DETECTION OF EGG DROP SYNDROME 1976 VIRUS BY POLYMERASE CHAIN REACTION AND STUDY OF ITS PERSISTENCE IN EXPERIMENTALLY INFECTED LAYER BIRDS

N.S. KUMAR[1], J.M. KATARIA[1*], M. KOTI[1], K. DHAMA[1], R. TOROGHI[2]

[1]Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar 243 122, Bareilly, U.P., India; [2]Department of Research and Diagnosis of Poultry, Razi Vaccine and Serum Research Institute, Teheran, Iran

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Summary. – Polymerase chain reaction (PCR) assay was developed for the detection of Egg drop syndrome 1976 (EDS-76) virus in tissues, namely in the uterus, spleen and buffy coat. It was also used to study the persistence of the virus in tissues of experimentally infected layer birds. The PCR assay could detect as little as 10 fg of purified EDS-76 viral DNA. It also amplified the DNA of Fowl adenovirus serotypes 4 (FAV-4) and 8 (FAV-8). The virus persisted in the uterus up to day 21 post infection (p.i.). Detection of EDS-76 viral DNA in the buffy coat could be useful for studying the occurrence of the respective disease in layer bird flocks.

Key words: egg drop syndrome; Egg drop syndrome 1976 virus; polymerase chain reaction; diagnosis; buffy coat; persistence

Introduction

EDS is one of important infectious viral diseases of poultry since it causes major economic losses due to direct effect on egg production. The disease is characterized by sudden and severe drop in egg production at the onset or peak production laying period with high frequency of shell defects. The disease is caused by EDS-76 virus, a hemagglutinating adenovirus that is an unassigned virus of the *Adenoviridae* family. The virus has a dsDNA genome of 33,213 bp of known sequence (Hess *et al.,* 1997). No serological differences among EDS-76 viral isolates have been reported and only one serotype is prevalent worldwide. It is usually diagnosed by hemagglutination-inhibition test for detection of antibody in serum. Other conventional tests like AGPT, enzyme-linked immunosorbent assay, FAT and SNT are also being used but not routinely (Smyth and McFerran, 1989). Recently, PCR is being routinely used for precise and quick diagnosis of various avian pathogens (Jestin and Jestin, 1991; Poulsen *et al.,* 1991; Lee *et al.,* 1992; Kwon *et al.,* 1993; Nguyen *et al.,* 1994; Raj *et al.,* 2001).

In this study we describe the standardization of PCR assay for detection of EDS-76 virus in various samples, namely in infected chicken embryo liver cell culture fluid, duck allantoic fluid, uterus, spleen tissues and buffy coat.

*Corresponding author. E-mail: jmkataria@rediffmail.com; fax: +91581-2303284.

Abbreviations: CAV = Chicken anemia virus; CEL = chick embryo liver, CPE = cytopathic effect, EDS = egg drop syndrome; EDTA = ethylene diamine tetraacetate; FAV = Fowl adenovirus, IBDV = Infectious bursal disease virus; NDV = Newcastle disease virus; PCR = polymerase chain reaction; p.i. = post infection; SDS = sodium dodecyl sulfate; TV = trypsin-versene

Materials and Methods

Viruses. EDS-76 viral isolates, FAVs, Chicken anemia virus (CAV), Newcastle disease virus (NDV), Infectious bursal disease virus (IBDV) and reovirus used in this study were maintained in this Institute. EDS-76 virus was propagated in chick embryo liver (CEL) cell cultures. The latter were prepared following the met-

hod of Adair *et al.* (1979) with some modifications. Briefly, livers from 15-day-old embryos were minced and washed with Hank's Balanced Salt Solution (HBSS) and trypsinized with trypsin-versene (TV) for 3–5 mins. The product was filtered through a muslin cloth. The filtrate was centrifuged at 2,500 rpm for 10 mins and the cell pellet was washed twice with HBSS and resuspended in a growth medium (M-199 with 15% of calf serum). The cells were dispensed in 75 cm^2 culture flasks (Corning, USA) and incubated at 37°C. Confluent monolayers formed within 72 hrs were infected with 0.5 ml of a virus inoculum for 1 hr. Then maintenance medium (M199 with 2% of calf serum) was added and the cultures were kept at 37°C and observed for cytopathic effect (CPE).

Extraction of viral DNA from culture cells was carried out according to Shinagawa *et al*. (1983). In brief, the infected cells from cultures showing 50–60% CPE were collected in TE buffer (10 mmol/l Tris and 1 mmol/l ethylene diamine tetraacetate (EDTA), pH 7.5) and treated with 1 mol/l NaCl and 0.5% sodium dodecyl sulfate (SDS) overnight at 4°C. The lysate was shaken with phenol, centrifuged and the resulting aqueous phase with the interphase were precipitated with 1.5 volume of ethanol at -20°C overnight. The precipitate was washed twice with 70% ethanol, resuspended in TE buffer and treated with 1 mg/ml proteinase K in the presence of 0.5% SDS and 0.5 mol/l NaCl at 37°C overnight. The extract was shaken with phenol and phenol/chloroform and then precipitated with 2.5 volumes of ethanol at -20°C for 2 hrs. The precipitate was centrifuged at 13,000 x g for 10 mins, washed with 70% ethanol and dissolved in 50 µl of TE buffer. Purity and concentration of DNA were determined spectrophotometrically in a standard way. The purified DNA was used for optimization of the PCR assay.

DNA extraction from tissues infected with EDS-76 virus was carried out by triturating 100 mg of tissue in 1 ml of TNE buffer (TE buffer supplemented with 200 mmol/l NaCl). The triturated suspension was centrifuged and 500 µl of the supernatant was treated with 1% SDS and 500 µg/ml proteinase-K at 56°C for 1 hr and extracted with phenol/chloroform (1:1) and chloroform. The aqueous phase was collected and ethanol-precipitated. The precipitated DNA was pelleted, washed with 70% ethanol, air-dried and dissolved in 50 µl of TE buffer.

DNA extraction from cell culture fluid (500 µl) was carried out with 0.5% SDS and 100 µg/ml proteinase K at 56°C for 1 hr followed by treatment with phenol/chloroform and chloroform and ethanol precipitation as described above.

DNA extraction from buffy coat and allantoic fluid. Infected duck allantoic fluids (100 µl) were first mixed with of TNE buffer (400 µl per sample). Buffy coats obtained from blood samples were resuspended in 500 µl of TNE buffer per sample. These samples were treated with SDS and proteinase K as for DNA extraction from tissues described above.

The primers E5 (24-mer, forward) and E6 (23-mer, reverse) were designed from the published nucleotide sequence of the complete genome of EDS-76 virus (Hess *et al*., 1997). Their sequences and positions are given in Table 1. They flanked a 1925 bp region of the hexon gene of EDS-76 virus (Fig. 1). The primers were supplied by Life technologies, USA. The primers had partial similarity with the primers used by Raue and Hess (1998) for the amplification of the hexon gene.

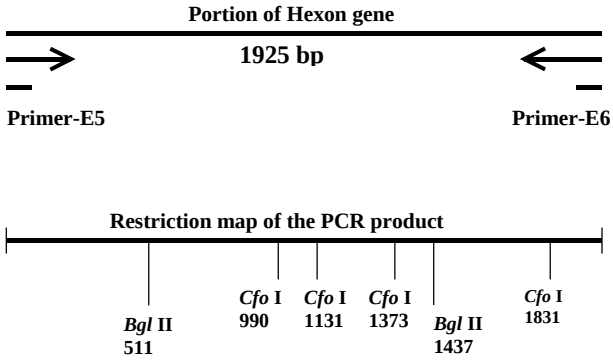

Fig. 1

Part of the EDS-76 viral genome amplified by PCR and restriction map of the PCR product

Optimization of PCR was done according to Xie *et al.* (1999). Various components of the reaction mixture were tested in different concentrations, namely 1–3 mmol/l $MgCl_2$, 5–40 pmoles of each primer, 1–5 U *Taq* polymerase, 50–300 mmol/l NTPs and 55–63°C as annealing temperature. The amplification was carried out in 25 µl (total reaction volume) using a programmable thermal cycler (PTC-200, MJ Research, USA). Tenfold serial dilutions of purified viral DNA, obtained from a low melting agarose gel by means of the Qiaex II gel purification kit (Qiagen, Germany), contained from 100 ng to 10^{-3} fg of viral DNA were used to test the sensitivity of the PCR assay. The specificity of the PCR assay was tested using 100 ng of DNA or 5 µl of cDNA prepared from 1 µg of RNA extracted from a known positive sample. The uninfected control tissues, different avian viruses as FAV-1, FAV-4 and FAV-8, CAV (the *Circoviridae* family, the *Circovirus* genus), NDV, a reovirus and an IBDV (the *Birnaviridae* family, the *Avibirnavirus* genus) and bacteria as *Escherichia coli*, *Salmonella gallinerum, Pasturella multocida* and *Mycoplasma gallisepticum* were tested.

Confirmative test for the PCR product. The EDS-76 virus-specific product of 1925 bp amplified by PCR was tested by its size by electrophoresis in 1% agarose gel using suitable molecular size markers and restriction analysis. The PCR product was purified using the Wizard PCR purification system (Promega, USA) and digested with the restriction endonucleases *Bgl*II (2 sites) and *Cfo*I (4 sites).

Experimental infection. Thirty 22-week-old white leghorn laying birds free from EDS-76 viral specific antibodies were obtained from the experimental layer farm of the Indian Veterinary Research Institute, Izatnagar. Fifteen birds were kept as control and another fifteen birds were infected oro-nasally with 3 x 10^6 (LD_{50} of EDS-76 virus) in 0.5 ml. Three chicks from infected and control groups were sacrificed on days 7, 10, 15, 21 and 28 p.i. and tissues as the uterus, spleen and blood for the buffy coats were collected.

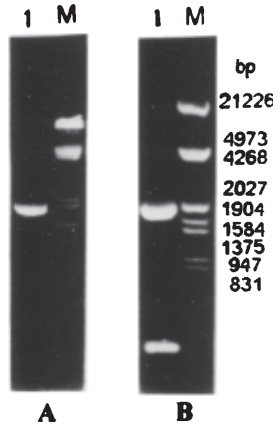

Fig. 2

Optimization of annealing temperature for PCR

A. Specific PCR product of 1925 bp obtained at annealing temperature of 55°C (lane 1); B. Specific and non-specific PCR product obtained at annealing temperature of 63°C (lane 1). DNA size marker (lanes M).

Results

The PCR assay was standardized using the purified viral DNA. Optimum and specific DNA amplification was achieved under the following conditions and concentrations of reaction components: initial denaturation of viral DNA at 95°C for 4 mins, subsequent 30 cycles the denaturation at 94°C for 1 min, the annealing at 63°C for 1 min and the extension at 72°C for 2.5 mins, and final extension at 72°C for 10 mins (Fig. 2). The optimum amplification was achieved in a reaction mixture containing 1 U of *Taq* polymerase, 1.5 mmol/l $MgCl_2$, 200 mmol/l dNTPs and 10 pmoles of each primer.

The amplified PCR product of about 1.9 kbp was separated by electrophoresis in 1% agarose gel using a DNA size marker (a digested lambda DNA, Promega, USA) and by digesting the purified PCR product using *Bgl*II and *Cfo* I. *Bgl*II had 2 restriction sites and yielded three fragments (Fig. 3), while *Cfo*I had 4 restriction sites yielding four visible fragments (Fig. 4); one small fragment of less than 50 bp was not resolved in the gel.

The developed PCR successfully amplified all the seven Indian viral isolates and the European reference virus (BC-14) and detected as little as 10 fg of viral DNA in a sample (Fig. 5). Among various viruses and bacteria tested for specificity only the FAV-4 and FAV-8 were amplified (Fig. 6). The developed PCR assay could detect the viral DNA extracted from CEL cell culture fluid, duck embryonic allantoic fluid, buffy coat cells and tissues as uterus and spleen. No PCR

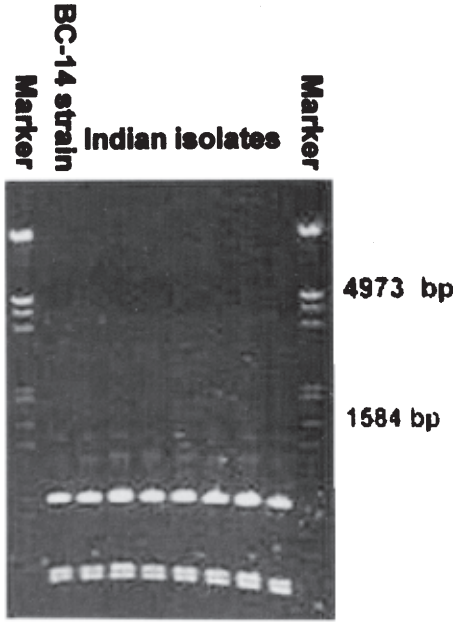

Fig. 3

***Bgl*II digestion of PCR products from seven Indian EDS-76 virus isolates and reference strain BC-14**

Agarose gel electrophoresis.

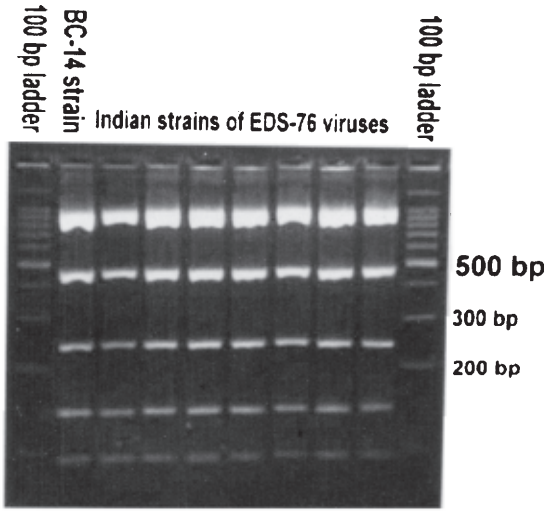

Fig. 4

***Cfo*I digestion of PCR products from seven Indian EDS-76 virus isolates and reference strain BC-14**

Agarose gel electrophoresis.

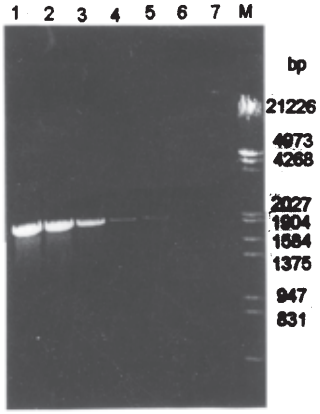

Fig. 5

Sensitivity of the PCR assay

Agarose gel electrophoresis. Serial 10-fold dilutions of purified EDS-76 viral DNA used. DNA amounts: 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg (lanes 1–7). DNA size marker (lane M).

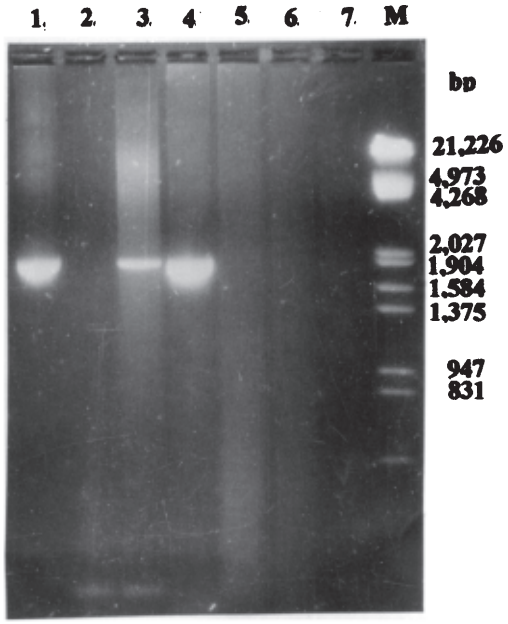

Fig. 6

Specificity of the PCR assay tested with DNAs from different avian pathogens

Agarose gel electrophoresis. Purified EDS-76 viral DNA as positive control (lane 1); cDNA from NDV (lane 2); FAV serotype 4 (lane 3); FAV serotype 8 (lane 4); *E. coli* (lane 5); *Pasteurella multocida* (lane 6); *Salmonella gallisepticum* (lane 7); DNA size marker (lane M).

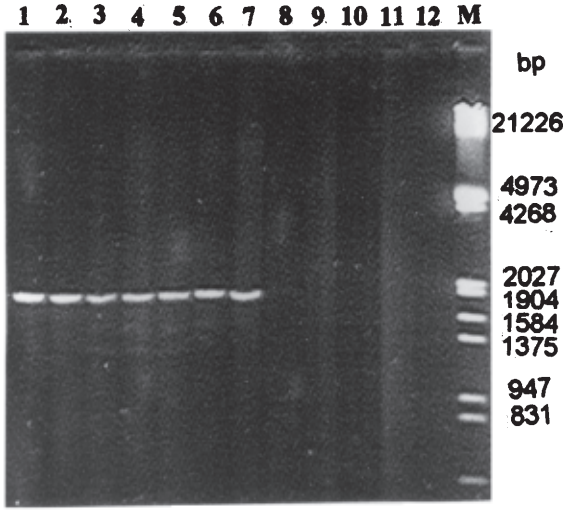

Fig. 7

Detection of EDS-76 virus in the uterus, spleen and buffy coat by PCR

Agarose gel electrophoresis. The uterus on 10, 15, 21 and 28 days p.i. (lanes 1, 4, 7 and 10, respectively); the splen on 10, 15, 21 and 28 days p.i. (lanes 2, 5, 8 and 11, respectively); the buffy coat on 10, 15, 21 and 28 days p.i. (lanes 3, 6, 9 and 12, respectively).

amplification was obtained with uninfected control samples as CEL cell culture fluid, allantoic fluid, buffy coat and tissues, which were tested in a similar manner.

The experiments on the virus persistence in experimentally infected layer birds showed that the virus could be detected from day 3 to 21 p.i. in the uterus and from day 3 to 15 p.i. in the spleen and buffy coat (Fig. 7).

Discussion

The primers used in this study were designed from the published complete nucleotide sequence of the EDS-76 virus strain 127 (Hess *et al.,* 1997). They specifically amplified the hexon gene. The primers amplified successfully the amplicon of seven Indian isolates of EDS-76, the European reference virus (BC-14) and FAV-4 and FAV-8. These findings are in contrast to the earlier reports that the EDS-76 virus and FAVs had little or no homology at the genomic level as estimated by restriction and nucleotide sequence analysis (Zsak and Kisary, 1981b; Zakharchuk *et al.,* 1993; Hess *et al.,* 1997; Jadhao, 1998). However, a cross-amplification among serotypes of FAVs in PCR has been reported. Namely the primers designed from the hexon gene sequence of FAV-10 amplified FAV-4 and FAV-12 isolates (Ganesh *et al*., 2000; Rahul, 2003). In the present study, the primers were designed to amplify a part of hexon gene,

which codes for the major capsid protein having a conserved pedestral regions (P1 and P2) and variable surface loops (L1-L4) (Roberts *et al.,* 1986). Hess *et al.* (1997) have completely sequenced the EDS-76 virus genome and found that the EDS hexon protein had only 52% and 51% amino acid identity with hexon proteins of FAV-1 and FAV-10, respectively.

L1 loop has been reported to have six of seven hexon hypervariable regions. Concerning L4 it has been speculated that it could determine the species specificity. These L1 and L4 regions of EDS-76 and FAV-1 have only 28% and 38% amino acid identity, respectively. Due to clear distinctness of EDS-76 virus and FAVs, cross amplification in our experiments was not expected. However, a contrast result was surprisingly obtained. There was similarity in certain regions even though the amino acid homology between EDS -76 virus and FAVs was very low, namely around 50%. These products could be differentiated by restriction analysis, by hybridization using type-specific probes. Also nucleotide sequencing could be used, but it was not attempted in our study. However, the cross-reactivity will not represent a problem in interpretation of the results while attempting the EDS-76 diagnosis, because whereas EDS-76 infects laying hens FAV-4 and FAV-8 cause a disease mainly in young broiler chickens.

FAV-1 (Celo virus) is a cell culture contaminant and normal inhabitant of the avian respiratory tract (McFerran, 1991). However, it was not amplified in our PCR assay. Hence, the developed PCR could successfully be used for the amplification of hexon gene and detection of the EDS-76 viral DNA in various samples as the uterus, spleen, buffy coat, cell culture fluid and duck allantoic fluid. To our knowledge, this is the first report on detection of EDS-76 virus from different tissues by PCR. Zhang *et al.* (1996) have reported the EDS-76 DNA detection in cloacal swabs, serum and soft shell egg. Raue and Hess (1998) developed three different PCR assays combined with restriction analysis for detection and differentiation of all 12 fowl adenovirus serotypes and EDS-76 virus.

We used the PCR technique to study the persistence of this virus as it has a high sensitivity in detecting even small quantities of viral DNA in tissues. Tissues as the uterus, spleen and buffy coat were examined since these might be the probable sites of viral latency.

To study the persistence of the virus, 22-week- old white leghorn layer birds were infected oronasally with 1 ml of infected duck allantoic fluid at the third passage level. The experimental infection produced no clinical disease in birds but resulted in abnormal eggs and gross lesions as described earlier (Lutticken and Baxendale, 1980; Taniguchi, 1981; McCracken and McFerran, 1978; Van Eck, 1986).

Studies on infected laying hens by PCR revealed that the virus persists at most for 21 days in the uterus and for 15 days in the spleen and buffy coat. It supported the earlier data of Heffels *et al.* (1982) that the virus was isolated from internal organs within at most 21 days p.i. Baxendale (1978) has recovered the virus from the buffy coat within at most 16 days p.i. in laying hens. The lateral transmission of EDS-76 virus is recently gaining importance due to its dissemination by carrier water fowls and through infected eggs. The infected egg is considered one of important sources in lateral spread of EDS-76 virus in laying hen, as they are readily ingested and the birds themselves become infected. In vertical transmission, EDS-76 virus becomes latent in chicks during their growing period and is reactivated at the time of laying, when the virus is passed into eggs and excretions (Smyth and McFerran, 1989). Thus the screening of layers in commercial breeder farms is very important for the prevention and control of the disease.

It may be concluded that the developed PCR technique can be successfully used for the detection of EDS-76 virus DNA in the uterus, spleen, allantoic fluid, cell culture fluid and buffy coat. The virus detection in the buffy coat appears to be most useful for screening the layer flocks for this disease. Similarly, study of the virus persistence in vertically infected young chicken could be useful for avoiding the production losses due to reactivation of latent virus at the time of laying and dissemination of the virus from the parent stock to their off springs.

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References

Adair MB, McFerran JB, Connor TJ, McNulty MS, McKillop ER (1979): Biological and physical properties of a virus (strain 127) associated with the egg drop syndrome 1976. *Avian Pathol.* **8**, 249–264.

Baxendale W (1978): Egg drop syndrome 76. *Vet. Record* **102**, 286.

Ganesh K, Suryanarayana VVS, Raghavan (2000): Detection of Fowl Adenovirus Associated with Hydropericardium Hepatitis by a Polymerase Chain Reaction. *Vet. Res. Commun.* **26**, 73–80.

Heffels U, Khalaf SED, Kaleta EF (1982): Studies on the persistence and excretion of egg drop syndrome 1976 virus in chickens. *Avian Pathol.* **11**, 441–452.

Hess M, Blocker H, Brand P (1997): The complete nucleotide sequence of EDS virus- an intermediate between Mastadenoviruses and Aviadenoviruses. *Virology* **238**, 145–156.

Jadhao SJ (1998): Molecular characterization of Indian isolates of fowl adenovirus associated with inclusion body hepatitis in chickens. *Ph.D. Thesis*, Indian Veterinary Reserach Institute, Izatnagar (U.P) India.

Jestin V, Jestin A (1991): Detection of Newcastle disease virus RNA in infected allantoic fluids by in vitro enzymatic amplification (PCR). *Arch. Virol.* **118**, 151–161.

Kwon HM, Jackwood MW, Gelb JJ (1993): Differentiation of infectious bronchitis virus serotypes using polymerase-chain reaction and restriction fragment length polymorphism analysis. *Avian Dis.* **37**, 194–202.

Lee, LH, Yu, SL, Shieh, HK (1992): Detection of infectious bursal disease virus infection using the polymerase chain reaction. *J. Virol. Methods* **40**, 243–254.

Lutticken D, Baxendale W (1980): Egg production drops caused by experimental infection with a haemagglutinating adenovirus (EDS virus) in different breeds of fowl. *6th European Poultry Conference* **2***,* 400–407.

McCracken RM, McFerran JB (1978): Experimental reproduction of the egg drop syndrome 1976 with a haemagglutinating adenovirus. *Avian Pathol.* **7**, 783–490.

McFerran JB (1991): Adenoviruses. In Calnek BW (Ed.): *Diseases of Poultry.* 9th ed., Iowa State University Press, Ames, pp. 552–563.

Nguyen AV, Khan MI, Liu Z (1994): Amplification of salmonella chromosomal DNA using the polymerase chain reaction. *Avian Dis*. **38**, 119–126.

Poulsen DJ, Burton CRA, O'Brian JJ, Rabin SJ, Keeler CL Jr (1991): Identification of the infectious laryngotracheitis virus glycoprotein gB gene by the polymerase chain reaction. *Virus Genes* **5**, 335–347.

Rahul S (2003): Molecular characterization of Fowl Adenovirus serotype-4 isolates associated with Hydropericardium syndrome and its pathogenicity in chicken co-infected with Chicken Infectious Anaemia Virus. *M.V.Sc. Thesis*, Deemed University, IVRI, Izatnagar (U.P.), India.

Raj DG, SivaKumar S, Sudarsan S, Mohan AC, Nachimuthu K (2001): Genomic characterization of Indian isolates of egg drop syndrome virus 1976. *Avian Pathol*. **30**, 21–26.

Raue R, Hess M (1998): Hexon based PCR combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. *J. Virol. Methods* **73,** 211–217.

Roberts MM, White JL, Grutter MG, Burnett RM (1986): Three-dimensional structure of the adenovirus major coat protein hexon. *Science* **232**, 1148–1151.

Shinagawa, M, Matsuda A, Inshiyama T, Goto H, Sato G (1983): A rapid and simple method for preparation of adenovirus DNA from infected cells. *Microbiol. Immunol.* **27**, 817–822.

Smyth JA, McFerran JB (1989): Egg drop syndrome. In Pandey R (Ed.): *Progress in Veterinary Microbiology and Immunology – Nononcogenic Avian Viruses.* Karger, Basel, Switzerland, pp. 83–108.

Taniguchi T, Yamaguchi S, Maeda M, Kawamura H, Horiuchi T (1981): Pathological changes in laying hens inoculated with the JPA-1 strain of egg drop syndrome-1976 virus. *Natl. Inst. Anim. Health Q. Jap*. **21,** 83–93.

Van Eck JHH (1986): Egg drop syndrome 1976 (EDS-76) in fowl – aspects of manifestation, pathogenesis, histopathology, pathophysiology, immunology, epizootiology and incidence in the Netherlands. *Thesis*, Utrecht University.

Xie Z, Fadly AA, Girshick T, Khan MI (1999): Detection of Avian adenovirus by polymerase chain reaction. *Avian Dis.* **43**, 98–105.

Zakharchuk AN, Kruglyak VA, Akopian TA, Naroditsky, Tikchoenko TI (1993): Physical mapping and homology studies of egg drop syndrome-76 (EDS-76) adenovirus DNA. *Arch. Virol*. **128,** 171–176.

Zang Zijun, Hu Muzhi, Jin Yaixix, Zhang ZJ, Hu, Jin YX (1996): Detection of EDS-76 virus by polymerase chain reaction. *Chinese J. Virol.* **12**, 156–161.

Zsak L, Kisary J (1981b): Studies on egg drop syndrome (EDS) and chick embryo lethal orphan (CELO) avian adenovirus DNAs by restriction endonucleases. *J. Gen. Virol*. **56**, 87–95.