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E3 Gene-Based Genetic Characterization of Canine Adenovirus-2 Isolated from Cases of Canine Gastroenteritis in India Revealed a Novel Group of the Virus

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Abstract
Canine adenovirus (CAV) circulates as two distinct serotypes, CAV-1 and CAV-2, which are antigenically related but differ in their clinical manifestations. CAV is one of the important viral agents in the etiology of canine gastroenteritis. Here, we report the molecular surveillance and genetic characterization of CAV from clinical cases of canine gastroenteritis. A total of 302 fecal/rectal swabs were collected from dogs presented with gastroenteritis at various clinics in and around Hyderabad, India during 2018–19. These samples were tested for CAV using polymerase chain reaction with primers designed for the CAV *E3* gene and the virus was isolated from positive samples. CAV-2 nucleic acid was present in 4.9% of the test samples. The partial sequence analyses of the *E3* gene of the CAV-2 isolates revealed a frameshift mutation by insertion of nucleotide "G" at 1077 position of *E3* gene, which resulted in an extension of the polypeptide chain by eleven amino acids. As a result, isolates from the current study formed a novel group, and the virus that was previously subdivided into two groups worldwide is now categorized under three. The study identifies a novel group of CAV-2 circulating in India providing an updated information regarding CAV-2.

Introduction

Gastroenteritis in canines is a frequently encountered problem leading to severe dehydration and death. There is multiple etiology for the cause, and canine adenovirus (CAV) infection is one among them [1]. CAV is a non-enveloped icosahedral virus that belongs to the genus *Mastadenovirus* of the family *Adenoviridae* [1]. The size of the CAV virus is about 70–90 nm and the genome is made up of double-stranded DNA of size 36–44 kb [2]. CAV consists of five early (E) transcriptional units E1A, E1B, E2, E3, and E4, two intermediate units IX and IVa2, and five late units L1 to L5. The early expressed regions were known to be involved in viral gene expression, replication of virus, and regulation of host-cell functions.

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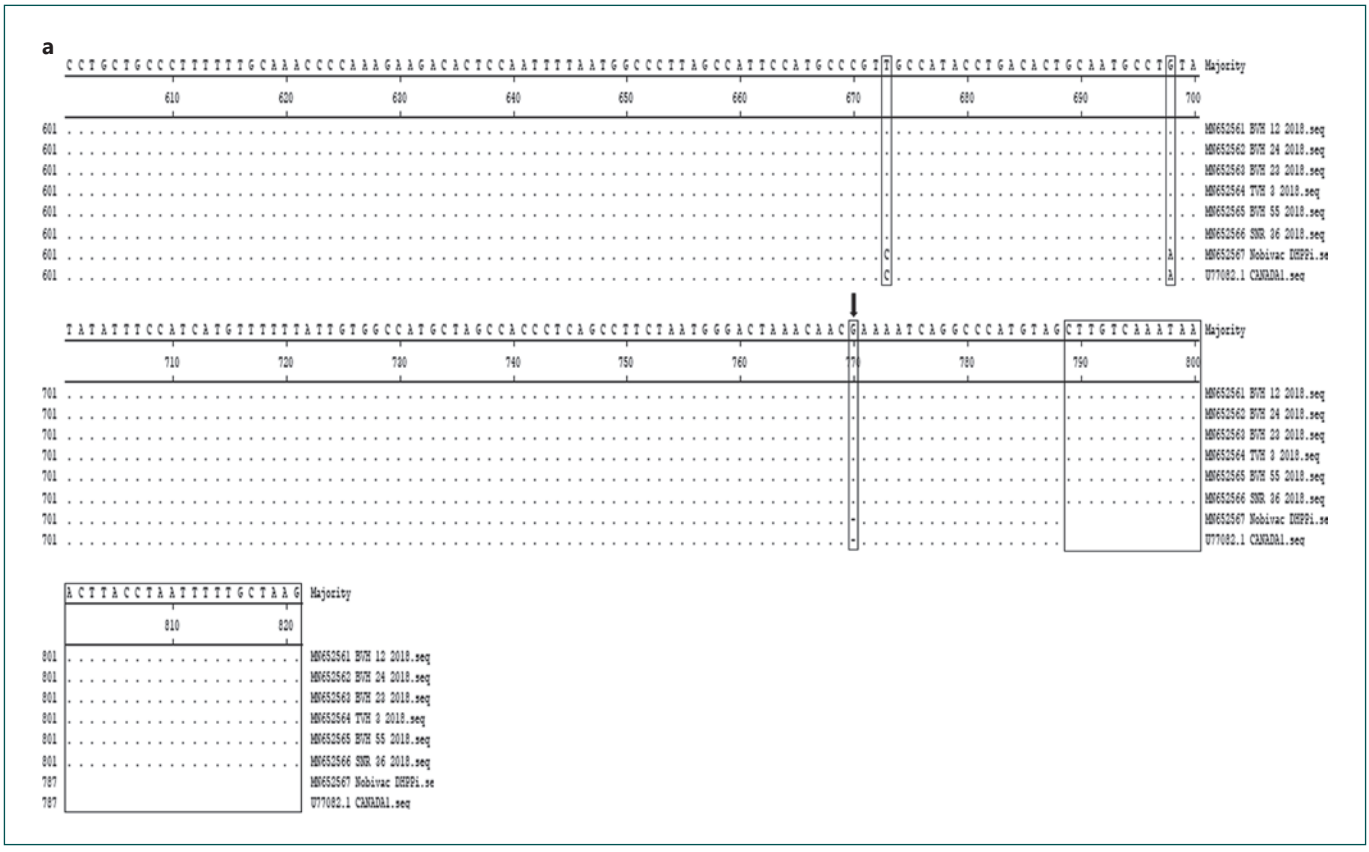


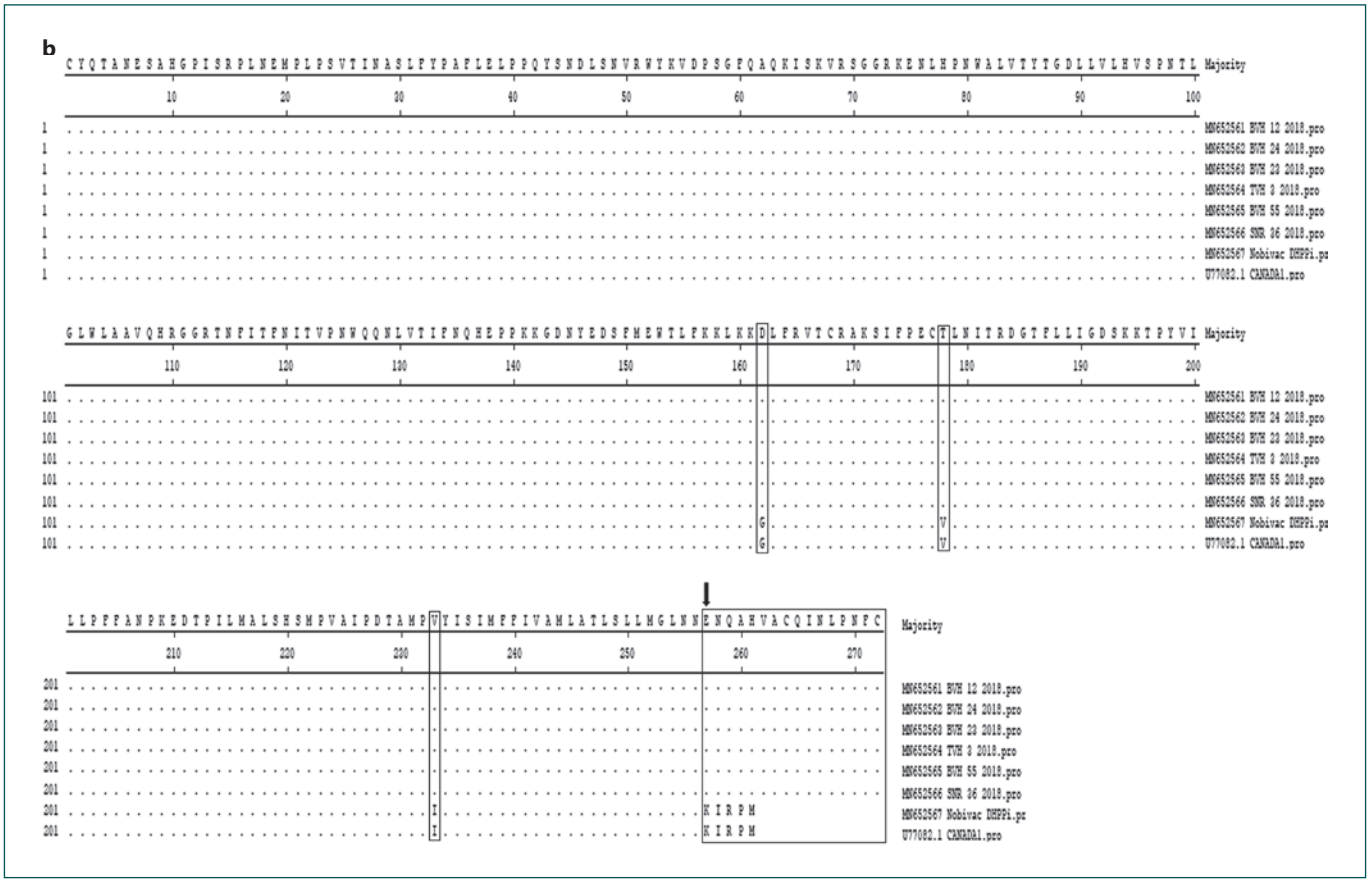
Fig. 1. Nucleotide/amino acid multiple sequence alignment of the current CAV-2 isolates against reference isolates. **a** At position 1077 bp of *E3* gene, a nucleotide insertion (indicated with black arrow) that resulted in frameshift mutation can be noticed. **b** The polypeptide chain shows amino acid variation after insertion (indicated with black arrow) and the chain was extended by 11 amino acids (shown in rectangle).

(Figure continued on next page.)

The late region encodes most of the virions [2]. E3 protein was reported to modulate the host response to adenovirus infection by interacting with immune defense mechanisms [3]. CAV circulates as two distinct serotypes, CAV-1 and CAV-2, which majorly cause infectious canine hepatitis and infectious canine laryngotracheitis, respectively, in dogs [4, 5]. CAV-1 and CAV-2 are closely related by nucleotide identity of about 75% [6, 7]. A polymerase chain reaction assay can effectively distinguish between the two [8]. There were few reports of CAV isolation from India [9, 10]; however, its molecular evolutionary patterns are not clear. The current study was aimed to isolate the circulating variants of CAV (involved in gastroenteritis) during the year 2018–19 and understand their molecular evolutionary relationship against the reference isolates from different geographical regions based on the *E3* gene. In this study, swab samples collected from dogs presenting with gastroenteritis at various clinics were tested using PCR targeting the *E3* gene region to obtain information about the possible role of CAV in enteritis, and the partial *E3* amplicons were sequenced for molecular characterization of local CAV strains.

Materials and Methods

A total of 302 fecal/rectal swabs were collected from various clinics in and around Hyderabad, Telangana, exhibiting clinical gastroenteritis. Briefly, the sample was emulsified in 1 mL of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/mL benzylpenicillin, 100 μg/mL streptomycin sulfate), centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and boiled at 96 °C for 10 min followed by chilling on ice. Then the samples were centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was used as a template for PCR. The samples were screened by real-time PCR as described before [11]. This assay amplifies a 166-bp fragment of CAV genome containing almost 47 nucleotide substitutions between CAV-1 and CAV-2 and as a result, there will be a



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difference in melting temperature (Tm) greater than 4 °C between the two viral types. Hence, the samples showing amplification with a Tm of 81 °C–84 °C on the melt curve were considered positive for CAV-2, and 74 °C–76 °C for CAV-1 [11].

The positive fecal samples/rectal swabs were propagated for virus isolation in the Madin-Darby canine kidney (MDCK) cell line. Briefly, 1 mL of the filtered fecal sample was added to the 70% confluent monolayer and kept for 1 h adsorption at 37 °C. The infectious material was replaced with fresh maintenance medium (DMEM with 1% FBS), incubated at 37 °C and observed for any rounding of cells every 24 h until 5 days. For subsequent passages, 0.1 multiplicity of infection of virus was used for infection as described above. The Nobivac DHPPi (distemper, hepatitis, parvo, parainfluenza) vaccine having CAV-2 strain is the most commonly used vaccine and hence it served as an effective positive control. For sequencing, PCR was performed targeting the open reading frame 2 (ORF 2) of the *E3* gene of CAV with DNA as template and primer pairs targeting the *E3* gene as described previously [9] using Emerald Amp® GT PCR Master Mix (TaKaRa). The purified PCR products were sequenced at the sequencing facility, Xcelris Labs Ltd, Gujarat using *E3* gene-specific primers used for amplification.

The sequences obtained are aligned with the reference sequences from NCBI using Clustal W from MEGA 6.0 [12]. The changes were compared with the peaks from the chromatogram using Chromas v.2.0. The sequence editing if needed was performed in Lasergene software (DNASTAR Inc., Madison). After retrieving the sequences from the chromatogram, the sequences were aligned against the other isolates from the NCBI database using Clustal W algorithm from MEGA6. The unrooted phylogenetic tree was generated using the neighbour joining method, Tamura 3-parameter model with a bootstrap value of 1,000 in MEGA 6.0 [13]. The neighbour joining method takes a matrix of pairwise evolutionary distances between the given sequences to build the evolutionary tree. Tamura 3-parameter model corrects for multiple hits, taking into account differences in transitional and transversional rates and G+C content bias [13].

Results and Discussion

The preliminary screening of fecal/rectal samples by real-time PCR revealed that 15 were positive for CAV nucleic acid out of a total 302 screened. This accounts for the morbidity of 4.9%. All the samples that showed amplification had Tm in the range of 81.01 °C to 83.56 °C categorizing the virus as CAV-2. All the 15 positive samples were propagated in MDCK cells to recover the virus. Six isolates could be obtained upon three passages in the

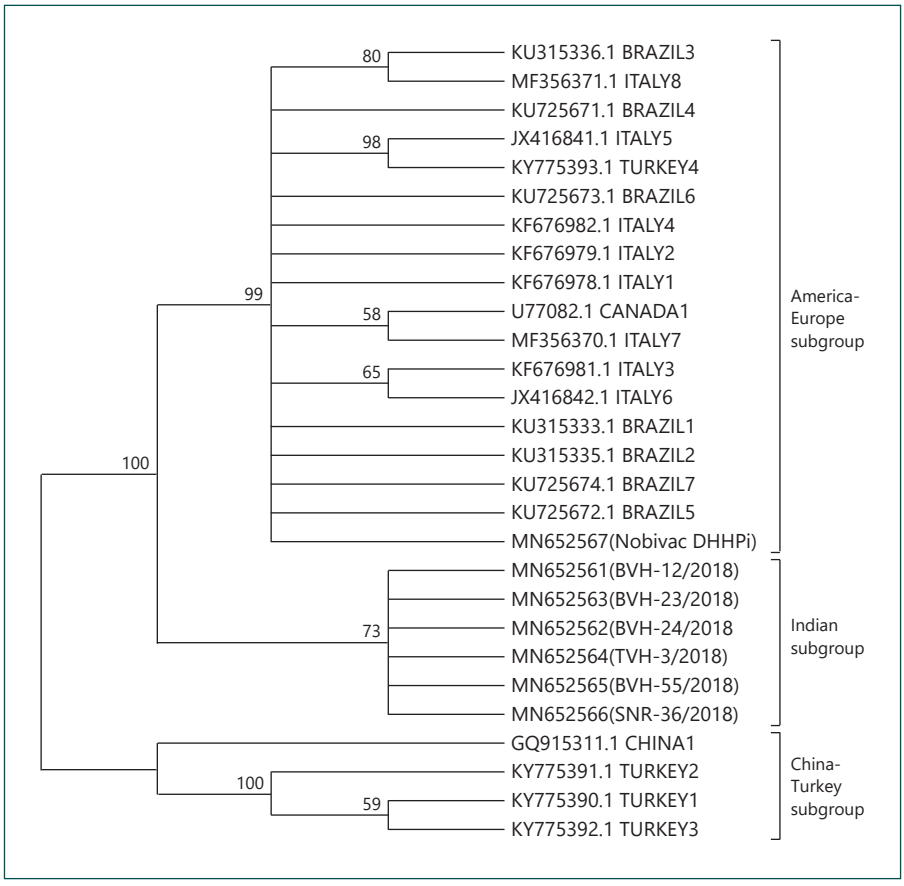

Fig. 2. Phylogenetic analysis of circulating isolates of CAV-2 during 2018–19 in India. Phylogenetic tree is based on 821 nucleotide sequences of 28 strains of CAV-2 generated using the neighbour joining method algorithm. Test of phylogeny is the bootstrap method with 1,000 replicates, using the MEGA 6.0 software program. The numbers at the branch point are the bootstrap values that indicate the number of occurrences for the same branch when the phylogenetic reconstruction was repeated for 100 times. The Indian isolates formed a novel group when compared with other isolates across the world.

MDCK cell line and were named BVH-12/2018, BVH-23/2018, BVH-24/2018, TVH-03/2018, BVH-55/2018, and SNR-36/2018. Partial *E3* (ORF 2) gene was amplified by PCR from the six CAV-2 isolates, and the Nobivac DHPPi vaccine. The positive samples with an expected amplicon of size 1,030 bp specific to CAV-2 were sequenced. The sequences were extracted from the chromatogram for the six isolates and the sequences were submitted in the GenBank of NCBI database. The accession numbers for the same are: BVH-12/2018 (MN652561), BVH-23/2018 (MN652563), BVH-24/2018 (MN652562), TVH-03/2018 (MN652564), BVH-55/2018 (MN652565), SNR-36/2018 (MN652566), Nobivac DHPPi vaccine (MN652567).

The multiple sequence alignment of the six isolates show that they are similar to each other but have a nucleotide insertion ("G") at 1077 position of *E3* gene (ORF 2) when compared to the reference isolate (U77082) or vaccine control; as a result, there is a shift in frame (Fig. 1a). Furthermore, the protein alignment showed that there is a change at the C-terminal end of the amino acid chain. The protein differed in amino acid sequence after the frame-shift mutation, resulting in the elongation of polypeptide chain by eleven amino acids (Fig. 1b). We also noticed substitutions in amino acid sequences at other locations of E3 when compared with reference or vaccine sequence/s. The changes include G266D, K361E, I362N, R363Q, P364A, and M365H (Fig. 1b). The phylogenetic tree reconstructed by the neighbour joining method using Tamura 3-parameter model for the six isolates against other isolates collected from NCBI database revealed that the six isolates from the current study formed a separate group, which is more closely related to the America-Europe group than that of the China-Turkey group (Fig. 2).

In the recent past, a reported increase in cases of canine gastroenteritis despite routine vaccinations is posing a potential challenge for veterinary clinicians across the globe. CAV is one of the most important viruses involved in gastroenteritis of canines that circulates as two distinct serotypes; CAV-1 and CAV-2 [4, 5]. Although the virus has long been known to exist, there are only a few reports available on the isolation of CAV from canine gastroenteritis cases [14]. Moreover, the molecular evolutionary dynamics are least explored [15–17], with no reports from India

to date. To the best of our knowledge, this is the first report on the isolation of CAV-2 from clinical cases of gastroenteritis in India with an emphasis on the understanding of the evolutionary dynamics of the virus with reference to other isolates from the world. In this study, all the 15 isolated CAV samples were categorized as CAV-2. It is interesting to note that only CAV-1 was found in the fecal samples tested before from India [10]. This emphasizes the dynamic nature of the etiology of gastroenteritis.

An optimized SYBR Green real-time PCR method was employed for the detection and discrimination of the two CAV types through the melting curve analysis. In melting curve analysis, the double-stranded DNA was dissociated by increasing the temperature. The temperature at which 50% of DNA dissociates is called Tm. This assay amplifies a 166-bp fragment of CAV genome containing almost 47 nucleotide substitutions that produces a difference in Tm greater than 4 °C between the two viral types; CAV-1 (76.37 °C ± 0.16) and CAV-2 (81.18 °C ± 0.08) [11]. Here, we observed a consistent Tm in the range of 81.01 °C to 83.56 °C for CAV-2.

In the present study, the *E3* gene (ORF 2) that is highly variable was targeted for understanding molecular epidemiology. Furthermore, E3 protein is known to modulate the host response to adenovirus infection and gives direct insight into the host-virus interactions [3]. The multiple sequence alignment for all the isolates revealed that the amino acid and nucleotide sequences were identical within the isolates of the current study. It was noted that our isolates had 95% nucleotide similarities with the reference strain and 100% similarities among the strains in the study. We further noticed a major insertion at 1077 position of nucleotide sequence in comparison to the vaccine or the reference isolates that resulted in a frameshift mutation. There were no previous reports of the frameshift mutation occurring in the *E3* gene (ORF 2) of the CAV-2; this is the first of its kind to be reported. It is interesting to note that this mutation extended the peptide chain by 11 amino acids, and it would be noteworthy to further elucidate the functional significance of the same. CAV-2 is known to be divided into two major groups (America-Europe and China-Turkey) worldwide, based on *E3* gene-based phylogeny [16]. Another major significant finding of our study is that the frameshift mutation along with several nucleotide substitutions noticed at other locations of *E3* have separated the Indian group from the other two groups.

However, our study was limited to *E3* gene-based molecular epidemiology, and there is a need for investigating the other gene segments and/complete genome of current isolates to further strengthen this finding. It is also not clear whether the changes are specific to a geographical area. Hence, a wider sample throughout the country can provide better insights into this aspect. The molecular epidemiology of CAV-2 is least known. Our work suggests there are a lot of areas that need to be explored with respect to CAV for the development of effective vaccine strategies and for installing proper control measures.

Statement of Ethics

The current study does not involve human/animal experimentation; hence, ethical approval is not needed. The work has been performed in accordance with standard operating procedures adopted by the Department of Veterinary Microbiology and Biotechnology, PVNRTVU, Hyderabad. Further, although dogs were used in this study for collection of rectal swabs, they were not subjected to any form of treatment or experimentation.

Disclosure Statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author Contributions

Kalyani Putty, Narasimha Reddy Yella conceived the study. Ashwini Ramidi, Bhagyalakshmi Buddala performed research. Kalyani Putty, Vishweshwar Kumar Ganji, Gnana Prakash Manthani analyzed the data. Kalyani Putty, Vishweshwar Kumar Ganji wrote the manuscript, which was read, edited, and approved by all authors.

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