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ORIGINAL ARTICLE

Genetic characterization of equine herpesvirus 1 isolates from abortion outbreaks in India

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Abstract Equine herpesvirus 1 (EHV1) is a common pathogen of horses that causes upper respiratory tract disease, abortion, neonatal death and neurological disease. The neurological form of disease is called equine herpesvirus myeloencephalopathy (EHM). During the past decade, the incidence of EHM has been on the rise in Europe, North America, Australia and Asia. Some EHV1 isolates causing EHM exhibit a single-nucleotide polymorphism (SNP) in the DNA polymerase gene (ORF30) at position 2254 (A_{2254} to G_{2254}). Further, based on polymorphism in the ORF68, EHV1 isolates have been classified into different groups. The aim of the present study was to estimate the genetic diversity of EHV1 and to determine the prevalence of the neuropathogenic genotype of EHV1 in India. Out of 133 clinical specimens from abortion cases in northern India, 56 were positive for EHV1 infection. Analysis of the A/G SNP by real-time PCR and sequence analysis revealed that 54 of 56 samples (96.43 %) were of the non-neuropathogenic genotype (A_{2254}), while two (3.57 %) had the neuropathogenic marker (G_{2254}). Sequence analysis of the polymorphic region of ORF68 of EHV1 isolates (n = 9) from India indicated that the Delhi/1998, Tohana-2/2013, Hisar-2/2014 and Hisar-15/1990 isolates belonged to group 4, while the Jind/1996, Rajasthan/1998, Delhi-3/2007 and Tohana-5/1996 isolates clustered within group 5. One isolate (Hisar-7/1990) exhibited SNPs at positions C_{710} and C_{713}, forming a separate group. Here, we report for the first time the detection of neuropathogenic genotypes of EHV1 in India and show that Indian EHV1 isolates cluster within groups 4 and 5.

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Introduction

Equine herpesvirus 1 (EHV1) is enzootic in horse populations worldwide and is a significant cause of economic loss to the horse industry. The virus causes a range of clinical signs including respiratory disease in young horses, late-term abortion, neonatal foal mortality, and equine herpesvirus myeloencephalopathy (EHM), resulting in paresis/paralysis [1]. During the past decade, the incidence of abortion and rhinopneumonitis due to EHV1 has been declining, possibly due to widespread vaccination practices. At the same time, reports of EHM have been increasing in frequency [2–12].

EHV1 is an enveloped, double-stranded DNA virus belonging to the genus *Varicellovirus,* subfamily *Alphaherpesvirinae*, family *Herpesviridae* [13]. The viral genome is a linear, double-stranded DNA molecule of 150,223 kbp that contains 76 open reading frames (ORFs) [14]. EHV1 isolates from different outbreaks differ in their pathogenic potential. The genetic basis for differences in pathogenicity between different EHV1 isolates is still poorly understood. Single-nucleotide polymorphism (SNP) at position 2254 of the DNA polymerase gene (encoded by ORF30) is associated with neurovirulence [2, 15–17]. EHV1 isolates possessing guanine (G_{2254}) at this site are considered to have neuropathogenic potential, whereas those with adenine (A_{2254}) are thought to be non-neuropathogenic.

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Table 1 Details of EHV1 positive clinical specimens used in the study

Sample no.	Isolate name	Place of outbreak	Disease condition	Year of isolation
1	Hisar-7/1990*	Hisar, Haryana	Abortion	1990
2	Hisar-15/1990*	Hisar, Haryana	Abortion	1990
3	Hisar-2/1990	Hisar, Haryana	Abortion	1990
4	Tohana-5/1996*	Tohana, Haryana	Foal mortality	1996
5	Jind/1996*	Jind, Haryana	Abortion	1996
6	Delhi-1/1998*	New Delhi, Delhi	Abortion	1998
7	Rajasthan/1998*	Bikaner, Rajasthan	Abortion	1998
8	Delhi-3/2007*	New Delhi, Delhi	Abortion	2007
9	Tohana/2007	Tohana, Haryana	Abortion	2007
10	Hisar/2007	Hisar, Haryana	Abortion	2007
11	Delhi/2008	New Delhi, Delhi	Abortion	2008
12	Hisar-1/2010	Hisar, Haryana	Abortion	2010
13	Hisar-2-2010	Hisar, Haryana	Abortion	2010
14	Ambala/2010	Ambala, Haryana	Abortion	2010
15	Gurgaon/2011	Gurgaon, Haryana	Abortion	2011
16	Tohana-2/2013*	Tohana, Haryana	Abortion	2013
17	Hisar-2/2014*	Hisar, Haryana	Abortion	2014
18	Hisar/2014	Hisar, Haryana	Abortion	2014
19	Ab4	Suffolk, United Kingdom	Abortion & neurological disease	1981
20	V592	Glasgow, United Kingdom	Abortion	1985

* EHV1 isolates included for ORF68 sequencing

Similarly, genetic variations reported in the EHV1 ORF68 region, which is homologous to the human herpesvirus 1 US2 region, have been used as a genetic marker for classification of EHV1 isolates into different groups [15], as this region has the highest variation (2 %). EHV1 isolates have been divided into 6–10 groups based on SNPs in the polymorphic region of ORF68 and number of G residues in the homopolymeric G tract (nt 732-739) [10, 15, 18]. In India, EHV1 has been associated with cases of abortion, stillbirth, neonatal foal mortality, and paresis [19–21]. There is limited information available about genetic diversity in EHV1 isolates from India [22]. In this study, we analyzed the genetic diversity among EHV1 isolates from India by sequence analysis of ORF30 and ORF68.

Materials and methods

Cells, virus isolates and clinical samples

RK13 cells were grown in Eagle's minimal essential medium (EMEM; Sigma Aldrich, New Delhi, India) supplemented with 10 % fetal bovine serum, 100 units of penicillin, 100 µg of streptomycin and 0.25 of µg amphotericin-B per ml. Viruses (n = 18) used in this study were isolated at National Research Centre on Equines from clinical cases of abortion and neonatal foal mortality in different parts of India since 1990 and stored in liquid nitrogen. Reference EHV1 strains Ab4 and V592 maintained in our laboratory were also used as controls (Table 1). All isolates were cultured in RK13 monolayer cell cultures in 25-cm^2 flasks. After a cytopathic effect (CPE) was observed in 90 % of the cell monolayer, the flasks were frozen and thawed three times, and the supernatant was clarified by centrifugation at $10000\times g$ for 15 min. Clinical samples used in the study were collected from cases of equine abortion/neonatal foal mortality reported during 2013-14 from the northern Indian states of Haryana, Rajasthan and Delhi. The samples included aborted fetal tissues (n = 14), mare blood samples in EDTA (n = 93), and mare vaginal and nasal swabs (n = 4 each). Peripheral blood mononuclear cells (PBMCs) from 5.0 ml of blood were separated by Histopaque-1077 (Sigma Aldrich, USA) and suspended in 1.0 ml of PBS.

EHV1 nested PCR (nPCR)

Viral DNA was extracted from 200 µl of clarified supernatant from virus-infected cell culture, a 10 % homogenate of aborted fetal tissues, or 100 µl of PBMCs using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden,

Table 2 Reference sequences included in ORF68 analysis

Sample no.	Isolate name	Place	Group	Accession no.
1	GB80_1_2 (Ab4)	UK	1	DQ172353
2	US89_1_1	USA	2	DQ172408
3	GB01_1_1	UK	3	DQ172334
4	FR_00_1_1	France	4	DQ172326
5	US01_1_2	USA	5	DQ172375
6	GB85_1_1 (V592)	UK	6	DQ172359
7	82_03	Hungary	7	HQ654059
8	04_03	Hungary	8	HQ654067
9	83_01	Hungary	9	HQ654060
10	04_04	Hungary	10	HQ654069

Germany) as per the manufacturer's instructions. All viral isolates and clinical specimens were tested by nested PCR for EHV1/EHV4 as reported previously [23].

EHV1 real-time PCR (rPCR)

A duplex real-time PCR was standardized for allelic discrimination at position 2254 of ORF30 with modifications of methods adopted by Smith et al. [24] using two probes (5HEX-ATCCGTCAACTACTCG-TAMRA and 6 ~ FAM-ATCCGTCGACTACTCG-BHQ1). A total of 133 clinical specimens (EHV1 archived virus isolates [n = 18], aborted fetal tissues [n = 14], mare blood samples in EDTA [n = 93], vaginal swabs [n = 4] and nasal swabs [n = 4]) were analyzed by rPCR. Plasmid DNA carrying a 654-bp (nt 2036-2689) region of the ORF30 gene of the reference isolates Ab4 (G_{2254} genotype) and V592 (A_{2254} genotype) were used as controls for A/G allelic discrimination by real-time PCR. The reactions were carried out in Bioer LineGene 9600 (Bioer Technology Co. Ltd, China). The rPCR assay was done using a Quantitect Multiplex PCR NoRox Kit (QIAGEN, Hilden, Germany). The 25-µl reaction mix consisted of 12.5 µl of 2X Quantitect Multiplex Master Mix, 1 µl each of forward and reverse primer (400 nM each primer), 1 µl of each probe (200 nM each), 3.5 µl of nuclease-free water, and 5 µl of DNA template. The amplification was done with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/amplification at 58 °C for 30 s. Results were interpreted based on Ct values, where Ct <39 was considered positive.

PCR amplification, cloning and sequencing

A 654-bp ORF30 region (nt 2036-2689) and a 925-bp region of ORF68 were amplified by PCR using primers and amplification conditions reported previously [15]. A total of 18 EHV1-positive clinical specimens (eight archived EHV 1 isolates and 10 EHV1-positive clinical samples) were employed for ORF 30 sequence analysis (Table 1). For ORF30 amplification, the PCR master mix (50 µL) consisted of 5 µL of 10X PCR buffer, 2.0 µL of magnesium sulphate (50 mM), 1.0 µL each of forward and reverse primer (10 µM), 1.0 µL of dNTP (10 mM), 0.2 µL of High-Fidelity Platinum Taq DNA Polymerase (Life Technologies, USA), 34.8 µL of nuclease-free water and 5 µL of template DNA. The PCR products were gel purified using a PureLink Quick Gel Extraction Kit (Invitrogen, USA) and cloned into PTZ57R/T vector (Thermo Scientific, USA) as per the manufacturer's protocol. The plasmids carrying the desired insert were sequenced commercially. The consensus sequences of duplicate clones were assembled and edited using BioEdit software version 7.2.5. The sequences were aligned using CLUSTAL W algorithms available in the MEGA 6.0 software programme [25], and a phylogenetic tree was constructed in MEGA 6.0 using the UPGMA algorithm.

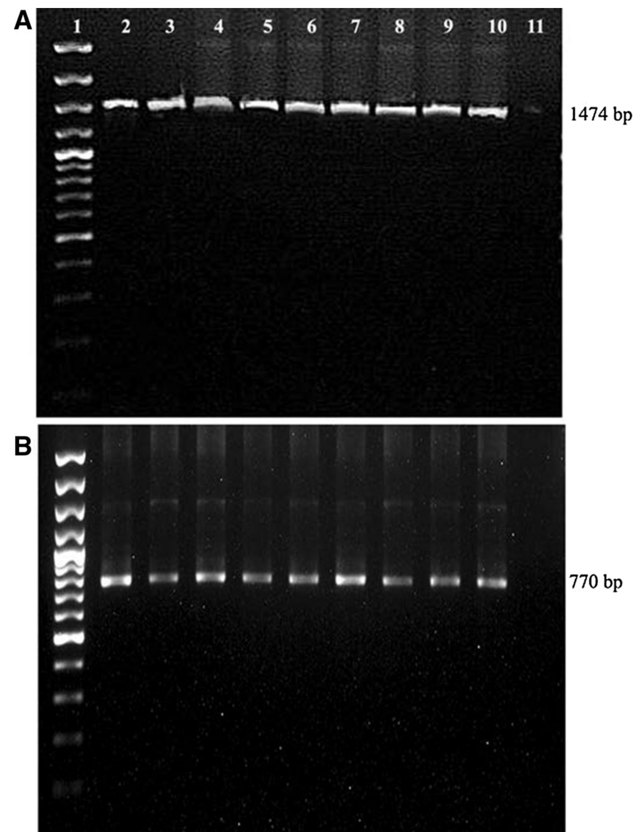

Fig. 1 Nested PCR of clinical specimens (n = 8) for detection of EHV1. Panel A, first-round PCR product (1474 bp); panel B, nested PCR product (770 bp). Lane 1, 100 bp plus DNA ladder; lane 2, Hisar-7/1990; lane 3, Hisar-15/1990; lane 4, Tohana-5/1996; lane 5, Delhi-3/2007; lane 6, Delhi/2008; lane 7, Gurgaon/2011; lane 8, Tohana-2/2013; lane 9, Hisar-2/2014; lane 10, positive control (V592); lane 11: no-template control

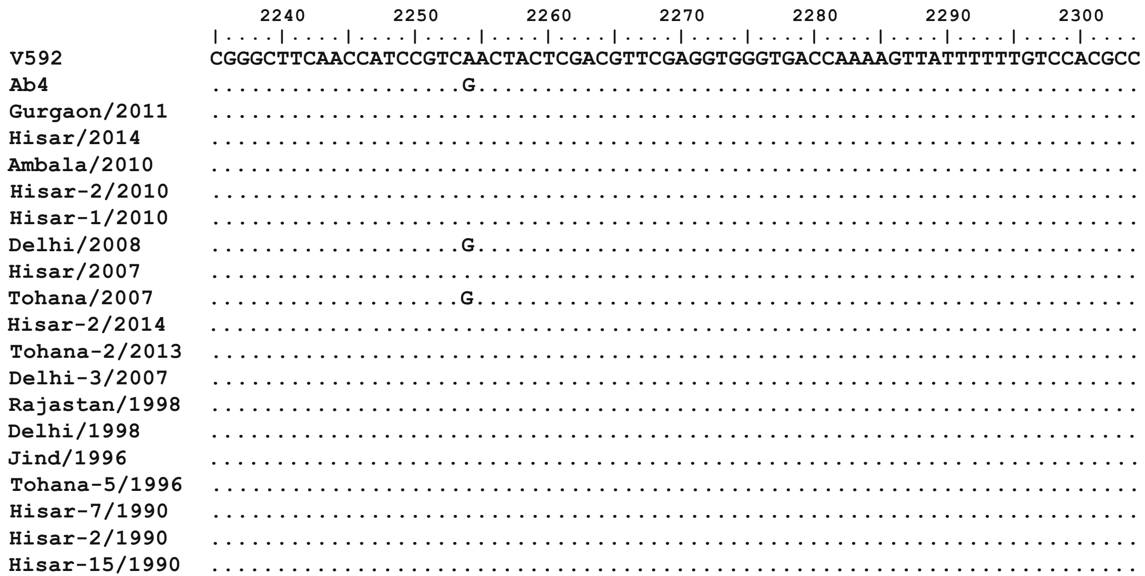

Fig. 2 Sequence analysis of partial ORF30 of EHV1 isolates. The figure shows the presence of the G2254 polymorphism in two isolates (Tohana/2007 and Delhi/2008). Dots indicate sequence identity in the alignment as compared to the EHV1 V592 isolate

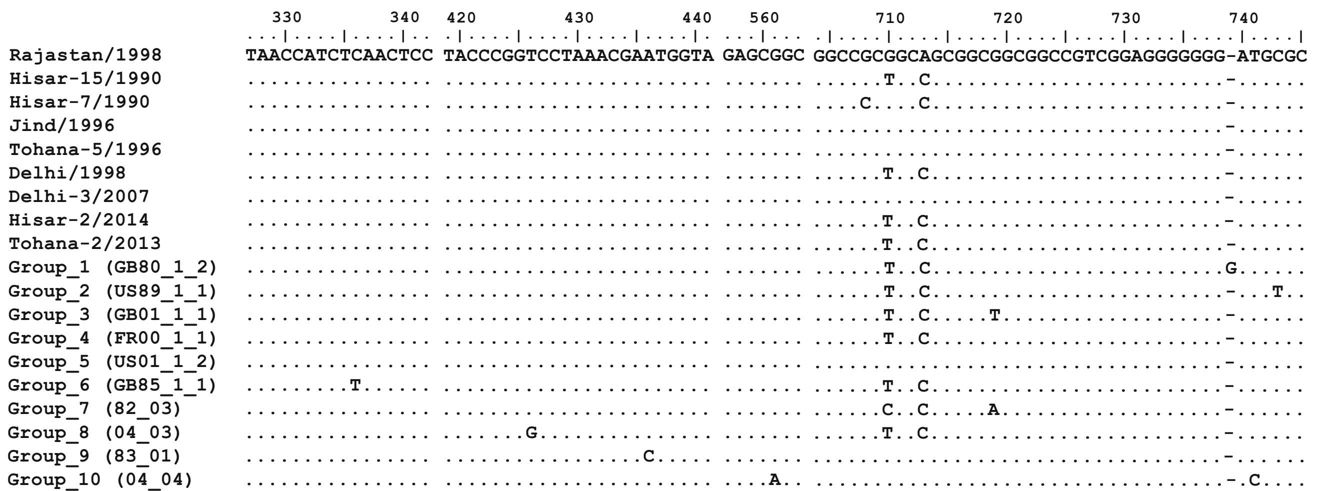

Fig. 3 Sequence analysis of partial ORF68 of EHV1 isolates. Dots indicate sequence identity, and dashes indicate gaps in the alignment. Nucleotide positions are indicated above the alignment in accordance with the Rajastan/1998 sequence

For ORF68 analysis, nine EHV1 isolates were selected (Table 1). The GC-rich ORF68 amplicons were generated with Q5® Hot Start High-Fidelity Master Mix (New England Biolabs, USA). The Master Mix consisted of 10 µL of 5X Q5 reaction buffer, 10 µL of 5X Q5 high GC enhancer, 1 µL of dNTP (10 mM), 1 µL each of forward and reverse primer (10 µM), 1 µL of Q5® Hot Start High-Fidelity DNA Polymerse, 22 of µL nuclease-free water, and 5 µL of template DNA. Cloning and sequencing was carried out as described above. Grouping of isolates was carried out as described by Nugent et al. [15] by comparing the sequences with EHV1 reference isolates (Table 2).

Results

EHV1 nPCR and rPCR

A total of 38 of 115 clinical specimens from abortion cases in mares and 18 archived EHV1 isolates tested positive for EHV1 by nested PCR, yielding amplicons of 1474 bp and 770 bp in the first round and nested PCR, respectively (Fig. 1). On testing specimens for the A/G SNP by rPCR, two (3.57 %) clinical specimens (Delhi/2008 and Tohana/2007) were found to contain G_{2254} genotypes, while the remaining 54 (96.43 %) belonged to the A_{2254} genotype.

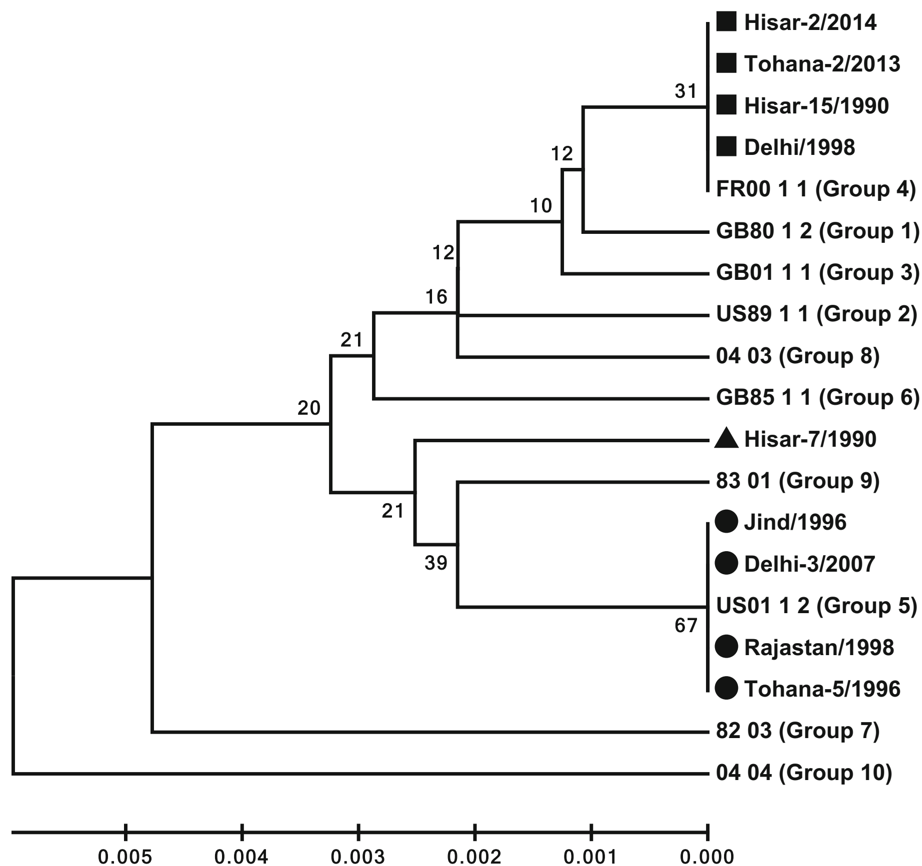

Fig. 4 Phylogenetic tree based on a portion of the ORF68 region. Evolutionary analysis was conducted in MEGA 6.0. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the maximum composite likelihood method and are in units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

ORF30 and ORF68 sequence analysis

The ORF30 sequences of 18 EHV1-positive clinical specimens (accession no. KM285370-KM285385, KR812328-KR812329) were compared with those of EHV1 reference isolates (DQ172353 and DQ172359). Sequence analysis revealed that two out of 18 clinical specimens (Delhi/2008 and Tohana/2007) had the nucleotide substitution ‘G’ at position 2254 of ORF30, while the remaining 16 had the A_{2254} genotype (Fig. 2).

ORF68 sequence analysis of nine EHV1 isolates (KM285386-KM285392, KT180205-KT180206) revealed that the EHV1 isolates from India contained SNPs that were characteristic of EHV1 isolates belonging to group 4 and group 5 (Fig. 3). Based on the SNP characteristics in the ORF68 sequence, the isolates Delhi/1998, Tohana-2/2013, Hisar-2/2014 and Hisar 15/1990 clustered into group 4, while Jind/1996, Rajasthan/1998, Delhi-3/2007 and Tohana-5/1996 clustered into group 5, which is also evident from the phylogenetic dendrogram (Fig. 4). Hisar-7/1990 isolate showed some variations in its sequence as compared to isolates of group 4 ($T_{710} \rightarrow C$) and group 5 ($A_{710} \rightarrow C$ and $G_{713} \rightarrow C$) and formed a separate clade in the phylogenetic dendrogram.

Discussion

In the present study, clinical specimens from abortion cases of equines from different parts of India during 1990-2014 were analyzed to assess their genetic diversity. The A/G_{2254} SNP was detected in EHV1 isolates/samples by employing a real-time PCR assay as well as sequence analysis, and the two tests showed 100 % agreement. The neuropathogenic genotype (G_{2254}) was detected in 3.57 % of total clinical specimens analyzed. The incidence of the neuropathogenic genotype from neurological cases of EHM has been reported to range between 20 % and 86 % [2, 4, 5, 7, 10], while in cases of abortion, it ranges from 1.5–25.8 % [4–8, 10, 12] .The prevalence rate of neuropathogenic genotype in abortion cases in this study is similar to those reported from Poland [12], Japan [8] and Australia [10].

SNPs located at position 336, 426, 561, 628, 629, 710, 713 and 741 in the polymorphic region of ORF68 and the number of G residues in a homopolymeric tract (nt positions 732–739) helps in placing EHV1 isolates into different groups [15]. Sequence analysis of the ORF68 region revealed that viruses from groups 4 and 5 were most common among Indian EHV1 isolates included in the

study. One of the isolates (Hisar-7/1990) exhibited SNPs at position 710 and 713, forming a new group/clade. This virus was isolated from northern India from an abortion outbreak in 1990 and has been passaged extensively in RK-13 cells for use as a vaccine candidate [21]. The observed SNPs in this isolate might be due to repeated passages in cell culture. Although there is evidence that certain groups are geographically restricted, the existence of multiple EHV1 ORF68-based genetic groups in a particular geographic area is not uncommon [15, 18]. The group 5 genotype was reported to be predominant in North America [15], group 4 was the predominant genotype reported in Europe and Africa [11, 26], and the group 2 genotype was reported from Japan [8]. This is the first study reporting the genetic grouping of EHV1 isolates from India. In conclusion, this study establishes that neuropathogenic and genetically diverse EHV1 genotypes are circulating in India. These findings may have implications for studying the pathogenesis and molecular epidemiology of EHV1 infections in this region.

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Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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