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PURIFICATION OF INFECTIOUS BRONCHITIS VIRUS PROPAGATED IN EMBRYONATED CHICKEN EGGS AND ITS CONFIRMATION BY RT-PCR

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The Massachusetts strain (M41) of avian infectious bronchitis virus (IBV), was obtained in freeze dried ampoule and reconstituted in 1 ml phosphate buffered saline (PBS) (pH 7.4), of which 0.2 ml was inoculated in ten 9 day old specific pathogen free (SPF) embryonated chicken eggs (ECE). At 48 hours of incubation, the embryos were chilled and the allantoic fluid harvested with titre of the virus determined to be $10^{3.108}$ EID_{50}/0.1 ml, $10^{3.508}$ EID_{50}/0.1 ml, and between $10^{4.508-5.508}$ EID50/0.1 ml of allantoic fluid after 1st, 2nd and 3rd passages, respectively. Characteristic signs of IBV like curling and dwarfing of the embryos were obtained at 7 days post infection. The virus growth was also confirmed by *in vitro* specific amplification of RNA from the infected allantoic fluid using reverse transcription - polymerase chain reaction (RT-PCR). The high titred virus at the third passage level was used as a seed inoculum for bulk virus propagation in fifty 9 day old ECE, following the protocol described earlier. Along with the allantoic fluid, the chorioallantoic membrane (CAM) was also carefully collected separately from each inoculated embryo and washed once with PBS, and centrifuged to remove RBCs. The pooled CAMs were homogenized thoroughly and the homogenate clarified by centrifugation at 1500 rpm for 10 minutes. For purification of the virus, 20 ml of the allantoic fluid and CAM suspension were layered separately over 5 ml of discontinuous 30% and 55% sucrose in GNTE buffer (0.025M Tris HCl, 0.2M Glycine, 0.002M EDTA; pH 7.0) and centrifuged at 90,000 g for 4 hrs at 4°C. The virus specific band was collected from the interface of sucrose gradient and diluted 1:2 in GNTE buffer. Further, the above suspension was layered over 15 ml of continuous gradient of 30% and 50% sucrose and centrifuged at 90,000 g for 6 hrs. The virus band was collected, diluted as described before and pelleted at 90,000 g for 3 hrs through a 5 ml of 30% sucrose cushion. The virus pellet was resuspended in GNTE buffer and the protein concentration estimated at 260/280 nm optical density (OD) ratio and the virus preparation having an OD of 1.2 was considered as pure in nature. IBV purified by density gradient ultra-centrifugation from allantoic fluid had a protein concentration of 2.56 mg/ml and the CAM virus had 4.36 mg/ml. Higher yield of the virus was obtained from the CAM membranes than from the allantoic fluid. The presence of the virus was confirmed using RT-PCR testing of the purified virus suspension. Further work on the use of the purified IB virus as an antigen in ELISA is underway.

Infectious bronchitis is an acute and highly contagious viral respiratory disease of chickens characterized by tracheal rales, coughing and sneezing. IBV is the prototype species in the family *Coronaviridae* and the virus is placed in group-3 of genus *Coronavirus* belonging to the order Nidovirales and its distribution is worldwide. The disease primarily occurs in young chicks from 2 days to 3 weeks of age. Mortality rates as high as 40 to 90% were observed in affected chicks (Fabricant, 1998). Mortality in broilers often peaks in the last two weeks of life, commonly at 5-6 weeks of age, usually complicated by secondary bacterial infections like *E. coli* and *Mycoplasma spp*. following damage to respiratory tract caused by IBV. Some nephropathogenic strains cause mortality upto 30% in young chicks. IB in mature hens can result in decrease of 10 to 50% or more of production, increased number of deformed eggs, altered egg shell colouration, failure of oviduct to develop properly and the production never return to the normal level (Dhinakar Raj and Jones, 1997).

IBV grows well in the developing chicken embryos as compared with chicken kidney or tracheal organ cultures (Cook *et al*., 1976). Upon initial inoculation of embryos, dwarfing of a few embryos with survival of 90% eggs through 18 to 19 days of incubation is characteristic of IBV growth (De Wit, 2000). The embryo gets curled into a spherical form, with feet deformed and compressed over the head with a thickened amnion adhered to it. The virus titres are highest in embryos at 24 to 48 h post-inoculation (PI), although embryo lesions are seen only after 5 to 6 days P.I. (De Wit, 2000).

IBV strains differ in their density in sucrose gradients, particles with a full complement of spikes have a density of 1.18 g/mL, and lesser-spiked particles (lacking RNP) may be as low as 1.15 g/mL. Centrifugation forces of greater than 100,000 g should be avoided, as loss of spikes can occur. Those of IBV Beaudette strain appear to be especially unstable; incubation at 37 °C sometimes results in the loss of the spike subunit (Stern, D.F and Sefton, B.M., 1982).

In India, Verma and Verma first reported the prevalence of IBV infections in chickens in 1964. Mahalingam *et al*. (1973) tested 1784 pooled serum samples of which 17 were tested positive for IBV antibodies. They have also reported a higher incidence in birds over 6 months of age. Tripathy and Kar (1973) examined 158 pooled serum samples from 8 poultry farms in

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Orissa, and found 17% of samples positive for IBV by AGPT. They reported the incidence almost the same both in young and adult age groups. Chandramohan *et al.* (1995) compared the efficacy of different diagnostic tests like AGPT, direct negative phase contrast microscopy (DNCEM) and immunoelectron microscopy (IEM) for detection of IBV. The speed and accuracy of IEM was preferred over the other two methods. Kumar *et al.* (1984) found no serum to be positive for IBV in Haryana and the adjoining states between 1971 and 1982. Nath *et al.* (1978) reported the prevalence of IB in the twin cities of Hyderabad and Secunderabad. Rajeshwar (2002) reported the prevalence of mass serotype IBV in Tamil Nadu by serum neutralisation tests and Kumanan *et al.* (2003) confirmed their findings using a panel of Mabs and IP staining. The aim of the present study is to purify the virus after growing in the allantoic cavity of ECE by density gradient ultra centrifugation and confirmation of the virus by reverse transcription polymerase chain reaction.

MATERIALS AND METHODS

Virus: The vaccine virus Massachusetts strain (M41) was obtained in freeze dried ampoule (passage level not known) and the ampoule was dissolved in 1 ml PBS (pH 7.4). 0.2 ml of the inoculum was inoculated in five 9 day old SPF (Specific pathogen free) chicken eggs (VHL Pune) and the eggs candled daily for 48 hrs. Those, which died within 24 hrs, were discarded as non-specific death and those, which survived, were chilled and the allantoic fluid was collected, centrifuged at 3000 rpm for 10 min at room temperature and stored at -20 °C till use. The procedure was repeated twice till the characteristic signs of IBV like curling and dwarfing of the embryos were obtained (Fig. 1). The virus growth was confirmed by *in vitro* amplification of the extracted RNA from the 3rd passage infected allantoic fluid using RT-PCR.

Titration of virus: Virus titration was carried out in embryonated chicken eggs following the procedure of Verma *et al.* (1964). Serial ten-fold dilution of the virus material was inoculated in 0.1 ml amounts into each of the 3 ECE per dilutions ranging from 10^{-2} to 10^{-8}. Infection of virus was presumed from the occurrence of dwarfing and curling among the embryos. Embryo deaths within 24 hours post-inoculation were regarded as due to nonspecific cause and discounted while calculating the end point titres. Three passages were done and titration was done after each passage in ECE. Endpoint titres expressed as 50% embryo infective doses (EID_{50}) per ml was calculated by the method of Reed and Muench (1938).

Bulk production of virus stock from Embryonated chicken eggs: For the bulk production of virus stock fifty 7 day old ECE were obtained from Experimental Hatchery Unit of the Central Avian Research Institute, Izatnagar. The 9-day-old embryos each were inoculated with 0.1 ml of virus inoculum M41 (Massachusetts strain) containing $10^{4.508}$ EID_{50}/0.1 ml and candled daily for 48 hrs. As mentioned earlier those, which died in 24 hrs, were discarded as non-specific death and those, which survived, were chilled and the allantoic fluid was collected, centrifuged at 7000 rpm for 30 min at 4°C and stored at -20 °C till use. The presence of the virus was confirmed by RT-PCR.

From Chorio allantoic membrane (CAM): The Chorio allantoic membrane was also collected carefully from each inoculated embryo and washed once with PBS by centrifugation at 3000 rpm for 10 minutes to remove the RBCs. The pooled CAMs were homogenized thoroughly using a homogenizer (Dupont instruments). The homogenate was centrifuged once at 1500 X for 10 minutes to remove the coarse particles. The supernatant collected was used for virus purification stored at -20 °C till further use. The presence of the virus was confirmed by RT-PCR.

Purification of the virus: The virus purification was performed as per Marquardt *et al.*(1981) employing sucrose density gradient ultra centrifugation. Briefly, 20 ml of allantoic fluid/CAM suspension was layered over 5 ml of discontinuous 30% and 55% sucrose in GNTE buffer pH 7.0 (0.025M Tris HCl, 0.2M Glycine, 0.002M EDTA) and centrifuged at 90,000 x g for 4 hrs at 4 °C. The virus band was harvested from the interface of 30% and 55% sucrose gradient and diluted 1:2 in GNTE buffer. The above suspension was further layered over 15 ml of continuous sucrose gradient of 30% and 50% sucrose and centrifuged at 90,000 X g for 6 hours. The virus band was collected and diluted as described earlier. The virus was finally pelleted at 90 000 X g for 3 hrs through a 5ml 30% sucrose cushion. The virus pellet was resuspended in GNTE buffer and the protein concentration was estimated using UV spectroscopy.

Estimation of protein concentration: Optical density of the purified virus was taken at 260 nm and 280 nm in the spectrophotometer. The protein content in the sample was determined using the formula: Protein (mg/ml) = $(OD_{260} \times 1.55)$-$(OD_{280} \times 0.77)$. Ratio of absorbance at 260/280nm was calculated to check the purity of the virus sample. The virus preparation having an OD value of 1.2 was considered as pure.

Confirmation by RT-PCR: For the detection of virus by RT-PCR, total RNA from the infected allantoic fluid was extracted by Trizol reagent (Life-Technologies Inc. USA), as per the manufacturer's protocol. In brief, 250 μl of allantoic fluid suspension was taken, adding 750 μl of TRIzol. Sample was incubated at room temperature for complete cell lysis and then mixed with 200 ml of chloroform. After incubation at room temperature for 5 min, the mixture was centrifuged at 12000 x g for 10 min and the aqueous phase was collected. The RNA in aqueous phase was precipitated by adding 0.5 ml of isopropanol and was resuspended in 20 μl of nuclease free water after washing with 70% ethanol.

Reverse transcription was carried out using 8 μl of total RNA extracted, following the protocol as has been standardized in our laboratory. In a 20 μl reaction volume, cDNA was synthesized using 50 ng random hexamer primers and 200 U of MMLV reverse transcriptase (Promega) at 37°C for 1 hour. For Polymerase Chain Reaction (PCR), 5 μl of cDNA was subjected

to amplification using 10 picomoles of each IBV S1 gene specific primers described earlier, 200 mM of dNTPs mix, 3 U of Taq DNA polymerase and 0.9 mM Mg^{++} in 1x PCR buffer (all Promega). The PCR conditions used were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C, primer annealing at 43°C and extension at 72°C of 1.5 min each. Primers used to amplify the S1 gene includes forward 5'TGAAAACTGAACAAAAGACA3' nucleotide position 20302-20321 and reverse primer 5' CATAACTAACATAA GGGCAA3' nucleotide position 22002-22021, designed from Massachusetts M41 serotype to amplify the S1 whole gene (Kwon *et al*., 1993). The generated PCR product was analysed in 1.5% agarose gel, demonstrating a band of 1.7 Kbp. Authenticity of the primary PCR product was confirmed by Restriction endonuclease digestion (RE) also (Fig. 2).

RESULTS AND DISCUSSION

IBV grows well in the developing chicken embryo. Optimum age of embryo, temperature and length of incubation for maximum infectivity titer of IBV Beaudette following allantoic cavity inoculation have been thoroughly studied and reviewed (Jordan, F.T.W and Nassar, T.J., 1973). Following inoculation of approximately 10^7 EID_{50}, similar peak titres in allantoic fluid (AF) of embryos inoculated at 10-11 days old were achieved after 12 hr and 24 hr at 37 °C and 32 °C, respectively. Virus titers of chorioallantoic membranes were higher than those of AFs. Virus induced embryo death was first observed at 24 hr and 48 hr after incubation at 37°C and 32 °C, respectively. Incubation at 42 °C resulted in earlier mortality (12 hr) and lower titres. In a different study, a less egg adapted strain (20-30 embryo passages) attained maximum titers after 24-30 hr at 37 °C, irrespective of the inoculum dose (Gelb, J. and Killian, S.L., 1987). In general, inocula of about 10^3 tracheal organ culture infectious doses or 10^4 EID_{50} should give near maximum titres by 36-40 hr at 37 °C.

Dwarfing of a few embryos with survival of 90% through the 19th day of incubation is characteristic of IBV field material upon initial inoculation in 10 to 11 day-old embryonated chicken eggs. Embryo mortality and dwarfing increase as the number of serial passages increases, so that by the 10th passage most of the embryos are stunted, and up to 80% may die by the 20th day of incubation. Characteristic embryo changes are seen several days after inoculation of the virus. Only slight movement of a dwarfed embryo may be observed during candling. Upon opening the air cell end of the egg, the embryo is seen curled into a spherical form with feet deformed and compressed over the head and with the thickened amnion adhered to it. The yolk sac appears shrunken, and the membrane ruptures easily. An increased volume of usually clear allantoic fluid is present, a consistent internal lesion of the IB infected embryo is the persistence of the mesonephros containing urates. This lesion appears to be associated with the stunting of the embryo and is not specific for IB infection. Another lesion found in embryonated eggs inoculated with non-lethal isolates of IBV is the thickened amnion and adjacent layer of the allantois covering the stunted embryo. Evidence of this lesion can usually be detected on the 3rd day after inoculation.

The IBV Mass serotype M41 was revived and made to grow to high titres in chicken embryos by sequential passages in allantoic cavity of SPF embryos. The virus passage was done in SPF embryos for three times till the characteristic teratogenic changes of IBV like stunting, curling and dwarfing are observed. The titration results of the three passages were $10^{3.108}$ EID_{50}/ 0.1 ml, $10^{3.508}$ EID_{50}/0.1 ml and $10^{4.508\text{-}5.508}$ EID_{50}/0.1 ml after 1st, 2nd, and 3rd passages, respectively. A high virus titre of $10^{4.508\text{-}5.508}$ EID_{50}/0.1 ml was obtained after the 3rd passage in ECE. That titre of virus was used as a seed inoculum for bulk virus propagation in ECE to be used further for purification of the virus.

During the course of our study, we have observed, that IBV grows to a very high titre in ECE, when the amount of virus inoculum was diluted. When high titres of virus are inoculated, it causes mortality and obviously low titres of virus was obtained. Also we have found that the embryos, which are kept for more than 48 hrs, started producing DI particles (defective interfering particles) as evidenced by PCR detection. DI particles are incomplete virus particles, which are produced when the sufficient nutrients are not available (scanty availability of nutrients in allantoic fluid). The DI particle production is not beneficial in virus propagation, as they do not react well in ELISA and can give faulty results. Hence for virus propagation the production of DI particles in ECE has to be avoided. Therefore, the virus growth was optimized so that at 48 hrs and peak titres of the virus were obtained at that time period without any DI particles (Fig.3). A virus titre between $10^{4.508\text{-}5.508}$ EID_{50}/0.1ml was obtained from the inoculated SPF embryos.

IBV purified by density gradient ultra centrifugation from allantoic fluid had a protein concentration of 2.56 mg/ml and the CAM purified virus had 4.36mg/ml. Jadhao *et al.* (2000) while purifying the FAV serotype-4 by ultra centrifugation obtained a protein concentration of 3.56 mg/ml from Chicken embryo liver cultures. The extinction ratio (OD_{260}/ OD_{280}) of the purified virus was found to be 1.14 from allantoic fluid and 1.24 from the infected CAM fluid. In our study from 50 ECE we were able to obtain 600 ml of allantoic fluid. The virus yield was calculated to be 3.48mg and from the CAM it was 5.37mg of purified IBV virus. Thus higher yield of the virus was obtained from the CAM membranes than from the allantoic fluid. It is because the IBV virus multiplies in the layer underlining the CAM, hence the virus yield is higher in the CAM than in the allantoic fluid.

The virus thus purified by this method is free of defective interfering particles, which interfere in ELISA assays. The purity of the virus was also confirmed by RT-PCR assay. The purified virus from both the allantoic fluid and CAM will further analyzed in SDS PAGE and its suitability, as antigen in ELISA will be studied further. Thus, the purified virus will be useful in the sero-monitoring of the IBV infections in our country.

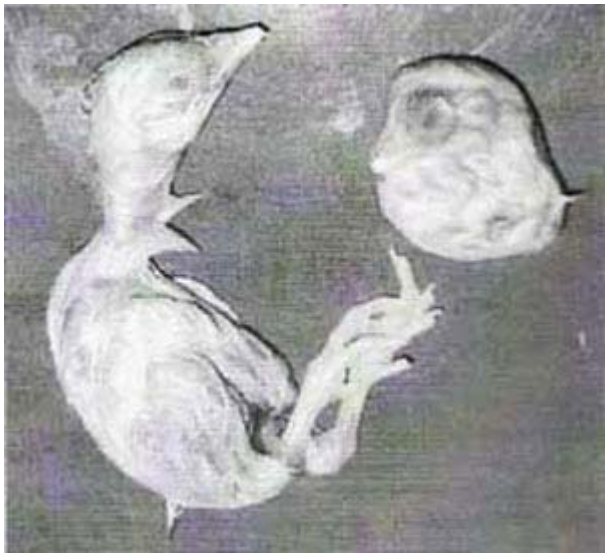

Fig. 1. C- Control uninfected 18 day old chick
I- Chick infected with M41Massachusettes vaccine strain showing characteristic teratogenic changes like curling and dwarfing at 20 day old embryos

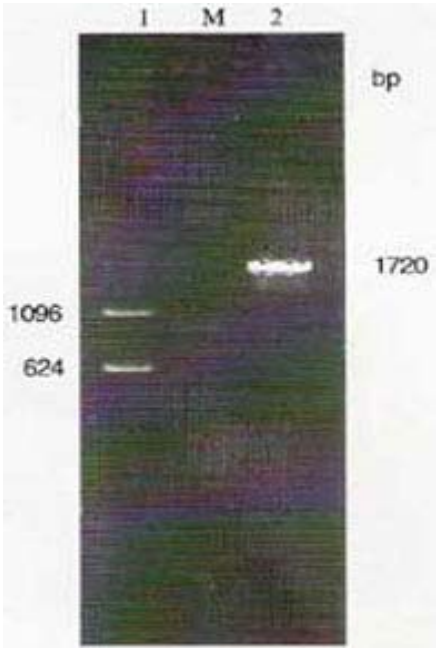

Fig. 2. Detection of IBV by RT-PCR and RE analysis in infected allantoic Fluid
M- PCR Marker
1- *Pst* I Digested Product
2- M41 Massachusettes Vaccine strain

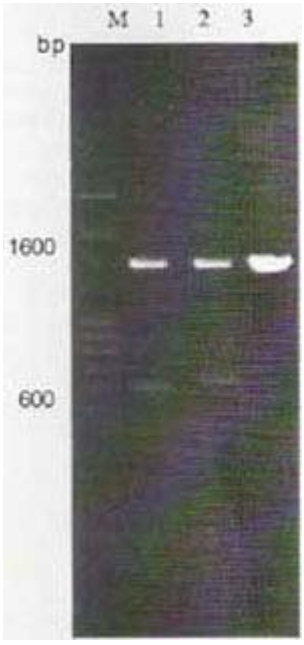

Fig. 3. Occurence of Defective Interferring Particles In Allantoic fluid of the infected embryos
M- 100 bp DNA ladder
1- At 96 hr interval
2- At 72 hr interval
3- At 48 hr interval

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