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Detection and Molecular Characterization of Avian Encephalomyelitis Virus in India: A Preliminary Report

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Tremovirus A, previously recognized as Avian encephalomyelitis virus (AEV) is, one of the viruses that have been reported to be associated with nervous and enteric disorders in young birds. In Kerala, there has been an increase in the cases of nervous and enteric diseases in chickens. The detection and characterization of other viruses associated with nervous and enteric viruses in chickens has been established in Kerala. While there have been reports of nervous and enteric diseases in chickens in the region, no previous research had focused on AEV as the causative agent. A total of 50 samples were tested to detect AEV by reverse-transcriptase polymerase chain reaction (RT-PCR) targeting the 5' untranslated region (5'UTR) of AEV. Of the samples tested, one (2%) was found to be positive for the virus. The most immunogenic protein of AEV is VP1 protein forming the major capsid protein. The VP1gene segment of the detected virus isolate was amplified and molecular characterization was done. The phylogenetic analysis of the VP1 gene sequence revealed that the virus was identical to AEV viruses detected in Hungary, China, and Hong Kong (64% similarity). The current isolate showed a nonsynonymous mutation of I184V which was noticed only in Hungary isolate. The results of the study indicate that AEV is rarely present in chickens of Kerala, India, and that its occurrence is very low. Since the virus is capable of inducing nervous signs, mortality, and economic losses, measures are to be taken to control the spread of the virus in chickens in Kerala, India.

Keywords: Avian encephalomyelitis virus; 5'UTR; VP1; polymerase chain reaction; phylogeny.

1. INTRODUCTION

Avian encephalomyelitisvirus (AEV) is a significant viral pathogen affecting mainly young birds of chicken, pheasant, turkey, and quail between 1 or 2 weeks of age. It is a single-stranded, non-enveloped, positive stranded RNA virus coming under *Tremovirus* genus of the *Picornaviridae* family [1]. The route of transmission in poultry is through the fecal-oral route, and it has the potential to be transmitted to embryos, resulting in distinctive neurological symptoms [2]. The symptoms include ataxia and rapid tremors, especially of head and neck region, giving rise to its colloquial name, "epidemic tremor." Avian encephalomyelitis (AE) was initially detected in the United States in 1932. The viral particle has a diameter ranging from 24 to 32 nm. Its icosahedral shape is created by 32-42 capsomeres [3]. The genome, which is 7 kb nucleotides in length, includes a 5′-untranslated region (5′-UTR) followed by an extensive open reading frame responsible for encoding a large polyprotein P1, P2, and P3. The P1 region encodes four structural proteins: VP4, VP2 (VP0), VP3, and VP1. Meanwhile, the P2 and P3 regions are responsible for encoding non-structural proteins 2A, 2B, 2C, and 3A, 3B, 3C, and 3D [4].

The coat proteins of AEV, namely VP1 and VP2 encoded by the P1 region, serve as the principal antigen proteins. Prokaryotic expression and activity assessment of AEV's coat protein VP1 demonstrated its main antigenic activity, indicating its potential for the development of subunit vaccines [5].

There exist two strains of Tremovirus A (AEV): a natural enterotropic strain and a highly neurotropic embryo-adapted strain known as the Van Roekel strain, which is highly neurophilic [6]. The enterotropic strain spreads horizontally through the oral route and is disseminated through feces [7]. This particular Van Roekel strain induces neurological issues in vulnerable chicks but remains nearly asymptomatic in birds aged 3-4 weeks or older. However, it can lead to a decline in egg production among breeders and laying hens, along with the potential for vertical transmission through eggs [8]. The embryo-adapted strain is generated through repeated passages via intracerebral inoculation in chicks or inoculation in embryonated eggs by yolk-sac route [9]. These adapted strains elicit severe neurological changes in birds of all ages after intracerebral or parenteral inoculation. Notably, the ability for fecal transmission is lost after oral ingestion due to the virus's incapacity to multiply in the digestive cells of birds [10]. Enteric strains are transmitted horizontally through feces and vertically through infected embryonated eggs in natural conditions [11]. The duration of virus excretion is age-dependent, with birds under two weeks typically excreting the virus for two weeks, while those over three weeks excrete it for about five days [12]. Vertical transmission takes place when breeders, lacking immunological activation against the virus, get infected during the laying period and subsequently transmit the virus to

their offspring [13]. Avian encephalomyelitis is present in almost every part of the world. Almost all chicken flocks become infected with the virus [9]. This disease has been recorded among European countries like England, France, Brion, Denmark, Sweden, the Netherlands, Germany, and Austria also reported in Australia [14].

In recent years, Kerala has witnessed a rise in nervous and enteric disorders, leading to a decline in chicken egg production and an increase in mortality. Therefore, this study reports the first molecular detection and characterization of AEV from Kerala.

2. METHODOLOGY

2.1 Sample Collection

A Total of 50 suspected tissue samples such as brain, sciatic nerve and intestinal tissue samples were collected from poultry flocks that showed nervous signs with mortality in chicks, reduced egg production in adults and enteric diseases from different districts of Kerala. Cloacal swabs were collected from the live birds which showed diarrhoea and reduced egg production. The samples were collected in viral transport medium, transported on ice and kept at -80°C until they underwent additional processing.

2.2 RNA Extraction

Total RNA was extracted from the tissue samples and cloacal swabs using a GeneJET RNA purification kit (Thermo Scientific, USA), as per the manufacturer's protocol. The extracted RNA was stored at -80 °C until further processing. cDNA was carried out from the extracted RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol.

2.3 Amplification by RT-PCR

The AEV genome in the clinical samples was detected by using RT-PCR using a published primer (Table 1) targeting a 286 bp fragment of 5'UTR [15]. The PCR reaction mixture consists of 12.5 µL 2X EmeraldAmp GT PCR master mix (2X) from Takara, 1 µL forward and reverse primers (10 pmol), 2 µL of cDNA, and the remaining volume made up by NFW to 25 µL. The cycling conditions involves initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and polymerization at 72°C for 30 seconds. The final extension step was performed at 72°C for 10 minutes [16].

2.4 Primer Designing

For the amplification of the VP1 gene of AEV, primer designing was carried out, partial P1 region of 1066bp that contains the most immunogenic part VP1 was designed. The complete genome of AEV, comprising 7040 bp nucleotides from foreign isolates (KF979338.1, AY517471.1, KT880668.1, NC003990.1, MF179107.1, and AJ225173.1) present in GenBank, were retrieved and aligned using MEGAX. The VP1 region has a total size of 843 bp. For the amplification of this region, a forward primer with 20 nucleotides and three degenerations, targeting positions 1839 to 1858 in the entire genome binding at the VP3 gene region was designed. A reverse primer with 20 nucleotides was designed to bind to positions 2886 to 2905 in the complete genome binding at 2A region of the virus. The primers we designed generate a product with a size of 1066 bp. The PCR reaction mixture, includes 12.5 µL of EmeraldAmp PCR master mix (2x), 1 µL of each primer set (10 pmol each), 2 µL of cDNA (500 ng), and NFW to reach a total volume of 25 µL. The PCR conditions were standardized: an initial denaturation at 95 ºC for 3 min, followed by 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 53 ºC for 1 min, and extension at 72 ºC for 1.5 min. After 35 cycles, a final extension was performed at 72 ºC for 5 min. The live vaccine of AEV sourced from Venkateswara Hatcheries Private Limited served as a positive control and was employed for the standardization of PCR protocols.

2.5 Sequencing and Phylogenetic Analysis

The PCR products were separated using 1.25% agarose gels and observed using a gel documentation system with UV light. The distinctive amplicons from the 5'UTR and VP1 gene amplification PCRs were purified using a GeneJET gel extraction kit (Thermo Scientific, USA) and sent to an external service for Sanger dideoxy chain termination sequencing. To verify the presence of AEV-specific sequences and compare them with existing AEV sequences in the GenBank database, BLAST analysis was conducted (http://www.ncbi.nlm.nih.gov/Blast). The obtained sequences were submitted to NCBI GenBank, and accession numbers (5'UTR - PP236062, VP1 gene - PP236063) were acquired.

Table 1. Primer details for AEV RT-PCR

Sl. No.	Gene	Primer	Sequence	Product size (bp)	References
1	AEM 5'UTR	AEM 5'UTR FP	5' -CTTTGCGTTTCACAGAACCATCC-3'	286	Goto et al. [15]
2		AEM 5'UTR RP	5' -AAATGCTACCCTTAATCTCTC-3'		
3	VP1 gene	AEV VP1 FP	5'- GCRCCTGCMAGTGTWTCACC-3'	1066	Designed in this study
4		AEV VP1 RP	5'- TCNACATGTACAACACCATC -3'		

The MEGAX software was employed for phylogenetic analysis of a 286-nucleotide sequence from the 5'UTR and an 843-nucleotide sequence of the full VP1 gene of the present isolate. Alignment of the 5'UTR and VP1 sequences, obtained in this study and those retrieved from the NCBI database (Supplementary Tables 2 and 3), was conducted using the Clustal W program within MEGA X. The evolutionary history was determined through the Maximum Likelihood method, and a bootstrap consensus tree from 1000 replicates was generated to illustrate the evolutionary relationships among the analyzed sequences. Evolutionary distances were determined through the application of the Hasegawa-Kishino-Yano method for the 5'UTR of AEV, and the Tamura 3-parameter with gamma distribution with invariant sites method was employed for the VP1 gene of AEV for inferring the phylogenetic tree, chosen based on the lowest Bayesian Information Criterion scores (BIC).

3. RESULTS AND DISCUSSION

3.1 Molecular Detection

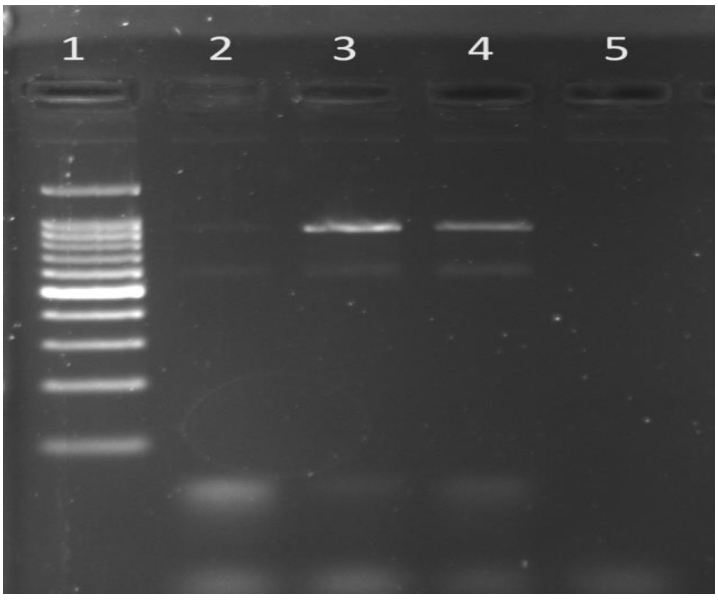
Of the 50 samples tested for the detection of AEV, one sample was positive in both brain and intestinal tissue for the virus targeting the 5'UTR. Specific amplicons of 286 bp were observed in the agarose gels. No amplification was observed in the negative control (Fig. 1). The positivity rate of AEV was determined to be 2 percent. The positive sample was used to amplify the VP1 gene of AEV and obtained a product size of 1066bp (Fig. 2). This study is the first detection and molecular characterization of AEV from India. Avian encephalomyelitis virus was identified in chickens of Japan through RT-PCR and observed a highly conserved sequence in the 5'UTR and developed novel PCR primers based on this conserved region for AEV detection [15] and the study successfully generated PCR products of the expected size of around 286 bp using the newly designed primers from eight clinical samples, six AEV isolates, and AEV vaccine strains [15]. In China RT-PCR was developed for the detection of AEV based on the VP2 gene of the structural protein P1 region of the AEV genome [17].

3.2 Sequencing

The two AEV positive amplicons were sent for sequencing after purification using GeneJET Gel Extraction Kit (Thermo Scientific USA) to GeneSpec Private Limited, Kochi. The nucleotide sequences of these isolates were confirmed to be that of AEV by sequencing and blast analysis and were submitted to NCBI GenBank and accession numbers were obtained. The accession numbers obtained were PP236062 and PP236063 for 5'UTR and VP1 gene respectively of the sample 394/MIB/2022. On BLAST analysis, the 5'UTR sequence obtained from sample 394/MIB/2022 (PP236062) revealed 97.90, 95.79, 94.76, and 94.41 % identity with AEV sequences from Hungary, China, Hong Kong, and Calnek Vaccine strain. The VP1 gene sequence (PP236063) was similar to sequences from Hungary (90.04 % identity), and China (86.14% identity). Details given in supplementary Table 3. The prevalence of AEV antibodies varied across different regions, with rates of 79% observed in Brazil [18], ranging from 45% to 91% in Bangladesh [19], 79.35% in the Tamil Nadu state of India [20], 14.6% in Saudi Arabia [21], and 57.1% in Sudan [22]. Avian Encephalomyelitis is a condition impacting the nervous system of young birds, leading to ataxia, weakness, and tremors in the head and neck [9]. This results in economic loss for poultry farmers as affected birds experience reduced food consumption and, consequently, a decline in meat conversion [7]. The highest vulnerability to this virus and the manifestation of clinical signs is observed during the initial two weeks of a bird's life [6], aligning with our findings that detected positive results in 7-day-old chicks. Nervous signs and diarrhea were some of the clinical signs observed in chicken flocks in which the virus was detected in this study.

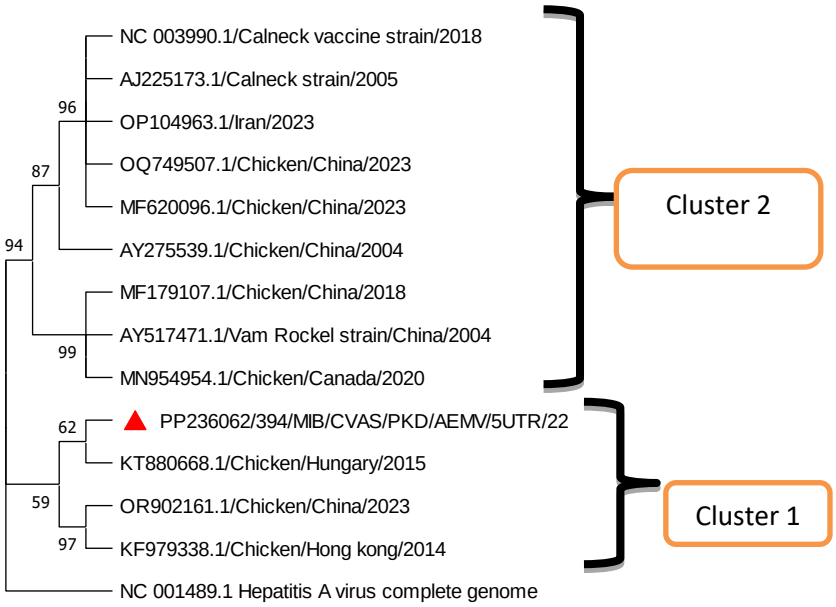
Lane 1 : 100 bp ladder
Lane 2 : Clinical sample (285 bp PCR amplicons)
Lane 3 : Positive control (VHL AE live vaccine)
Lane 4 : No template control

Fig. 1. Agarose gel showing 286 bp amplicons of partial 5'UTR of AEV



Lane 1 : 100 bp ladder
Lane 2,3 : Clinical sample (1066 bp PCR amplicons)
Lane 4 : Positive control (VHL AE live vaccine)
Lane 5 : No template control

Fig. 2. Agarose gel showing 1066 bp amplicons of partial VP1 of AEV



The symbols 'filled red triangle' depicts nucleotide sequence generated during this study. Boot strap replicates = 1000. Evolutionary distances between sequences were calculated by the Hasegawa-Kishino-Yano model.

Fig. 3. Maximum Likelihood tree constructed from nucleotide sequences of partial 5'UTR sequence of AEV

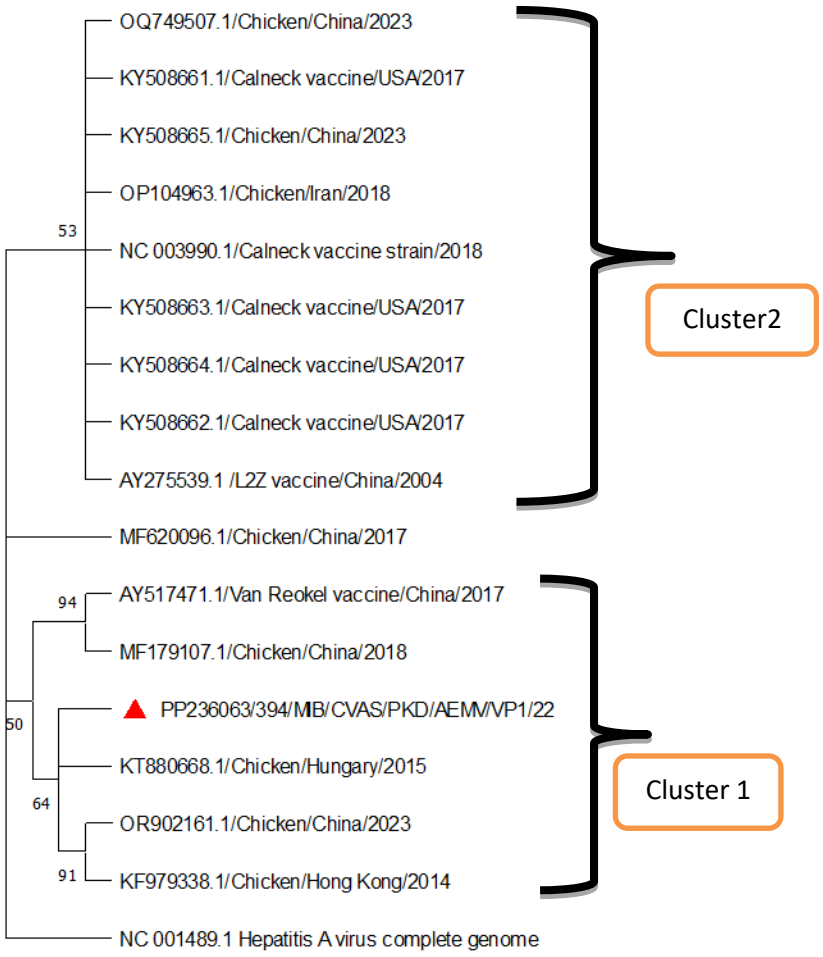
3.3 Phylogenetic Analysis

In the phylogenetic analysis of 286 nucleotides of the 5'UTR sequence, it was observed that the two major clusters were noticed. Cluster 2 consists of vaccine strains and isolates from Canada, the USA, and China. Isolates from Hungary, China, and Hong Kong were grouped into C1. The present isolate (PP236062) is grouped along with the isolate from Hungary, China, and Hong Kong under C1 (Fig. 3). In the phylogenetic analysis of AEV based on the VP1 gene, the present isolate (PP236063) is grouped along with the isolates from Hungary, China, and Hong Kong isolates (Fig. 4).

3.4 Amino Acid Analysis

The complete amino acid sequence of the VP1 protein of the current isolate, which consists of 281 amino acids was compared with other foreign isolates and the results revealed a 98.84% similarity to the Calnek vaccine strain (NC 003990), 99.13% to Hungary isolate (KT880668), 98.26% to the Chinese isolate (OR902161), and 98.26% to the Hong Kong isolate (KF979338). There was one nonsynonymous mutation and 121 synonymous mutations were observed when compared with the Calnek vaccine strain (Accession number NC003990). The one nonsynonymous mutation observed was I184V. Isoleucine was replaced by Valine. Valine is present only in the current isolate and Hungary isolate which was not present in any of the reference isolates. Details given in supplementary Table 4.

The primary indication of Avian Encephalomyelitis involves a tremor in the head and neck resulting from a nervous system disorder. Consequently, routine testing of these nervous organs is conducted for disease diagnosis. In this study, the virus was identified not only in the brains and sciatic nerves but also in the intestinal tissue. Hence, beside from the nervous system, the digestive tissue also can be targeted in diagnosing AEV. This aligns with the findings of [9], as the study revealed the presence of AEV in both nervous and digestive tissues, suggesting the importance of detecting the virus in these organs during Avian Encephalomyelitis outbreaks. Although infections by AEV may not manifest clinical signs that directly affect the health of adult birds, they cause an economic risk for poultry farmers. This risk includes decreased egg production [11], along with reductions in the hatchability and increased embryonic mortality in cases of



The symbols 'filled red triangle' depicts nucleotide sequence generated during this study. Boot strap replicates = 1000. Evolutionary distances between sequences were calculated by the Tamura 3-parameter with gamma distribution with invariant sites model.

Fig. 4. Maximum Likelihood tree constructed from complete VP1 sequence of AEV

breeder infections [23]. Additionally, it is important to consider that offspring from infected breeders may exhibit neurological symptoms associated with Avian Encephalomyelitis during the early weeks of life [8]. This study is the first detection and molecular characterization of AEV from India and this study could detect the presence of AEV in samples from chicks that have died of neurological symptoms in Kerala, India and the occurrence of the virus is very rare. The virus was found both in the nervous and intestinal tissues. Since the virus is reported to have neurological signs and due to the possibility of coinfection with other enteric viruses, steps have to be taken to prevent the virus from spreading in the poultry population of the State.

4. CONCLUSION

The results of the study indicate that AEV is rarely present in chickens of Kerala, India, and that its occurrence is very low. Since the virus is capable of inducing nervous signs, mortality, and economic losses, measures are to be taken to control the spread of the virus in chickens in Kerala, India.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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SUPPLEMENTARY

Supplementary Table 1. Details of isolates of AEV downloaded from GenBank for Phylogenetic analysis of 5ˈUTR

S. No	Accession No.	Host	Country	Isolation Year
1.	KT880668.1	Chicken	Hungary	2015
2.	OR902161.1	Chicken	China	2023
3.	KF979338.1	Chicken	Hong Kong	2014
4.	MF620096.1	Chicken	China	2023
5.	MF179107.1	Chicken	China	2018
6.	OQ749507.1	Chicken	China	2023
7.	OP104963.1	Chicken	Iran	2023
8.	AY517471.1	Chicken	China	2004
9.	NC_003990.1	Chicken	China	2018
10.	MN954954.1	Chicken	Canada	2020
11.	AY275539.1	Chicken	China	2004
12.	AJ225173.1	Chicken	USA	2005

Supplementary Table 2. Details of isolates of AEV downloaded from GenBank or Phylogenetic analysis of VP1 gene

S. No	Accession No.	Host	Country	Isolation Year
1.	KT880668.1	Chicken	Hungary	2015
2.	OR902161.1	Chicken	China	2023
3.	KF979338.1	Chicken	Hong Kong	2014
4.	MF620096.1	Chicken	China	2023
5.	MF179107.1	Chicken	China	2018
6.	OQ749507.1	Chicken	China	2023
7.	OP104963.1	Chicken	Iran	2018
8.	AY517471.1	Chicken	China	2004
9.	NC_003990.1	Chicken	China	2018
10.	MN954954.1	Chicken	Canada	2020
11.	AY275539.1	Chicken	China	2004
12.	AJ225173.1	Chicken	USA	2005
13.	KY508663.1	Chicken	USA	2017
14.	OR902161.1	Chicken	China	2023
15.	KY508664.1	Chicken	USA	2017
16.	KY508662.1	Chicken	USA	2017
17.	KY508665.1	Chicken	China	2023
18.	KY508665.1	Chicken	China	2023

Supplementary Table 3. Details of genetic variations that occurred in VP1 gene of AEV in comparison with the Calnek vaccine strain

Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV		Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV	
9	G	A	Transition	205	C	T	Transition
12	T	C	Transition	207	G	A	Transition
24	T	C	Transition	210	T	C	Transition
36	T	G	Transversion	216	C	T	Transition
48	A	G	Transition	228	C	A	Transversion
57	T	G	Transversion	231	T	C	Transition
66	G	A	Transition	243	A	T	Transversion
69	A	G	Transition	246	T	C	Transition
81	T	A	Transversion	249	A	G	Transition
82	T	C	Transition	258	A	G	Transition
84	G	A	Transition	261	T	C	Transition
87	C	T	Transition	276	A	C	Transversion
96	T	C	Transition	282	G	A	Transition
102	C	T	Transition	285	A	C	Transversion
111	A	G	Transition	288	A	G	Transition
126	C	T	Transition	289	T	C	Transition
135	C	G	Transversion	291	A	G	Transition
144	A	G	Transition	297	C	A	Transversion
153	A	G	Transition	300	T	C	Transversion
159	T	A	Transversion	303	A	G	Transition
162	A	G	Transition	306	G	A	Transition
165	A	G	Transition	315	G	T	Transversion
168	T	A	Transversion	318	G	A	Transition
174	T	A	Transversion	321	T	G	Transversion
177	A	G	Transition	327	T	C	Transition
180	T	A	Transversion	336	T	C	Transition

Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV		Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV	
183	A	G	Transition	339	A	G	Transition
186	T	C	Transition	351	A	G	Transition
192	G	T	Transversion	360	T	G	Transversion
195	C	T	Transition	363	T	C	Transition
198	C	T	Transition	366	T	C	Transition
369	C	T	Transition	568	C	T	Transition
375	T	C	Transition	570	G	A	Transition
396	A	G	Transition	576	C	T	Transition
420	C	T	Transition	579	C	T	Transition
432	G	A	Transition	582	A	G	Transition
435	T	C	Transition	585	C	A	Transversion
441	G	A	Transition	591	T	A	Transversion
447	G	A	Transition	594	A	G	Transition
456	G	A	Transition	613	T	C	Transition
462	G	A	Transition	618	G	C	Transversion
465	G	A	Transition	621	A	G	Transition
471	T	C	Transition	627	A	G	Transition
480	A	C	Transition	630	C	T	Transition
486	C	T	Transition	636	C	A	Transversion
501	A	G	Transition	642	C	T	Transition
504	T	C	Transition	645	T	C	Transition
525	A	G	Transition	648	T	C	Transition
540	C	G	Transversion	669	G	A	Transition
543	G	A	Transition	672	T	C	Transition
549	G	A	Transition	678	T	C	Transition
550	A	G	Transition	681	T	C	Transition
552	C	T	Transition	759	C	T	Transition
558	C	T	Transition	768	G	T	Transversion

Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV		Position	Nucleotide identity with Calnek vaccine strain	394/MIB/22/VP1 /AEV	
561	C	T	Transition	774	T	C	Transition
564	G	A	Transition	786	T	C	Transition
684	C	T	Transition	789	T	C	Transition
696	A	G	Transition	795	C	T	Transition
705	G	A	Transition	804	A	G	Transition
708	T	C	Transition	807	A	G	Transition
711	T	C	Transition	810	G	A	Transition
720	A	G	Transition	816	C	G	Transition
723	G	A	Transition	822	T	C	Transition
726	G	A	Transition	825	T	C	Transition
735	C	T	Transition	828	C	T	Transition
738	C	T	Transition	831	C	T	Transition
744	C	T	Transition	835	A	C	Transversion

Supplementary Table 4. Amino acid changes of VP1 gene of AEV with other isolates

POSITION	Foreign Isolates	Calnek Vaccine NC003990	394/MIB/22/VP1/AEV
3	G/E	E	E
9	F/S	S	S
72	F/S	F	F
83	E/K	K	K
103	D/G	G	G
153	K/E	E	E
184	T/I/(V only in Hungary)	I	V
200	T/A	A	A
255	I/T	T	T

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