Isolation of etiological agent of hydropericardium syndrome in chicken embryo liver cell culture and its serological characterization

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The virus causing hydropericardium syndrome was isolated in chicken embryo liver (CEL) cell culture from livers obtained from naturally infected broilers. The cytopathic effects characterized by rounding and degeneration of cells were visible 36 hr post infection in first passage. At 4th passage level, the infectivity titre was 5.24 log_{10} TCID_{50}/ml. In May-Grünwald and Giemsa stained cells, basophilic intranuclear inclusions ('bird eye' inclusion), typical of avian adenovirus infection, were observed. The specificity of inclusion was confirmed by indirect immunofluorescence. Various serological tests, such as agar gel precipitation test, counter immunoelectrophoresis, micro serum neutralization test and enzyme linked immunosorbent assay were also standardized to confirm the isolation of etiological agent of hydropericardium syndrome in CEL cell culture and to diagnose the disease in poultry.

Keywords: Chicken embryo liver cell culture, Hydropericardium syndrome, Serological characterization

Hydropericardium syndrome a highly contagious disease of domestic fowl has emerged as a serious problem in the broiler poultry industry of Indian subcontinent. It is mainly seen in 3-6 week old growing broiler chicks and results in up to 80% mortality. The disease, characterized by hydropericardium, hepatitis and enlarged kidneys, is caused by fowl adenovirus serotype 4, which can be propagated in primary cell cultures of chicken embryo liver (CEL) and chicken kidney (CK) origin. Although, isolation of hydropericardium syndrome virus in CEL cell culture has been reported, detailed cytopathological changes have not been studied.

The present paper describes isolation of virus from natural outbreaks in CEL cell culture, detailed cytopathic effects and its characterization by serological tests.

Materials and Methods

Natural outbreaks—An outbreak of disease was reported at a private poultry farm in Haldwani area of Uttarakhand. Post-mortem examination of birds revealed hydropericardium with accumulation of 5-15 ml of fluid in pericardial sac. The broilers were 7 weeks of age and mortality in 3 days was 11%. The samples of liver tissues from dead birds were collected in 50% glycerine saline for virus isolation.

Preparation of virus inocula—The samples of liver tissue were washed in Hank’s balanced salt solution (HBSS) and homogenized to make a 20% (w/v) suspension in HBSS. After adding penicillin (1000 IU/ml) and streptomycin (1000 μg/ml), the homogenate was centrifuged at 5000 rpm for 10 min and supernatant was collected and passed through membrane filter of 0.22 μm pore size. The inoculum thus prepared was stored at −20°C until used.

Chicken embryo liver cell culture—The primary cell culture was prepared according to the method of Adair et al., with slight modifications using chicken embryos of 14-16 days of age in Medium-199 (Hyclone laboratory, USA) supplemented with 15% (v/v) and 2% colostrum deprived newborn calf serum served as growth and maintenance media, respectively. Growth medium also contained 2% (v/v) tryptose phosphate broth (1% w/v).

Infection of monolayer and study of cytopathic effects—The confluent monolayers in tissue culture flasks and Leighton tubes were infected with 1.0 ml and 0.2 ml virus inocula, respectively, and incubated at 37°C. The infected monolayers were observed daily for cytopathic effects. The infected cover-slip cultures grown in Leighton tubes were also stained with May-Grünwald and Giemsa stain at different intervals post-infection (PI) and for immunofluorescence.
Preparation of hyperimmune serum—The hyperimmune serum against hydropericardium syndrome virus was raised in 3-week-old broiler birds as per Ravi Kumar et al. using 20% liver homogenate extract prepared from livers collected from natural outbreak of disease as source of antigen. The antiserum thus prepared was tested against reference virus as well as a virus associated with classical inclusion body hepatitis.

Preparation of gamma globulins - Gamma globulin fraction of hyper immune serum was precipitated by adding saturated ammonium sulphate (SAS) solution as per the method of Saifuddin and Wilks. The serum was first clarified by centrifugation at 10,000 g for 30 min at 4°C and then SAS was slowly added to precooled serum to a final concentration of 45% (v/v) with gentle stirring at 4°C. After overnight stirring, the serum-SAS mixture was centrifuged as above. The precipitate was resuspended in 25% SAS in distilled water and re-centrifuged. The precipitate was dissolved in phosphate buffered saline (PBS, pH 7.2) and clarified by centrifugation as mentioned above. After two further precipitations with 40% SAS, the final precipitate was dissolved in PBS to half the volume of original hyper immune serum and dialyzed at 4°C against 500 ml of PBS with four changes at 15 hr intervals. The globulin fraction was incubated with an equal volume of a 10% healthy chicken liver-kidney cell suspension for 1 hr at room temperature and then centrifuged at 10,000 g for 30 min at 10°C. The supernatant was collected and stored at -70°C.

Assay of viral infectivity—The infectivity assay of virus at different passage levels was carried out in CEL cell culture monolayer grown in 96 well microtitre cell culture plate. The 10-fold serial dilution of virus isolate was prepared in growth medium and 50 μl of each dilution was then dispensed in each well of microtitre cell culture plate in triplicate. Appropriate cell suspension (50 μl), containing 1.5×10^6 cells/ml was then added to each dilution of virus suspension. These plates were sealed with cello tape and incubated at 37°C for 6-7 days for development of CPE. The control wells received 50 μl of suspension and equal volume of medium.

Serological tests

Micro serum neutralization test (Alpha procedure)—Micro serum neutralization test was done as per the method of Erny et al. A serial 10-fold dilution of CEL cell culture harvest (4th passage) was prepared in serum-free Medium-199 and each dilution was dispensed in 5 wells @ 50 μl/well of microtitre plates. Antiserum (1:10) was added to each well @ 50 μl/well. A similar set of dilutions was prepared and virus inoculated to find out titre in presence of antiserum. Appropriate serum and virus controls were also included. Microtitre plate was then incubated at 37°C for 2 hr. Then primary chicken embryo liver cells prepared in growth medium were added to all the wells @ 100 μl/well. The plates were sealed, shaken gently for uniform distribution of the cells in the wells, incubated for 7 days at 37°C, and observed for cytopathic effects under inverted microscope.

The infectivity titre (TCID₅₀/ml) of the virus in the presence and absence of antiserum was calculated according to Reed and Muench. The difference between the two was expressed as neutralization index (log₅₀ NI) of the antiserum.

Agar gel precipitation test (AGPT)—Agar gel precipitation test was performed according to the method of Cullen and Wyeth with slight modifications. Agar gel was prepared by dissolving 1 g of noble agar in 100 ml of normal saline containing 8% NaCl in a boiling water bath. The molten gel was then poured in small petriplates and allowed to solidify at room temperature. The petriplates were then kept at 4°C for 30 min and three wells arranged in triangular fashion were punched in the gel at a distance of 3 mm and bottom sealed with molten agar gel.

The central well was filled with hyperimmune serum and peripheral wells were filled with concentrated (5-fold) CEL cell culture supernatant and uninfected liver homogenate (20% w/v). The petriplates were kept in moist chamber, and incubated at 37°C for 24 hr and examined for the precipitin lines. After 24 hr, the petriplates were transferred to refrigerator at 4°C for further development of lines.

Counter immunoelectrophoresis—Gel was prepared by dissolving 1% agarose in 0.1 M barbitone buffer. Two wells of 2 mm diameter were cut, sealed and charged with antigen and antiserum. The chambers of electrophoresis assembly were filled with 0.4 M barbitone buffer before placing the slides. Antiserum was kept on anodal side and current was passed at the rate of 10 mV per slide. The results were read after 2 hr.

Indirect fluorescent antibody technique (IFAT) —Indirect FAT was done for detection of viral antigen in infected CEL cell culture as per the method of...
Hudson and Hay. Infected CEL cell monolayers grown on coverslips were stained at 24, 48 and 72 hr post infection. The medium from Leighton tubes was decanted and coverslips washed with PBS (pH 7.2) and then fixed in chilled acetone for 30 min. The coverslips were then flooded with purified gamma globulins (1:50) and incubated at 37°C for 1 hr in a moist chamber. After incubation the coverslips were washed thrice each for 5 min and flooded with anti-chicken FITC-conjugate (1:200, Sigma) and again incubated for a hr at 37°C in moist chamber. The coverslips were washed thrice as mentioned above with PBS (pH 7.2), air-dried and mounted in glycerol. The stained coverslips were examined under UV light in fluorescent microscope.

Enzyme linked immunosorbent assay (ELISA) — ELISA was performed by single dilution method as per the procedure described by Chandrashekhar. The approximate concentration of viral antigen was determined by checkerboard titration. The antigen was diluted 1:50 in carbonate-bicarbonate buffer and 50 μl of this diluted virus was added to each well of 96 well ELISA plate (NUNC). The plate was incubated overnight at 4°C. Next day, antigen coated plates were washed thrice with PBS-T for 5 min each and tapped thoroughly. The unreacted sites were blocked by adding 100 μl of 2% bovine serum albumin to each well of the plate. The plates were incubated for 1 hr at 37°C. The plates were again washed as mentioned above.

The single dilution of the test serum was made in another 96 well microtitre plate by adding 200 μl of dilution buffer and 2 μl of test serum per well resulting into dilution of 1:100. Positive control serum (against local isolate of HPS virus) and negative serum were also diluted 1:100 in PBS-T.

The diluted negative control serum was added in 100 μl quantity to the first three wells of the first row and 100 μl of diluted positive serum was added to the first three wells of the last six wells of last row of ELISA plate and last three wells of the last row kept as blank. In remaining wells of antigen-coated plate, 100μl of test serum from dilution plate was transferred to each of the three corresponding wells.

The plate was incubated at 37°C for 1 hr and washed thrice with PBS-T. Then 100 μl of rabbit anti-chicken horse radish peroxidase conjugate (Sigma) diluted 1:5000 in PBS-T was added to each well and plates were incubated at 37°C for 1 hr. Each well of the plate was added with 100 μl of freshly prepared substrate solution ortho-phenylenediamine dihydro-chloride (OPD) in citrate buffer followed by H2O2 and then incubated at 37°C for 30 min in dark. The reaction was stopped by adding 100 μl of stop solution (1N H2SO4) in each well of plate. The plate was read at 492 nm in an ELISA reader (ECIL Microscan MS 5605).

Calculation of ELISA titre — The average absorbance of positive and negative control was calculated from the absorbance value of ELISA plate and corrected positive control (CPC) value was determined by subtracting average negative absorbance from average positive absorbance.

The specific value (Sp. Value) was calculated using following formula:

\[
\text{Sp. Value} = \frac{\text{Average absorbance of test sample} - \text{Average absorbance of negative control}}{\text{Corrected positive control}}
\]

The titre was calculated by

\[
\log_{10} \text{titre} = [1.464 \times \log_{10} \text{Sp. Value}] + 3.197
\]

Titre = Antilog of \(\log_{10} \text{titre}\)

Results

Clinical signs — The affected birds did not exhibit apparent clinical signs except sudden mortality. At the terminal stage of disease, however, some birds were dull, depressed, and reluctant to move for food, showed a characteristic posture, with their chest and beak resting on the ground, and closed eyelids.

Post-mortem lesions — Post-mortem examination of dead and moribund birds revealed accumulation of clear, straw coloured fluid in the pericardial sac (Fig. 1). The amount of accumulated fluid varied from 4 to 12 ml. The cone of heart was found floating in the pericardial sac filled with copious amount of fluid. In some birds, the pericardial fat showed yellowish discoloration.

The liver of necropsied birds was enlarged, swollen and contained pinpoint and diffused necrotic foci. There were large areas of focal/diffuse necrotic patches on the surface. The kidneys were pale, swollen, friable and contained haemorrhagic patches on the surface. Some birds exhibited deposition of urates in ureters. The lungs were oedematous and congested. The birds having mixed infection of HPS and IBD showed enlargement of bursa of Fabricius and haemorrhages in thigh and breast muscles.

Isolation of virus in chicken embryo liver cell culture — The CEL cell culture inoculated with 20%
Fig. 1—Accumulation of straw coloured clear fluid in the pericardial sac of a bird that died of natural disease.

Liver homogenate extract prepared from infected birds resulted in the isolation of HPS agent. The cytopathic effects characterized by rounding and degeneration of cells was evident from the first passage itself and appeared as early as 36 hr PI. By 72 hr PI, microplaques were evident. At second passage and onwards, the CPE was evident at 24 hr PI and by 48 hr PI, 60-70% of cells were involved (Fig. 2). By 72 hr PI, almost 70% of monolayer was detached. The yield of virus at 4th passage level was 5.24 log10 TCID50/ml. Uninfected monolayers did not exhibit any change.

May-Grunwald and Giemsa staining of infected chicken embryo liver (CEL) cell culture (4th passage) showed enlargement of nuclei of cell with occasional basophilic intranuclear inclusion bodies at 24 hr PI. At 48 hr PI, the number of nuclei having basophilic inclusions increased, and these inclusions filled almost whole nuclei except a narrow rim at the periphery (Fig. 3). The shape of nuclei also distorted and occasional inclusions were surrounded by a halo. The infected cells also showed varying degree of cytoplasmic vacuolation. Some intranuclear inclusions were granular and basophilic and were embedded in the eosinophilic matrix. At 72 hr PI, most of cells detached from the surface therefore, numbers of cells containing intranuclear inclusion bodies were less but percentage was higher.

Serological tests—Agar gel precipitation test performed to confirm the isolation of HPS virus in cell culture showed two precipitin lines between concentrated CEL cell culture antigen and homologous antisera pre-tested against reference virus. Counter-immunoelectrophoresis of the concentrated CEL cell culture antigen with antisera also showed one precipitin line after 1 hr of electrophoretic run followed by incubation at 37°C for additional one hr. Infected CEL cells examined for the presence of virus by indirect fluorescent antibody technique at 4th passage level revealed greenish yellow fluorescence of low intensity in the nuclei of CEL cells at 24 hr PI (Fig. 4). At 48 hr PI, almost all nuclei exhibited yellowish green fluorescence. Nuclei of these cells also showed numerous tiny granules of fluorescence in most of the cells. At 72 hr PI, these tiny granules of fluorescence coalesced and entire nucleus of the cell exhibited yellowish green fluorescence of greater intensity. At this passage level, most of the cells detached from the coverslip resulting into decrease in total number of fluorescing nuclei. However, there was no fluorescence in the cytoplasm of infected cells. The uninfected CEL cells did not exhibit nuclear or cytoplasmic fluorescence. The micro-serum neutralization test using constant serum and diluted virus, the antisera showed neutralization of CEL cell culture grown virus with a serum-neutralizing index of 4.12. Enzyme linked immunosorbent assay was performed to titrate antibodies at different days post infection in birds experimentally infected with cell culture adapted hydropericardium syndrome virus. In birds inoculated via oral route the antibody titres at 1, 2, 3, 5 and 10 days PI were 26.02, 28.96, 36.04 55.84 and 23.29, respectively. ELISA titres recorded in birds infected via I/M route at 1, 3, 5 and 10 days PI were 1.48, 19.45, 155.47 and 299.04, respectively.

Discussion

The outbreak investigated during present study revealed only mild clinical signs like dullness, depression, and reluctance to move and change in posture followed by sudden death, which is similar to other outbreaks reported earlier. The absence of clinical signs and sudden heavy mortality may be attributed to relatively short clinical course of HPS and accumulation of fluid in the pericardial sac leading to compromise in the functioning of heart. On
post-mortem also, accumulation of 4-12 ml of clear, straw coloured fluid in the pericardial sac and oedema of lungs together with severe impairment of liver and kidneys were recorded. These changes reported earlier by several investigators confirm the outbreak of HPS in the affected flock. However, the pinpoint white foci in pancreas and ventricular erosions as reported by Nakamura et al. were not seen in the present investigation. Some birds also showed lesions to IBD infection, which suggested that immunosuppression due to concurrent IBD infection precipitated the HPS. Toro et al. also suggested that synergism with other viruses or prior immunosuppression is necessary to produce IBH/HPS. Chicken embryo liver (CEL) and chicken kidney (CK) cell cultures are reported to support the growth of etiological agent hydropericardium syndrome. In the present study, the virus infected CEL cells showed cytopathic effects characterized by rounding and degeneration of cells and presence of large basophilic intranuclear inclusion bodies, within 24 hr PI in the first passage itself. Toro et al. also observed CPE in both CEL and CK cell cultures characterized by round refractile cells after the first passage. The rounding of cells and degenerative changes within 3-4 days PI with evidence of intranuclear inclusion bodies has also been recorded by Oberoi et al. Adenovirus causes severe condensation of and margination of the host cell chromatin, making nuclei appear abnormal, which is the basis for the characteristic inclusion bodies in adenovirus infected cells.

Agar gel precipitation test using concentrated CEL cell culture antigen gave two precipitin lines against pre-tested homologous antisera. In a study conducted by Khera et al. for the detection of inclusion body hepatitis (IBH), counter-immunoelectrophoresis (CIE) gave one precipitin line with CEL cell culture antigen. Khera et al. in their survey demonstrated that CIE was able to detect 30 out of 32 isolates of IBH. The test is rapid and sensitive and can be used for the rapid diagnosis of hydropericardium syndrome. The indirect fluorescent antibody technique (IFAT) performed with isolated HPS virus produced intense fluorescence with homologous serum. In CEL cell culture, intranuclear fluorescence was observed, which confirmed the presence of adenovirus in CEL.
also demonstrated intranuclear viral antigen in liver, pancreas and other tissues of chicks experimentally infected with IBH virus by IFAT. In micro-serum neutralization test, pre-tested homologous antiserum was able to neutralize CEL cell culture grown virus with neutralization index of 4.12, which confirms the identity of virus as avianadenovirus.23

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