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SEQUENCE ANALYSIS OF HEXON GENE OF THE INDIAN ISOLATES OF EDS-76 VIRUS OF CHICKEN AND JAPANESE QUAILS (*COTURNIX COTURNIX JAPONICA*)

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Abstract: The hexon genes of Indian isolates of EDS-76 virus, comprising of two chicken isolates (CEDS-1& CEDS-2) and one quail isolate (QEDS) and the reference strain BC-14 were amplified, targeting the variable region (L1 to L4). The 1901bp PCR product was cloned and sequenced. The nucleotide and the amino acid sequences were analysed in comparison to the published sequences of the European EDS-76 isolate (EEU) and Chinese isolate of quail origin (QEC), which revealed the differences of 0.1% in nucleotide and 0.2% in amino acid sequences existing amongst the Indian isolates and with that of the reference strain BC-14. The CEDS-1 was showing more variations from other two Indian isolates (CEDS-2 and QEDS) and the reference strain that is 0.4% in nucleotide and 0.6% in amino acid sequences. The Indian isolates and the BC-14 strain had shown slight variation from that of the European chicken isolates and showing more than 5% variation from Chinese quail isolate. The BC-14 and EEU viruses were found of the same origin with 99.9% similarity. All the Indian EDS-76 virus isolates including the quail isolate had the similar lineage where as the Chinese quail isolate had a separate unique lineage as observed by Phylogenetic studies.
Keywords: Chicken EDS-76 virus, Quail EDS-76 virus hexon gene, sequencing Phylogenetic analysis.

Abbreviations: EDS-76 - Egg drop syndrome-76
CEDS – EDS-76 virus of chicken origin
QEDS- EDS-76 virus of quail origin
QEC- Chinese isolate of EDS-76 virus of quail origin
EEU- EDS-76 virus of European origin

Introduction

Egg drop syndrome-1976 (EDS-76) is a disease of chicken and quail, characterized by sudden drop in egg production of around 30% [1] (McFerran and Adair, 2003), with deterioration of the external and internal quality of eggs. The disease was first reported in Netherlands in 1973-74 [2](Van Eck, 1976). A haemagglutinating Aviadenovirus was isolated from the infected broiler breeder flocks showing the symptoms of egg drop syndrome [3] (McFerran, 1978). The Egg drop syndrome-76 virus was recently classified as Duck adenovirus-I of the Genus Atadenovirus [4] (Faquet *et al.*, 2005). The virus has a

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double stranded DNA of size 33.2 Kbps as its genome [5] (Hess *et al*., 1997). The virus is serologically not related to other Group-I and Group-II Fowl Adenoviruses. The EDS-76 virus isolates obtained from different parts of the world belong to a single serotype but they are classified into 3 genotypes based on the RE analysis of the whole DNA genome of the virus [6] (Todd *et al*., 1988). The restriction endonuclease analysis of the viral genome of the Indian isolates was done earlier by many workers [7,8](Dash, 1999 and Senthil Kumar *et al*., 2004) to observe the differences in restriction fragment length polymorphism (RFLP) pattern existing in the Indian isolates in comparison to the reference strain BC-14. But the genomic characterization leading to confirmation of taxonomic classification and to pinpoint the exact differences existing between the isolates can be done more accurately and precisely by nucleotide sequencing in comparison to RFLP.

The EDS-76 virus genome consists of several genes, encoding for structural and non-structural polypeptides and several unassigned open reading frames. Among these genes, the hexon gene encodes the hexon protein of 126 KDa is the major capsid protein of the virus and also one of the important immunogenic proteins carrying the group and type specific epitopes. The hexon protein consists of highly variable regions called as loops L1 to L4, which form the surface of the virus and two pedestral regions, P1 and P2 [5] (Hess *et al*., 1997). Therefore in the present study Sequence analysis of the variable region of the hexon gene was carried out in three Indian EDS-76 virus isolates of chicken and quail origin obtained from different geographical locations, along with the reference strain, BC-14 for necessary comparison. Further, the relationship existing between the EDS-76 virus isolates was derived by Phylogenetic analysis.

Materials and Methods

EDS-76 virus: Indian isolates of EDS-76 virus of chicken origin (CEDS-1 and CEDS-2), quail origin (QEDS) isolated from the poultry flocks of the organized poultry farms at different regions of India and the reference strain BC-14 mostly used as a vaccine strain obtained in the form of freeze dried infected duck embryo allantoic fluid from the virology laboratory of Avian diseases section, IVRI, Izatnagar, India, were used in this study. All the four isolates of EDS-76 virus were propagated in chicken embryo liver cell culture (CEL), according to the method of Adair *et al*., (1979) [9]. When the infected cell culture was showing 70% CPE, viral DNA was extracted by phenol chloroform isoamyl alcohol method.

PCR of EDS-76 viral DNA: PCR was done to amplify 1901 bp variable region of hexon gene using the published primer pair H5/H6 [10] (Raue and Hess, 1998). After confirming for

the size of the PCR product by agarose gel electrophoresis, the PCR products were concentrated and purified using gel extraction kit (Quiagen, Germany) for further use in cloning.

Cloning and Sequencing: The variable regions of the hexon gene of all the four virus isolates were ligated into pTZ57R/T plasmid vector having ampicillin resistant gene and gene for lactose metabolism. The recombinant plasmids were transfected into DH5α strain of *E coli* and grown in LB agar containing ampicillin. Recombinant clones, which appeared as white colonies, were selected and their plasmids were extracted by using plasmid extraction kit (Quiagen, Germany). PCR of the extracted plasmid was done using H5/H6 primers and the PCR products were observed after electrophoresis in 1.25% agarose gel containing ethidium bromide. Double digestion of the recombinant plasmid was carried out with the Restriction endonucleases *Eco* RI and *Sal* I followed by gel electrophoresis in 1.25% agarose to confirm the presence of the insert in the transformed plasmid. The recombinant plasmids were used to sequence the hexon genes of four EDS-76 virus isolates using the M13 forward primer, by primer walking method using the facilities available at Delhi University.

Sequence alignment and Phylogenetic analysis: The nucleotide sequences of variable regions of hexon genes were translated to amino acid sequences by using the EditSeq programme. The sequence of EEU with the accession number Y09598 [11] (Hess *et al*., 2005) and that of QEC with the accession number EF 093507 [12] (Huang *et al*., 2006) obtained from ncbi GenBank were also used for comparison. The DNA and deduced amino acid sequences were aligned by clustal method of Megalign program of DNA STAR software to establish the relationship existing amongst these Indian isolates and reference strains of EDS-76 virus.

Results and Discussion

The growth of EDS -76 virus isolates in the CEL cell culture produced cytopathic effects from 4th day post infection, which was characterized by the appearance of enlarged, round and refractile cells and sloughing off of some dead cells leaving empty spaces. The DNA extracted had a purity of OD_{260}/OD_{280} ratio ranging from 1.72 – 1.8 and concentration ranging from 0.37-0.48µg/ml. PCR amplification of extracted DNA produced a uniform product for each viral sample of 1901bp (Fig 1.) having the variable regions of the hexon gene carrying the loops L1 to L4. These findings were similar as observed by Raue and Hess (1998) [10]. After cloning, the presence of the insert was confirmed by PCR of the extracted plasmids, which showed an amplification product of 1901 bp. It was further confirmed by

double digestion of the plasmid DNA with *Eco*RI and *Sal*I to get a 1901 bp fragment of the hexon gene as the insert (Fig2).

The nucleotide and amino acid sequences of all the three Indian isolates along with BC-14 were compared with the EEU and the QEC. The variation in number of nucleotides and amino acids between various isolates are shown in the table 1 and 2 respectively. The nucleotide sequences of BC-14, CEDS-1, CEDS-2, and QEDS were published in the GenBank with accession numbers EU109793, EU109794, EU109795 and EU109796 respectively.

The percentage similarity and difference between nucleotide and amino acid sequences of different isolates are presented in Fig.3 and 4. The CEDS-1 carries the maximum variation in DNA sequences with that of the European isolate (0.3%), while CEDS-2 and QEDS have 0.1% each. In the amino acid sequence also CEDS-1 had maximum difference (0.5%), where as CEDS-2 and QEDS differed by 0.2% from European isolates. The CEDS-1 had the maximum variation in nucleotide sequence from QEC (5.5%), while CEDS-2 and QEDS differed from QEC by 5.2% and in case of amino acid sequence the CEDS-1 varied from the QEC by 1.3%, while the CEDS-2 and QEDS differed by 1% each. Earlier workers have reported a maximum of 4.6% difference among hexon gene sequence of different Indian EDS-76 virus isolates of chicken origin [13].

On the basis of the Phylogenetic analysis based on nucleotide and amino acid sequences, the Indian and European isolates of EDS –76 Virus formed a cluster where as the Chinese isolate came under another cluster. Since there is identity of 99.6% -99.9% between the Indian and European isolates this can be concluded that all the Indian EDS-76 viruses isolated from chicken and quail are originated from European virus. The Phylogenetic tree based on nucleotide and amino acid sequences are shown in the fig. 5 and 6 respectively. The Phylogenetic tree reveals that the Indian and European isolates have a common lineage but the Chinese quail EDS-76 Virus isolate had a separate unique lineage.

The presence of more number of changes in the DNA sequences of CEDS-1 compared to other Indian isolates of EDS-76 virus may be due to the result of point mutations. It may be assumed that the CEDS-1 might have affected Indian chicken flocks earlier than CEDS-2 and QEDS, leading to more changes of nucleotides of hexon gene and corresponding changes in amino acids. From the sequence analysis of hexon genes it was revealed that EDS-76 virus from quail origin showed highest degree of similarity with that of CEDS-2 indicating that QEDS might have originated from CEDS-2 as both isolates belong to

the same geographical region of India. It may be possible that the commercial layer chicken flocks are subjected to vaccination which may cause the virus to undergo mutation in the presence of antibodies to adapt themselves in the host for its long existence as the virus is vertically transmitted. This hypothesis is further supported with the evidence, that the vaccination against EDS-76 virus was being adapted in the poultry flocks, considerably for a longer period, in the region of India, from where the CEDS-1 was isolated. But the vaccination of the chicken against EDS-76 virus was recently adopted in the region from where CEDS-2 was isolated. But further studies on the sequence analysis of the hexon gene of more number of isolates obtained at different period of time should be done to confirm the presence of changes in the nucleotides and corresponding amino acids, which may be considered as Single Nucleotide Polymorphisms (SNP) being inherited in these isolates. Similar sequence analysis of the hexon or penton genes of the EDS-76 virus isolates of Indian origin need to be carried out at regular intervals to ascertain the changes occurring in their nucleotide and amino acid sequences and to be compared to that of the vaccine strain, so that the vaccine failure due to mutations at vital genes transcribing immunogenic proteins can be avoided easily. It will further facilitate the monitoring of the EDS-76 virus infection at molecular level in India. As this study has established the existence of a high rate of homology in the hexon genes of different Indian isolates as the hexon gene transcribed the hexon protein having neutralizing epitopes, all the Indian isolates of chicken and quail origin may be presumed as antigenically related.

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Table 1. Variation in the Number of nucleotides among different isolates of EDS-76 virus

	CEDS-1	CEDS-2	QEDS	BC-14	EEU	QEC
CEDS-1	----------	7	7	7	6	100
CEDS-2		------------	2	2	1	95
QEDS			-----------	2	1	95
BC-14				-----------	1	95
EEU					-----------	94
QEC						-----------

Table 2. Variation in number of amino acids among different isolates of EDS-76 virus

	CEDS-1	CEDS-2	QEDS	BC-14	EEU	QEC
CEDS-1	----------	4	4	4	3	8
CEDS-2		------------	2	2	1	6
QEDS			-----------	2	1	6
BC-14				-----------	1	6
EEU					-----------	6
QEC						-----------

Fig. 1. PCR amplified product of hexon gene.

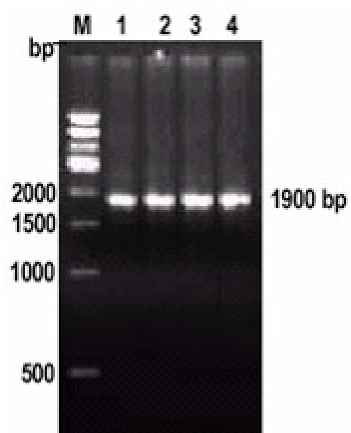
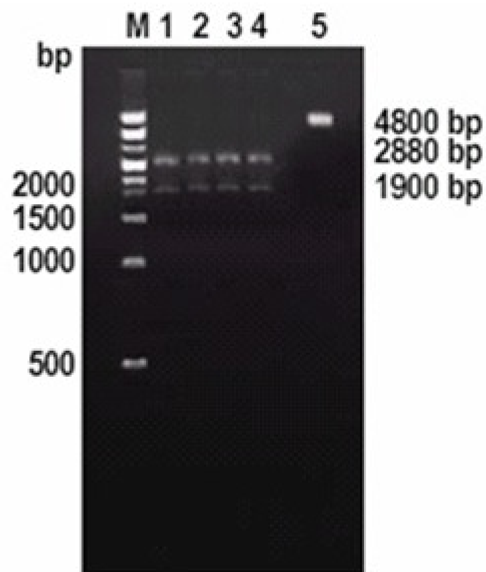


Fig. 2. DNA Fragments after double digestion of recombinant plasmid DNA



Double digestion of recombinant plasmid DNA with *Eco*RI and *Sal*I

Fig. 3: Sequence pair distance between nucleotide sequence of EDS-76 isolates

Percent Similarity (upper triangle); Percent Divergence (lower triangle)

	1	2	3	4	5	6	
1		99.7	99.9	99.9	99.9	95.0	1
2	0.3		99.6	99.6	99.6	94.6	2
3	0.1	0.4		99.9	99.9	94.9	3
4	0.1	0.4	0.1		99.9	94.9	4
5	0.1	0.4	0.1	0.1		94.9	5
6	5.1	5.5	5.2	5.2	5.2		6
	1	2	3	4	5	6	

Fig. 4 Sequence pair distance between amino acid sequence of EDS-76 isolates

Percent Similarity (upper triangle) / Percent Divergence (lower triangle)

	1	2	3	4	5	6	
1		99.5	99.8	99.8	99.8	99.2	1
2	0.5		99.4	99.4	99.4	98.7	2
3	0.2	0.6		99.7	99.7	99.1	3
4	0.2	0.6	0.3		99.7	99.1	4
5	0.2	0.6	0.3	0.3		99.1	5
6	0.8	1.3	1.0	1.0	1.0		6
	1	2	3	4	5	6	

Fig. 5 Phylogenic tree based on nucleotide sequence of EDS-76 isolates

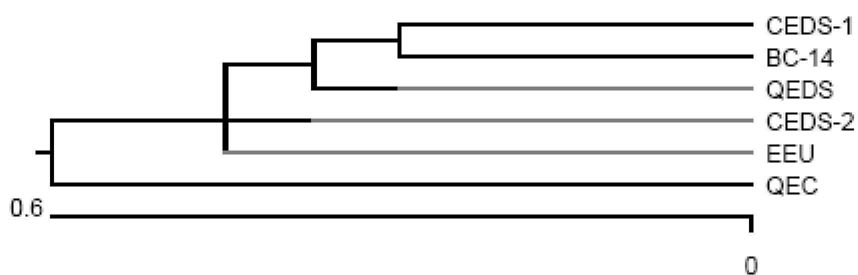


Fig. 6 Phylogenic tree based on amino acid sequence of EDS-76 isolates