partially purified virus. The presence of the virus in the final suspension was confirmed by AGPT and RT-PCR by amplifying 248 bp fragment of the VP2 hypervariable region of the virus. Further electron microscopy confirmed the IBD viruses as classical birna group of viruses on the basis of morphological structure.

Keywords: Cell culture, bursa, IBD, RT-PCR, electron microscopy

1. Introduction
The last three decades has shown a phenomenal increase in poultry production in India as poultry keeping has attained the status of a full fledged industry. However, such marked growth in poultry production has created a situation where the birds have become more susceptible to disease causing agent of diverse origin. Besides some latent infections, other infections have also been introduced in to the farms while importing the flock tested for high performance. These disease conditions have caused considerable economic downfall to poultry industry and have time and again threatened the progress made in recent years.
Infectious bursal disease (IBD), popularly known as Gumboro disease, is an acute, highly contagious viral infection that affects young chickens of 3-6 weeks of age since its key target is the bursa of Fabricius (BF). Infectious bursal disease virus (IBDV) is a small non enveloped virus belonging to the genus *Avibirnavirus* of the family *Birnaviridae*, which is characterized by a bisegmented dsRNA genome [1]. Diagnosis of IBD at an early stage of outbreak will help in the containment of the disease. Confirmation of a diagnosis of clinical IBD can be made at necropsy by examining the bursa of fabricius during the early stages of disease for characteristic gross lesions. But, in birds less than 3 weeks of age or in young chickens with maternal antibodies, IBD virus infections are usually subclinical. Thus, typical clinical signs are not present and diagnosis should be supported by histopathologic study of bursa of fabricus, serologic studies, or by virus isolation. Routine diagnosis of IBD relies on the demonstration of the virus in the bursa, or of virus-specific antibodies in the infected chicken. Both conventional tests and molecular techniques are being used for the detection of the virus or IBD virus-specific antibodies for the diagnosis of IBD. As compared to conventional virological and serological techniques, the molecular techniques are highly sensitive, extremely specific and versatile. Amplification of the VP2 hypervariable region by conventional RT-PCR, in combination with DNA sequencing of the PCR product, can differentiate classic, variant, and vvIBDV strains since variant and vvIBDV have characteristic nucleotide and amino acid substitutions [2]. In this study, effort was made to isolate and confirm the IBDV obtained from two different sources – cell culture adaptation and from bursal tissue homogenate. Further transmission electron microscopy of the isolated samples was done to confirm the presence of the virus and to reveal its morphology.

2. Materials and Methods

Embryonated chicken eggs (9 to 11 days-old) were procured from instructional poultry farm (IPF), GBPUA&T, Pantnagar, and utilized for production of primary embryo fibroblast (CEF) cell culture. Bursal sample from infected birds were obtained from field outbreak cases of IBD in Hisar, Haryana for isolation of virus. CEF adapted UA/BZ/2 strain of the IBDV was used as sample for isolation of virus from cell culture.

2.1 Preparation of chicken embryo fibroblast (CEF) culture

Primary CEF culture was prepared as per the method described by Cunningham [3] using 9-11 days old chicken embryos. Following candling and cleaning of egg, the embryos were picked out using a blunt curved forceps and placed in to the petridish containing minimum essential media without any serum. After removing the head, extremities and visceral organs, the remaining tissue was chopped finely using a pair of sterile scissors, then it was transferred into a conical flask and subjected to trypsinization using 0.25% trypsin with MEM (Minimum Essential Medium) in ratio of 2:1 for 20 minutes with constant stirring. The supernatant was filtered through a muslin cloth.

All filtered cell suspensions were finally pooled together and centrifuged at 2000 rpm for 10 min (4 °C). After washing cell pellet was added in MEM media and centrifuged. The pellet obtained thereafter was finally resuspendend in MEM media with 10% fetal calf serum and the cell concentration was adjusted to a final seeding rate of 1×10^6 per ml of the media and distributed in 25 cm^2 culture flasks and roux flasks at the rate of 0.3 ml of cells per cm^2. The seeded flasks were incubated in a CO_2 incubator at 37 °C and 5% CO_2 till confluent monolayers were formed. Usually the monolayers were found suitable for virus inoculation within 24-36 hours.

2.2 Subculturing of the primary cell culture:

The primary cell culture flask was open in viscinity of flame and the media was discarded. Four ml fresh media was then added on cover face of flask, rotated and discarded. Then trypsin was added (2.5 ml) to cover the whole surface and left for 15 second and the flask was rotated and trypsin was discarded. Then the flask was incubated for few minutes at 37 °C. After incubation 13 ml of fresh media and 1 ml of fetal calf serum was added and vigorous flushing was done so that cells were removed in patches. Then 7 ml of the suspension was poured in new flask. The seeded flasks were incubated in a CO_2 incubator at 37 °C and 5% CO_2 till confluent monolayers were formed. Subculture was completed within 48 hours from primary culture.

2.3 Propagation of virus in CEF culture

The culture flasks with 80-90% confluency were taken and the spent media was decanted and then inoculated with 0.5 ml $TCID_{50}$ of the virus inoculum prepared as per standard procedure per 25cm^2 flasks. These flasks were then incubated at 37 °C for 1 hour for adsorption of the virus then the inocula were decanted and fresh maintenance medium (MEM with 2% FBS) was added to the flasks and kept in incubator at 37 °C and 5% CO_2 for development of cytopathic effects. The inoculated monolayers were observed under phase contrast microscope daily for cytopathic effect (CPE) which is characterized by small round refractile cells [4].

2.4 Bulk production of virus in cell culture

The CEF were prepared in roux flasks and 75 cm^2 tissue culture flasks and were infected with CEF adapted UA/Bz/2 strain of IBDV. After appearance of CPE over 70% of the monolayer, the culture flasks were frozen. A total of 1000 ml tissue culture fluid was prepared by freeze-thawing three times quickly and the tissue culture fluid was stored at -20 °C for further use of purification of virus and its characterization.

2.5 Preparation of bursal tissue suspension

Preserved samples of the bursa of fabricius were washed with sterile phosphate buffered saline and triturated and homogenised to prepare 10% suspension (w/v) in PBS. The suspension was clarified by low speed centrifugation and was treated with gentamycin @ 500µg/ml for further processing.

2.6 Partial purification of virus

The procedure described by Marquardt and co-workers [5] was followed with few modifications. The culture supernatant stored in -20 °C was rapidly freeze-thawed thrice. The supernatant from both the sources was then clarified at 10,000 rpm for 30 minutes after which the viral particles were precipitated in 5% polyethylene glycol (6000) in presence of 2.2% sodium chloride. The precipitate was then resuspended in 4 ml of the TNE buffer and it was layered on top of 8 ml of sucrose cushions (40% w/v) in four beckman tubes and the virus particles were pelleted by ultracentrifugation at 1,40,000×g at 4 °C for 3.5 hrs in an ultracentrifuge (Beckman, Ireland) with 40T rotor. Following centrifugation the pellet was resuspended in 4 ml of TNE buffer.

2.7 Total protein estimation of Purified virus

The purified IBDV samples were checked for their total protein using Spectrophotometer (Eppendorf, Germany) and the OD values were read at 280 nm. Total protein was estimated by comparing the OD values with the standard values of bovine serum albumin.

2.8 Confirmation of Virus

The IBDV in the propagated and purified material was confirmed by AGPT and RT-CR.

2.8.1 Agar gel precipitation test

The method described by Nachimutthu and co-workers [6] was followed with some modification. About 4 ml of 1% molten agar gel prepared in 8% NaCl solution was carefully poured on each microscopic slide and allowed to solidify in a horizontal plane. Then the gel was kept in 4 °C for 5 min for solidification. Wells (5 mm diameter and 5 mm space in between) were made in pattern consisting of a central well surrounded by four peripheral wells using standard template and one slide with 3 well in a horizontal plane. The central well was charged with hyperimmune serum and the peripheral wells with equal volume of cell culture suspension and bursal tissue suspension (10%w/v in PBS). The slides were incubated in humid chamber for 24-72 hrs, and observed for precipitation lines.

2.8.2 Confirmation by RT-PCR

2.8.2.1 Isolation of total RNA

The total cellular RNA including viral RNA was isolated from cell culture as well as the bursal suspension [7, 8] with slight modifications. Media was discarded and cells were washed with 5-8 ml of ice cold phosphate buffer saline per 75

cm^2 culture flask. Thereafter, monolayer was detached from surface of tissue culture flask by treating the culture with 5ml of MLS. Similarly bursal suspension was also treated with MLS. After thorough mixing culture as well as the bursal suspension was transferred to the DEPC treated Oakridge tube and subjected to sonication for 30s of 10 cycles with 15s pause in each cycle. Then kept at room temperature for 15 minutes and transferred to the ependroff tubes of 1 ml each. To this 1ml of water saturated phenol and 200µl of chloroform-isoamyl alcohol (49:1) were sequentially added. Then it was mixed properly and incubated in ice for 15 min. There after, it was centrifuged at 12,000 rpm for 20 minutes at 4 ºC. Then upper aqueous phase was collected and 1ml of isopropanol was added to precipitate RNA. Then it was kept at -20 ºC for 1hour. Again it was centrifuged at 12,000 rpm for 20 min at 4 ºC and supernatant was discarded. Then the pellet was resuspended in 0.3 ml of 100% isopropanol and incubated at 20 ^{0}C for 30 minutes, centrifuged at 12,000 rpm for 20 minutes and the supernatant was discarded. Then RNA pellet was resuspended in 75% ethanol and incubated at room temperature for 10-15 minutes to dissolve residual amounts of guanidine. Again it was centrifuged at 12,000 rpm for 20 minutes at 4ºC and the supernatant was discarded. Lastly, RNA pellet was dissolved in 100µl of DEPC treated water and incubated for 10-15 minutes at 55ºC to ensure complete solubilisation of RNA and stored at -20ºC.

2.8.2.2 Quantification of RNA

Total RNA isolated from culture flasks was quantified using UV/ VIS spectrophotometer and reading absorbance at 260 nm.

2.8.2.3 RT-PCR

The RT-PCR was carried out in two steps. First reverse transcription of isolated RNA to cDNA and then polymerase chain reaction. The cDNA from total RNA was prepared by following the protocol prescribed by GeNei™ M-MuLV RT-PCR kit and finally chilled on ice and used for PCR or stored at -20ºC for later use. This was followed by amplification of a 248 bp specific fragment in the VP2 gene of IBDV (positions 804 to 1051, according to the numbering system of Bayliss and co-workers [9] using the forward primer 5`-GTR ACR ATC CTG TTC GC-3` and the reverse primer 5`- GATGTR AYT GGC TGG GTT ATC TC-3` [10].

The reaction conditions for amplifying the 248 bp fragment of vp2 gene of IBDV was standardized with RT-PCR with the following reaction mixture.

cDNA	:	2.5µl
Forward primer	:	1µl (12 pM)
Reverse primer	:	1µl (12 pM)
10 mM dNTP mix	:	1µl (2.5 mM each)
10X reaction buffer	:	2.5µl
Nuclease free water	:	16.5µl
Taq polymerase	:	0.5µl (3U/ µl)

The cDNA was denatured by keeping the mixture at 98ºC for 4 minutes. Then the amplification of vp2 gene was done for 30 cycles at 94ºC for 1 minute, 55 ºC for 1 minute (vp2 gene), 72 ºC for 1 minute, followed by final extension at 72^{0}C for 5 minutes. Once the cycles were completed the reaction mixture was checked for amplification by electrophoresis in ethidium bromide stained 1.5% agarose gel at 50 volts and documented in gel documentation system.

2.9 Electron microscopy of the virus

The samples were processed for observation under transmission electron microscopy by negative staining method using uranyl acetate. For negative staining, a drop of about 10 µl of the two different suspensions was applied to the hydrophobic surface of a parafilm square in a Petri dish. A formvar-coated copper grid was floated onto this drop for one minute, with the formvar side of the grid in contact with the liquid. The excess liquid was removed from the grid by touching its border with a cut piece of filter paper. The grid was immediately floated in a drop of 1% aqueous uranyl acetate. For better assessment of the samples, three grids were prepared. After staining for one minute, the excess stain was removed with filter paper and the grid was left to dry for a few minutes, before insertion into the microscope column for study of the virion morphology [11].

3. Results and Discussion

IBDV (UA/Bz/2) was propagated in CEF culture up to 8th passage. After 8th passage, cytopathic effects (CPE) in CEF cultures were noticeable in 48 hours after virus inoculation. Significant numbers of dead cells which are rounded were seen scattered in the monolayer. There were marked granulations in cell cytoplasm particularly around nucleus. Aggregates of tiny round refractile cells appeared which later spread to the entire cell sheet. The morphologically altered cells eventually detached from the surface of culture flask, leaving empty areas in the cell monolayer with the eventual destruction of the entire monolayer as compared to the control uninfected CEF cell culture. Similar observations were recorded by Khan [12] on inoculation of IBDV from third to seventh passage in CEF. These observations were also in agreement with the previous workers [13,14].

IBDV (UA/Bz/2) passaged in CEF at 8th passage was titrated in CEF culture and the $TCID_{50}$ was calculated at 24, 48, 72 h intervals. The calculated titre were found to be 3.16 x 10^4, 1.46 x 10^7, 2.63 x 10^7 $TCID_{50}$ per ml at 24, 48, 72 h intervals respectively.

Partially purified virus was obtained after ultracentrifugation of both the samples. Higher purity can be obtained, if the samples are subjected to CsCl cushions followed by isopycnic ultracentrifugation at 170,000 g for 40h and pelleting in a 20% sucrose cushion at 84,000 g for 2 hours [15]. However as the objective of the present study does not warrant such purity, the purification was done partially using 40% Sucrose cushion ultracentrifugation. The total protein content of purified cell culture passaged virus sample and the bursal tissue derived viral suspension was found to be 1.967 mg and 17.57 mg as observed by spectrophotometer.

AGPT of bursal homogenates obtained from infected birds as well as the cell culture fluid tested against the standard positive serum showed precipitation lines. This was in agreement with the observations made by earlier worker [16].

3.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR for 248 bp vp2 hyper variable region of IBDV was standardized using specific primers. The assay is very specific and amplification alone is usually sufficient to diagnose IBDV in a sample. Specificity of RT-PCR for IBDV was already compared with other viruses [17]. IBDV in bursa could be detected for a longer period after infection as could be evidenced from comparison of conventional assays and RT-PCR for IBDV detection in the bursa of fabricius of infected

birds [18]. RT-PCR performed for viral RNA prepared from infected bursa viz. and from CEF infected with strain UA/BZ-2 using specific primers resulted in amplification of 248bp vp2 hyper variable region of IBDV. The authenticity of amplified fragment was confirmed by comparing its size with 200 bp molecular marker. RT-PCR amplified 248 bp fragment bands of field bursal isolate as well as the cell culture adapted strain was observed (Fig.1). Similar observations were made by earlier workers [19, 20].

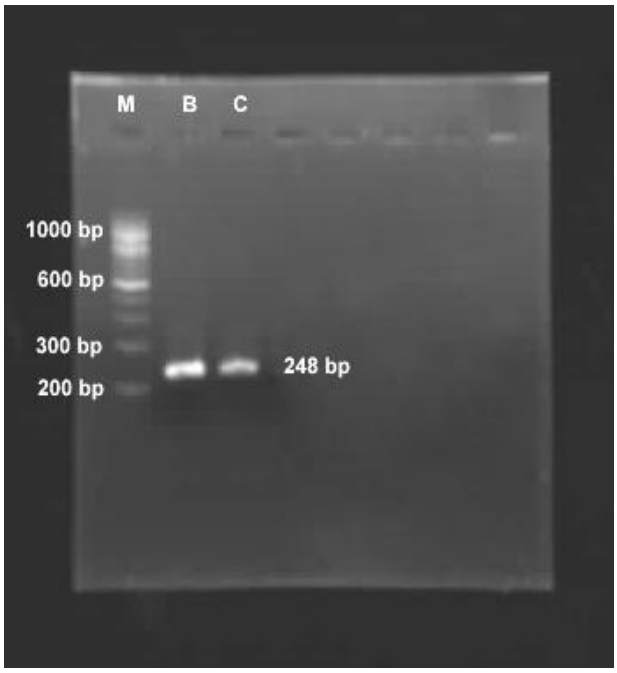

Fig 1: Agarose gel showing the PCR amplified 248 bp fragment of VP2 gene in bursa derived viral antigen (B) and cell culture adapted viral antigen (C)

3.2 Electron microscopy of the virus

The IBD viruses are classical birna group of viruses having icosahedron symmetry with no envelope. The size of the virus is approximately 60 nm in diameter having bisegmented ds RNA genome. The electron micrograph of the viral isolate derived from both the sources depicted the classical morphology of Birna group of viruses (Fig. 2, 3, 4, 5), which was in agreement with the observations made by previous workers [21, 22]. But during processing of bursa for virus isolation there is breakdown of some of the virions leading to difference in the size of the virions.

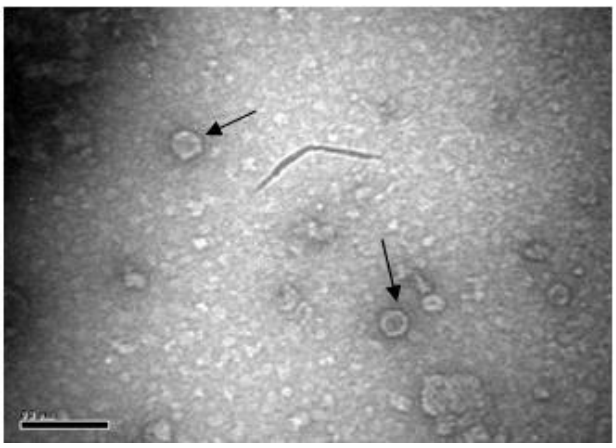

Fig 2: IBDV particles (cell culture adapted) under TEM (NS) × 1,00000x

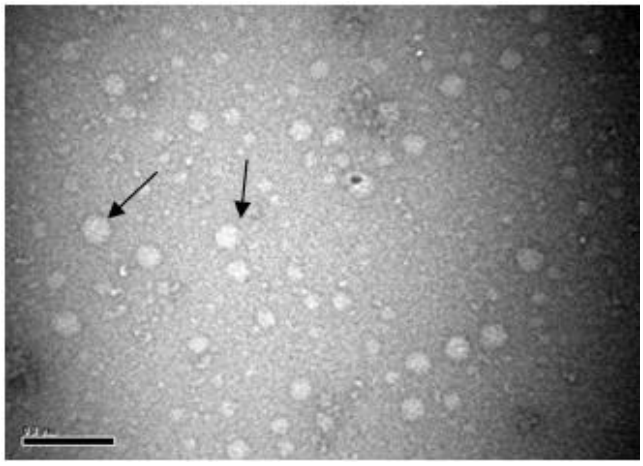

Fig 3: IBDV particles (bursa derived) under TEM (NS)×1,00000x

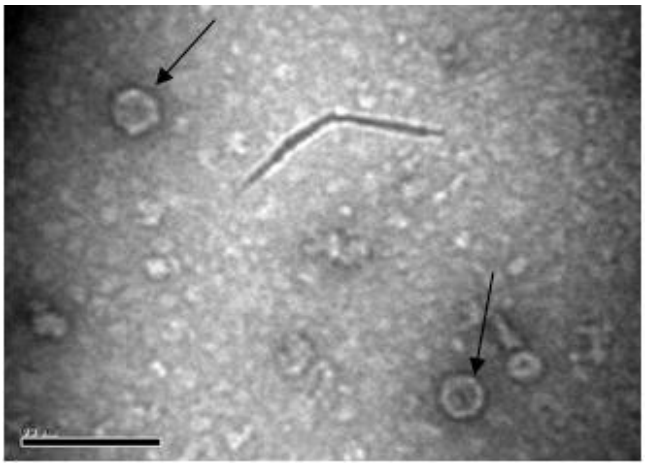

Fig 4: IBDV particles (cell culture adapted) under TEM (NS)×1,50000x

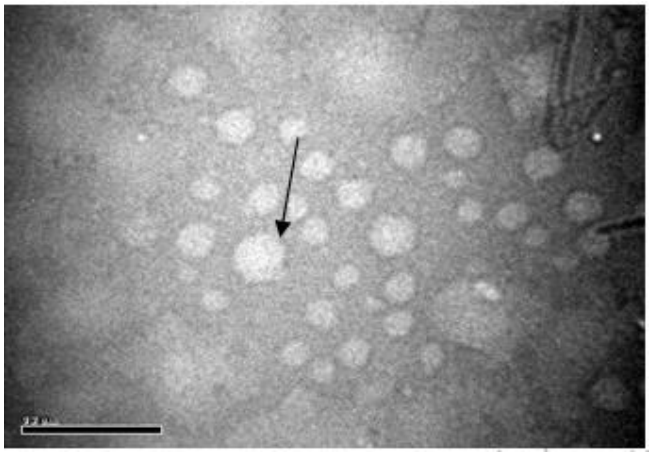

Fig 5: IBDV particles (bursa derived) under TEM (NS)×1,50000x

Conclusion

IBDV can be identified from both field bursal tissue suspension and chicken embryo fibroblast culture by AGPT and RT-PCR by amplifying 248bp vp2 hyper variable region of viral RNA. Further electron microscopy could confirm the morphological structure of virus as classical birna group obtained from both the sources.

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