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Application of polymerase chain reaction in detection of egg drop syndrome (EDS) virus

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ABSTRACT

Egg drop syndrome (EDS) is caused by a hemagglutinating adenovirus which has become a major cause of lost egg production and sever economic losses in breeder and layer chicken flocks throughout the world. A PCR assay was optimized for detection of EDS virus in inoculated allantoic fluids (AFs) of duck and chicken emberyonated eggs. Two strains of EDS viruses were propagated in allantoic cavity of eggs. Then the virus DNA was extracted. PCR test was designed and carried out by specific primers. The 1900 bp band was detected in agar gel electrophoresis. Serial two fold dilutions of infected allantoic fluid were prepared. The HA test and PCR assay were carried out for each dilution and finally results were compared to each other. The PCR assay could detect some negative HA titers. This study clearly indicates the superiority of PCR assay over HA test for detection of EDS virus in AFs.

Keywords: Egg drop syndrome virus, PCR, Hemagglutination test, Allantoic fluid

INTRODUCTION

Egg drop syndrome virus (EDSV) is a hemagglutinating adenovinus that causes an economically important disease in laying hens. The EDS virus is the sole member of the subgroup III avian adenoviridae and it is serologically unrelated to the subgroup I and subgroup II viruses, and only one serotype has been recognized (Adair *et al* 1979, McFerran 1998). The disease is characterized by drops in egg production accompanied by the production of soft shelled or shell-less eggs or by failure to reach peak production (McFerran & Adair 2003). Since its first description in 1976 in the Netherlands (van Eck *et al* 1976), this virus that is also called EDSV 76 has become a major cause of loss in egg production throughout the world (Baxendale 1978, McFerran & Adair 2003). Shortly after the first description of disease in Western Europe, suspicious cases occurred in broiler parent flocks in Iran, but the first confirmed report of the disease in Iran was done by Aghakhan and Khodashenas in 1990. They confirmed EDSV infection in broiler breeder flocks in Iran using hemagglutination inhibition (HI) test. EDS should be suspected whenever there is failure to achieve predicted egg production levels. Many infectious or non infectious causes can induce loss of egg production and while some signs of typical EDS 76 are suggestive, diagnosis must not be made on them

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alone but should be confirmed by serology before vaccination, virology and/or detection of viral antigen or DNA. Viral antigen can be detected in pouch shell gland epithelium by immunofluoresent or immunocytochemical staining, but its presence is transient and examination of randomly selected birds has usually been negative (McNulty & Smyth 2002). Serology can be useful for diagnosis but before vaccination. The HI test and detection of antibodies against EDSV before embarking on a vaccination program is the one of choice for diagnosis of EDSV infection and also for evaluation of vaccination in poultry flocks (McFerran *et al* 1977, Baxendale *et al* 1980, Cook & Darbyshire 1981, Cook 1983, Adair *et al* 1986, McFerran & Adair 2003, Banani *et al* 2005). Virus isolation is difficult because it is often not easy to identify the correct bird for taking the sample (McFerran & Adair 2003), further more new outbreak of EDS in poultry industry of Iran has not been observed after widespread EDS vaccination. Therefore, attempts to isolate the virus have not been successful even during outbreaks in Iran (Aghakhan & Khodashenas 1990, Aghakhan *et al* 1994). PCR has been routinely used for exact and quick diagnosis of different avian pathogens in the world. PCR assay had not been set up for detection of EDS virus before this study in Iran. Use of PCR could also help to explain the role of EDS virus in other disease conditions, such as its possible involvement in acute respiratory disease of goslings (Ivanics *et al* 2001). The purpose of this study was to set up a PCR test for rapid detection of EDSV in allantoic fluid samples of inoculated embryonated chicken and duck eggs.

MATERIALS AND METHODS

Viruses. A live HI antigen strain of EDSV 76 obtained from Venzie - Padova institute of Italy and strain 127 of EDSV 76 as standard strain described by McFerran *et al* (1977) obtained from CVL of England were used in this study.

Duck eggs. One day-old embryonated duckeggs free from some avian pathogens were obtained from Couovir de la seigneuriter company of France. The eggs were free of *Mycoplasma*, *Chlamydia*, *Salmonella*, adenoviruses, Newcastle disease and influenza viruses and the mean antibody HI titers of their yolks against EDS virus were below than 6 based on log2. Duck eggs were incubated for ten days in a special incubator (Masallas, HS 36).

Chicken eggs. 11-day-old SPF embryonated chicken eggs (Valo, Lohmann and Cuxhaven, Germany) were used in this study.

Propagation of virus. One part of original viral seed was diluted with 100 part of phosphate buffer solution (PBS) pH=7.2, then inoculated in allantoic sac of duck and chicken embryonated eggs. The eggs were incubated at 37 ºC and examined daily. After 5 days the allantoic fluids were collected from embryos.

HA test. The HA activity of different dilutions of allantoic fluids (AFs) in PBS, were determined using standard micro-titer procedure. Serial twofold dilutions of the AFs suspension in PBS were mixed with equal volume of 1 percent chicken erythrocyte suspension. The results were read after incubation at 25 ºC for 30 minutes. The reciprocal of the highest dilution showing hemagglutination was taken as the titer (HAU/0.025 ml). After demonstrating HA activity the agent responsible for such HA must be determined. Common hemagglutinating agents for birds include avian influenza virus (AIV), Newcastle disease virus (NDV) and also hemagglutinating bacteria. In order to ascertain the presence or absence of NDV or AIV, antisera against these viruses were used in microtiter hemagglutination inhibition (HI) testes. A loopful of the AFs were cultured for bacteria and fungi using standard media such as blood agar, PPLO broth and Sabouraud dexterose agar. Electron microscopy studies of AFs were carried out by electron microscope (Philips 400), and using 2.5 percent phosphotungstic acid for negative staining.

Viral DNA Extraction by Phenol/Chlorophorm method. 100 μl of harvested allantoic fluid was added to 100 μl lysis buffer and tube was placed in a 56 ºC bath for 4 hours. Then 200 μl saturated phenol was added and tube was centrifuged (13000 rpm or 15700 g) for 20 min. Upper phase was transferred to other tube and equal volume of mixed phenol / chlorophorm (1:1) was added. After centrifugation at 13000 rpm for 20 min the aqueous phase was transferred and added to equal volume of pure chloroform and was centrifuged (13000 rpm) for 5 min. Upper phase mixed with 1/10 volume of acetate sodium and were precipitated with 2 fold volume of cool and pure ethanol. After final precipitation using by 70% ethanol the DNA was dried and resuspended in 50 μl T.E. buffer at 4 ºC and used for PCR.

Viral DNA Extraction by Boiling method. The allantoic fluid sample was centrifuged (3000 rpm) for 10 min. The supernatant was incubated in a boiling bath (100 ºC for 10 min). After that it was transferred to a -70 ºC and was kept for 10 min. The released DNA was used for PCR.

Primers. In this study two primers H5 (19-mer,forward)): 5´-TTC TGT CAC CGA TAA AGG T-3´ and H6 (19-mer, reverse) : 5´-AGT TAT TCC AAA TGG GCA T-3´ which have designed from the published nucleotide sequence of the complete genome of EDS-76 virus (Hess *et al* 1997) were used. They flank and amplify a 1901 bp region of the hexon gene of EDS-76 virus (Raue & Hess 1998). The primers had imperfect resemblance with the primers used by Kumar *et al* (2003) that flank a 1925 bp region of the hexon gene of EDS-76 virus.

PCR Parameters and Optimization. DNA amplifications were carried out in a total volume of 25 μl containing 1 μl DNA, 0.2 μl of each primer, 0.5 μl dNTP mix (10mM) [CinnaGen Inc.], 1.5 μl $Mgcl_2$ (25mM) [CinnaGen Inc.], 2.5 μl PCR buffer (10X) [CinnaGen Inc.], 0.2 μl Taq DNA polymeras (5 unit / μl) [Cinna Gen Inc.] and 19 μl H_2O (Autoclaved distilled water). Reaction mixtures were thermocycled 30 times beginning with an initial denaturation step of 4min at 95 ºC. The temperature and time profile of each cycle was as follows: 94 ºC for 1min (denaturation), 57 ºC for 1min (annealing), and 72 ºC for 2.5 min. PCRs were finished with a final extension step of 10 min at 72 ºC. PCR products were stored at 4 ºC. PCRs were carried out using two programmable thermal cyclers (Primus and Mastercycler gradient). Positive and negative controls were included in all tests and separated by electrophoresis in an 1% agarose gel with 0.5 μl/ml ethidium bromide (100 volts for 1 hr) following U.V transillumination.

Specificity and Sensitivity of the test. The sensitivity of the PCR procedure was carried out by testing the DNA extractions of serial log dilutions of AFs. The specificity of the PCR was confirmed by testing DNA extracted of *Mycoplasma gallisepticum*, *Salmonella entritidis*, fowl pox virus, sterile SPF allantoic fluid and water.

RESULTS

HA activity. The mean titers of non diluted AFs of duck and chicken eggs were 14 and 9 based on log2 respectively. The titers of diluted AFs were decreased up to undetected HA reaction. More two fold dilutions after negative HA activity of embryonated duck AFs were prepared and compared to PCR test.

Purity. Serological, virological and other microbiological testes showed purity from contaminations. Results of the culture of the virus pool were negative for bacteria, fungi and the other viruses. The HI test with NDV and AIV antisera showed no hemaggulutination inhibition but complete inhibition was done using EDSV antiserum. Electron microscopy studies of the two strains of the virus propagated in chicken and duck eggs fulfilled the morphological description of adenoviruses (Mc Frran & Adair 2003, Banani *et al* 2005).

PCR. No difference was seen between phenol and boiling methods of DNA extraction. This PCR test successfully amplified the DNA of both strain of EDS virus. The amplified PCR product of about 1.9 kbp was separated by electrophoresis in 1% agarose gel using 100bp DNA ladder and this PCR product was not observed for negative controls (Figure1). The PCR assay detected some negative results of HA test (Table 1 and Figure 2).

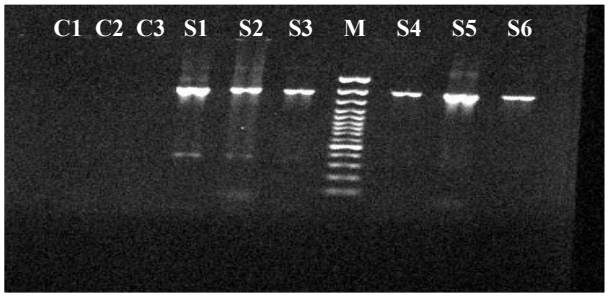

Figure 1. Electrophoresis of PCR products. The PCR assay could detect five more two fold dilution after the first negative result of HA test.(Table 1, Figure 2). **S1:** HI antigen strain of EDSV, in duck egg, HA titre=17, phenol extraction. **S2:** HI antigen strain of EDSV, in chicken egg, HA titre=12, phenol extraction. **S3:** strain 127 of EDSV, in chicken egg, HA titre=7, phenol extraction. **M:** Marker (100bp DNA ladder) **S4:** strain 127 of EDSV, in duck egg, HA titre=15, phenol extraction. **S5:** HI antigen strain of EDSV, in duck egg, HA titre=17, Boiling extraction. **S6:** strain 127 of EDS virus, in duck egg, HA titre=15, Boiling extraction. **C1:** negative control, H_2O **C2:** negative control, DNA of Mycomplama gallisepticum **C3:** negative control, SPF allantoic fluid of chicken egg.

former methods are laborious and time-consuming (Hess 2000).

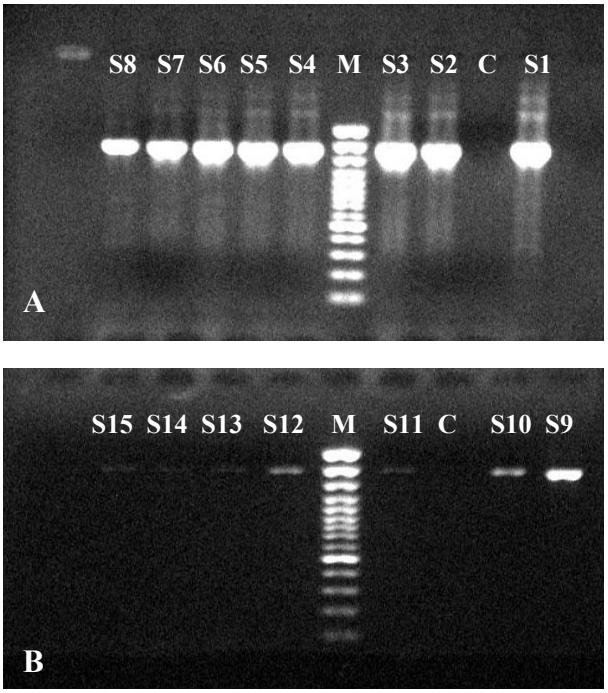

Figure 2. Sensitivity of PCR assay. Samples: Serial two fold dilutions (1, 1/2 , to 1/1000 000) of EDS virus; passaged in duck egg; M: Marker (100 bp DNA Ladder), C: Negative Control (H_2O). S1: 1/1 (pure) AF, HA titer of AF before dilution was 17 based on log 2. S2: 1/64, S3: 1/128, S4: 1/256, S5: 1/512, S6: 1/1000, S7: 1/2000, S8: 1/4000. **B:** S9: 1/8000, S10: 1/16000, C: Negative Control (H2O), M: Marker, S11: 1/32000. S12: 1/64000, S13: 1/128000, S14: 1/256000, S15: 1/512000

DISCUSSION

EDS is characterized by otherwise healthy birds producing thin shelled or shell-less eggs. These eggs are often missed and even after observing them in a flock, it is not easy to find out which birds have laid these abnormal eggs (McFerran 1998, McFerran & Adair 2003). For a long time, the diagnosis of EDS virus infection was restricted to isolation and identification of causative agent or antigen and also detection of specific antibodies that especially

Transient presence of the virus in target organ of infected birds and lack of obvious clinical signs in those birds are some difficulties in the virus isolation. It is highly recommended that in order to isolate the virus, abnormal eggs from suspected flocks should be fed to antibody negative adult laying hens. Then the virus could be isolated from the oviduct of these experimental infected birds (McFerran 1998, McFerran & Adair 2003). Routine systems that usually are used for isolation of other avian viruses are not suitable for EDS virus isolation. The most sensitive systems for the primary isolation

of the EDS virus from the oviduct are eggs or tissue cultures from waterfowls at least free from EDS virus or antibody (McFerran 1998, Zsak *et al* 1982). At least two passages are necessary to sure that virus will grow or not. Following isolation, the virus in the allantoic fluids or cell culture supernatants is normally checked by HA for chicken erythrocytes. After demonstrating HA in AF, the agent responsible for such HA must be determined by other methods (McFerran 1998, McFerran & Adair 2003). In Iran there is no report of the EDS virus isolation and in recent years after mass EDS vaccination of almost all commercial layer and breeder flocks, no EDS outbreak has reported in Iran poultry industry (Aghakhan & Khodashenas 1990, Aghakhan *et al* 1994, Banani *et al* 2005).

Because of the problem of virus isolation, serological methods had been used for detecting of EDS virus antibody for several years in Iran and throughout the world. Adair *et al* (1986) compared sensitivity of five serological tests. They showed that there were false positive reaction in the ELISA, IFA, or DID tests but not in the HI or SN tests. Recent progress in diagnosis of avian adenoviruses has mainly been done at a molecular level. Several PCRs have been published, completing the diagnostic scheme for EDS virus (Hess 2000). Complete nucleotide sequence of EDS virus was published by Hess *et al* in 1997. The virus has a dsDNA genome of 33,213 nucleotides of known sequence (Hess *et al* 1997). In 1998 for the first time a PCR assay was developed in order to detect EDSV by Raue and Hess. They used H5/H6 primers to amplify a 1901 bp fragment of nucleotides of hexon gene of EDS virus DNA. These primers were EDS virus specific and oligonucleotides H5/H6 located in the variable regions of EDS virus hexon gene that do not detect any of all 12 fowl adenovirus serotypes. Dhinakar Raj *et al* detected 5 Indian isolates of EDS virus using H5/H6 primers in 2001 in India. In another study of Dhinakar Raj *et al* in 2001 they used other EDS virus specific primers for amplification of the 238 bp of J fragment of EDS virus DNA. Xie *et al* detected all three groups of avian adenoviruses using MK90/MK89 primers in 1999. An avian adenovirus-specific 421-bp DNA product was amplified by those primers from avian adenoviruses but not from the mammalian adenoviruses. So those primers can not use to differentiate EDS virus from other avian adenoviruses (Xie *et al* 1999).

Table 1. Comparison PCR assay and HA test results for detection of EDS virus.

Dilution of allantoic fluid before DNA extraction	HA titer	PCR result
1 (pure)	17	+
1/2	15	+
1/4	14	+
1/8	13	+
1/16	12	+
1/32	12	+
1/64	10	+
1/128	9	+
1/256	7	+
1/512	6	+
1/1000	5	+
1/2000	4	+
1/4000	3	+
1/8000	2	+
1/16000	0	+
1/32000	0	+
1/64000	0	+
1/128000	0	+
1/256000	0	+
1/512000	0	+

Before this study there was not any report of using PCR assay for detection of EDS virus, in Iran. In this study we used H5/H6 primers for PCR assay. The EDS virus specific PCR set up in this study is a rapid, sensitive, and specific test to detect two different strains of EDS virus. According to other works (Raue & Hess 1998, Dhinakar Raj *et al*) the primers used in this study can detect all different strains of EDS virus but do not detect any of other fowl adenoviruses. The need for this kind of test for laboratory diagnosis of EDS has long been recognized.

Our findings clearly indicates the superiority of PCR assay over HA test for detection of EDS virus in allantoic fluids (AFs). This Study showed PCR could detect EDS virus in highly diluted AFs that HA test can not detect virus particles. In addition, HA test needs confirmation methods but PCR is specific and it does not need to use specific antisera. PCR has many benefits like high sensitivity and high specificity, it needs a short time in comparison with isolation method, and it does not need the alive virus to amplification. In order to facilitate the virus isolation, it is recommended that boiled aliquoted tissue homogenates/swab extracts from suspected cases use directly in PCR test for EDS virus genome detection. If they are found to be negative they can be passaged once in embryonated duck eggs followed by PCR of the allantoic fluids before a negative result is established.

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