



Molecular Detection and Genotypic Characterization of Fowl Adenovirus Associated with Inclusion Body Hepatitis (IBH) in Broiler Chickens of Nagpur

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ABSTRACT

Background: Fowl adenovirus (FAdV) is the causative agent of inclusion body hepatitis (IBH) in broiler chickens and is responsible for increased mortality and reduced production in the poultry sector. Molecular detection of the hexon gene is the primary focus for antigenic determination and genotyping of FAdV. The present study intended to explore the prevalence and genotype characterization of fowl adenovirus serotypes circulating in the poultry population of Nagpur.

Methods: Two hundred liver tissue samples collected from broiler chickens were subjected to molecular detection of the hexon gene by conventional PCR, followed by a Sanger sequencing approach to determine prevailing FAdV serotypes.

Result: A total of 105 (52.5%) samples were recorded as positive for fowl adenovirus by hexon gene-based PCR. The representative nucleotide sequences showed 98.44-99.88% homology with serotype 11 and 99.17% with serotype 8b of FAdV D and E species, respectively. This study imparts crucial information regarding the circulating serotypes of fowl adenovirus in broiler chickens of Nagpur and emphasizes the need for monitoring of FAdVs to formulate better immunization strategies to control the dissemination of the disease.

Key words: Fowl adenovirus, Hexon gene, Inclusion body hepatitis, PCR, Phylogenetic analysis.

INTRODUCTION

Fowl adenoviruses (FAdVs) are non-enveloped, linear, double-stranded DNA viruses belonging to the genus *Aviadenovirus*, family *Adenoviridae*. They cause several sporadic diseases in chickens, including inclusion body hepatitis (IBH), hydropericardium-hepatitis syndrome (HHS) and adenoviral gizzard erosion (AGE), which lead to significant financial losses globally (Marek *et al.*, 2010; Niu *et al.*, 2018; Schachner *et al.*, 2018). Inclusion body hepatitis (IBH) was first identified in the USA in 1963 (Helmholtz *et al.*, 1963). Since then, reports of this disease have been documented in numerous countries (Batista *et al.*, 2024; Qiao *et al.*, 2024). In India, many outbreaks of IBH have been reported in earlier works (Sandhu *et al.*, 1994; Kataria *et al.*, 2013). IBH primarily infects broiler chickens up to 5 weeks of age and spreads through both vertical and horizontal transmission (Harrach *et al.*, 2012; Grafl *et al.*, 2012; Schachner *et al.*, 2018). Affected birds exhibit dullness, ruffled feathers, huddling and respiratory distress. Sudden mortality rates approaching 30% have been reported in affected flocks (Choi *et al.*, 2012; Schachner *et al.*, 2018; Santander-Parra *et al.*, 2023). FAdVs are divided into five species (A-E) and 12 serotypes (1-8a and 8b-11) (Dutta *et al.*, 2023; Sohaimi *et al.*, 2021; Li *et al.*, 2022). The main structural proteins include hexon, penton and fiber. The hypervariable region of hexon gene plays a significant role in the antigenic determination of virus type, group and sub-group and serves as a reliable marker for molecular detection and genotyping (Wang *et al.*, 2023). Molecular approaches, such as polymerase chain reaction,

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which amplifies the hexon gene, are commonly used for the rapid identification of FAdVs (Islam *et al.*, 2023). PCR combined with Sanger sequencing has often been utilized for FAdV genotyping (Cizmecigil *et al.*, 2020). Inclusion body hepatitis is emerging as the primary acute disease of broiler chickens, causing a surge in mortality and potential financial losses for the poultry industry (Choi *et al.*, 2012; Bertran *et al.*, 2021). Recent studies in India have reported serotype 11 as the major cause of IBH alongside the active

circulation of serotype 8b (Chavan *et al.*, 2023; Shankar *et al.*, 2022). Numerous outbreaks of IBH have been reported in India, but the disease is still not fully understood and there is a lack of information regarding FAdV serotypes circulating in Central India. This study investigated the prevalence and genetic diversity of predominant FAdV serotypes circulating among broiler chickens of Nagpur.

MATERIALS AND METHODS

Study area

The present study was conducted from 2022 to 2024 in the Department of Veterinary Microbiology at Nagpur Veterinary College, Nagpur. The research primarily focused on the Nagpur region, with broiler poultry farms as the main research subjects.

Collection of samples

A total of 200 liver tissue samples (2 g) were collected from broiler chickens suspected to be affected with inclusion body hepatitis, which were brought in from various broiler poultry farms for postmortem examination in the Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur. Before further processing, the tissue samples were stored at -20°C in a sterile zip-lock plastic bag.

DNA extraction

Liver tissue samples were weighed (10 mg) and homogenized in 1 mL of sterile phosphate buffered solution (PBS) (pH 7.4) using a commercial homogenizer. The homogenates were kept for freeze drying at -80°C for 2-3 hours before thawing at room temperature, repeating this process three times before subjecting the samples to centrifugation at 10000-12000 rpm for 5 mins. The supernatant was collected and filtered using a 0.22 µm

pore size syringe filter, followed by DNA extraction using the HiPurA® Multi-Sample DNA Purification Kit. The purity of the DNA was checked by spectrophotometer and was stored at -20°C until further analysis.

Molecular detection of FAdVs

The samples were subjected to conventional PCR amplifying the fragment of loop 1 of the hexon gene utilizing published primer sequences (Meulemans *et al.*, 2001). A reaction mixture of 20 µl was set up comprising of 10 µl of 2X Emerald Amp PCR Master Mix, 0.75 µl of forward and reverse primers (10 µM) each, 1.5 µl of DNA template and 7 µl of nuclease-free water to make up the reaction volume. The amplification was carried out in a thermocycler and the cycling conditions for PCR were as follows: Initial denaturation at 95°C for 5min, followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 61°C for 1 min, extension at 72°C for 1min and a final extension step at 72°C for 10 min. PCR products were analysed by running on a 1.5% agarose gel containing ethidium bromide dye, 3 µl/40 ml of 1X TAE buffer. Visualization of amplification bands was performed under a UV gel documentation system (Syngene G box, UK).

Sequencing and phylogenetic analysis

A total of 15 representative positive amplicons were sent for Sanger sequencing (Barcode Biosciences (India) Pvt. Ltd., Bangalore). The sequences were checked for base call and trimmed using Bioedit software (<https://bioedit.software.informer.com/7.2/>) and were matched with the 38 fowl adenovirus nucleotide sequences retrieved from GenBank using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/bblast.cgi>). The sequences were aligned using ClustalW (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>). The

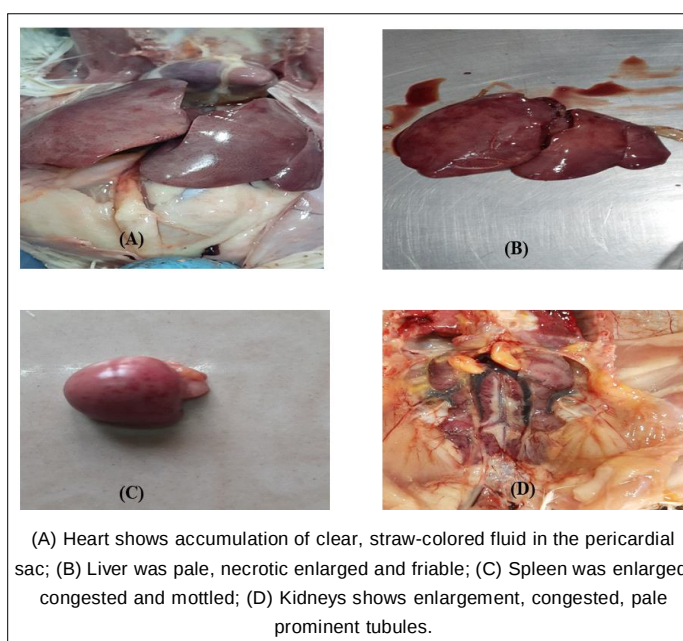


Fig 1: Gross postmortem lesions observed in different organs of affected broiler chickens.

phylogenetic tree was built following the Neighbour-Joining method with 1000 bootstrap replications. The evolutionary distances were computed using the Maximum Composite Likelihood method and were depicted in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion). Evolutionary analyses were conducted in MEGA11 software (Tamura *et al.*, 2021). Following this, the amino acids were deduced using expasy tool and per cent identity matrix was computed using CLUSTAL Omega software.

RESULTS AND DISCUSSION

Postmortem findings

The gross lesions observed during postmortem of the broiler chickens revealed accumulation of fluid in pericardial sac and necrotic liver with petechial haemorrhages, enlargement and congestion of spleen, pale and swollen kidneys (Fig 1). Similar postmortem lesions of hepatomegaly with hepatic necrosis and marked atrophy of thymus and splenomegaly were reported by (Thabet *et al.*, 2023).

Molecular prevalence of FAdVs

A total of 105 (52.5%) out of 200 samples were positive for the hexon gene of fowl adenovirus, yielding an amplicon size of 897 bp (Fig 2). The prevalence was higher in broiler chickens between 21-42 days of age in comparison to other age groups. Lower prevalence was noted in the 8-21 days old and above 42 days old age groups. Similar results

were reported by Kumar *et al.* (2022), who reported a 60% prevalence of FAdVs in commercial broiler chickens. Shankar *et al.* (2022) and Chavan *et al.* (2023) reported a prevalence of 42.85% and 48.28% of FAdV in broiler chickens in India. Niczyporuk *et al.* (2022) and Santander-Parra *et al.* (2023) reported a prevalence rate of 57.71% and 56.16% of FAdV in chicken flocks. Mittal *et al.* (2014) reported a higher prevalence of (82.5%) of FAdVs in poultry flocks. The broiler chickens in the present study were not vaccinated against fowl adenovirus, therefore greater prevalence of inclusion body hepatitis was observed.

Phylogenetic analysis

Phylogenetic analysis of the nucleotide sequences revealed that 14 of the representative sequences (FAdV-MT-02-2023, FAdV-BA-06-2023, FAdV-GO-19-2023, FAdV-KH-22-2023, FAdV-PI-29-2023, FAdV-KA-35-2023, FAdV-NC-48-2023, FAdV-KO-55-2023, FAdV-U-57-2023, FAdV-UM-67-2023, FAdV-WA-72-2023, FAdV-KD-79-2023, FAdV-MO-84-2023, FAdV-SO-86-2023) showed (98.44-99.88%) sequence identity to the serotype 11 of FAdV-D species (Accession no. OR690111.1 (India), Accession no. PQ466432.1 (India), Accession no. MH379242.1 (India), Accession no. MH379244.1 (India), Accession no. PP982456.1 (Pakistan), Accession no. MN447717.1 (India), Accession no. PQ466435.1 (India), Accession no. MN540444.1 (India), Accession no. OR371950.1 (India), Accession no. MN537891.1 (India), and Accession no. PQ466431.1 (India), whereas 01 nucleotide sequence (FAdV-BE-43-2023) showed 99.17% sequence identity to the 8b serotype of FAdV-E species (Accession no. MH379248.1 (India), Accession no. MW735942.1 (China) and Accession no. ON959146.1 (China) (Fig 3). The fowl adenovirus strains in the present study were closely clustered with the previously reported FAdV-11 serotypes from India and Pakistan. One FAdV strain in the present study was distantly placed and closely clustered with the FAdV 8b serotype from China. These findings indicate that FAdV-11 is predominant in cases of IBH in Nagpur, however, serotype 8b is also circulating in the affected broiler chickens. Shankar *et al.* (2022) and Sharif *et al.* (2020) also reported the highest prevalence of FAdV 11 in broiler chickens, followed closely by FAdV 8b. Serotypes 11 and 8b have the potential to be pathogenic and can cause serious clinical disease (Shankar *et al.* 2022). There have been reports of increased morbidity and mortality in broiler chickens due to FAdV 11 (Islam *et al.* 2023). The FAdV 11 and 8b serotypes of the present study showed 98.45%-99.88% nucleotide identity among themselves, indicating the distinctness of the viruses from one another. Several previous studies have reported that the main serotypes responsible for causing inclusion body hepatitis belonged to fowl adenovirus D and E (Schachner *et al.*, 2016; Wibowo *et al.*, 2019; Islam *et al.*, 2023). Additionally, studies have shown that the FAdV 8b serotype has emerged as the main cause of IBH in chickens with extensive tissue tropism and lower mortality (Huang *et al.*,

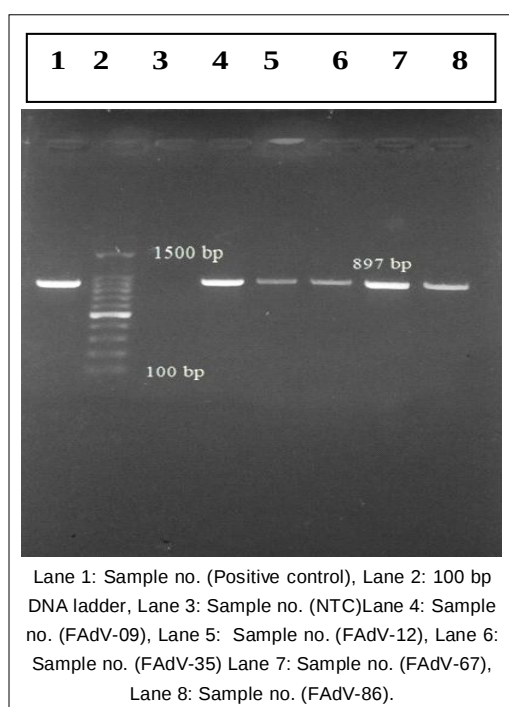


Fig 2: Amplification of loop 1 fragment of hexon gene by conventional PCR.

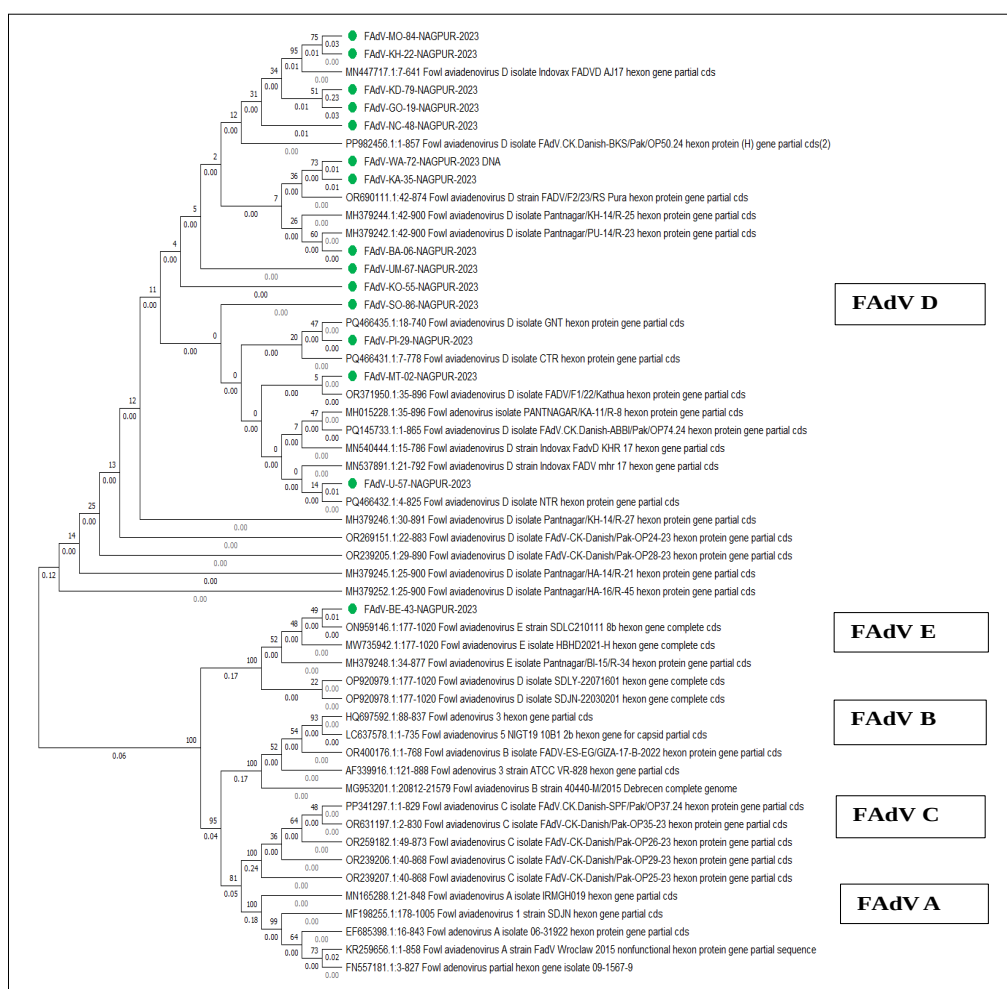


Fig 3: Phylogenetic tree of partial hexon gene sequences constructed using Neighbour-Joining method with 1000 bootstrap replicates based on maximum composite likelihood model in MEGA 11 software.

:AdV-Pi-29-NAGPUR-2023	100	97.5	96.3	98	98.3	97.5	97.1	97.1	97.5	97.5	97.5	97.54	97.5	97.5	97.5	97.5	97.5	90.98	93.81	94.3	89.57	93.42	
:AdV-KA-35-NAGPUR-2023	97.5	100	97.9	98.7	98.7	98.7	98.3	98.3	98.7	98.7	98.7	98.72	98.7	98.7	98.7	98.7	98.7	92.34	93.81	94.3	88.15	93.42	
:AdV-WA-72-NAGPUR-2023	96.3	97.9	100	97.2	98.3	98	98.4	98	98	98	98	98.4	98.78	98.8	98.4	98.4	98.4	98.8	92.71	93.81	94.3	89.57	93.42
:AdV-MT-02-NAGPUR-2023	98	98.7	97.2	100	99.6	98.8	98.4	98.4	98.4	98.8	99.19	99.2	98.8	98.8	98.8	98.8	99.2	91.5	93.81	94.3	89.57	93.42	
:AdV-SO-86-NAGPUR-2023	98.3	98.7	98.3	99.6	100	99.6	99.2	99.2	99.6	99.6	99.6	99.59	99.6	99.6	99.6	99.6	99.6	92.12	93.81	94.3	89.57	93.42	
:AdV-BA-06-NAGPUR-2023	97.5	98.7	98	98.8	99.6	100	98.8	98.8	98.8	98.8	99.2	99.59	99.6	99.2	99.2	99.2	99.6	91.9	93.81	94.3	89.57	93.42	
VBS4802.1_53-299	97.1	98.3	98.4	98.4	99.2	98.8	100	98.8	99.2	99.2	99.2	99.59	99.6	99.2	99.2	99.2	99.6	91.9	93.3	93.8	89.1	92.76	
JIO87096.1_2-248	97.1	98.3	98	98.4	99.2	98.8	98.8	100	99.2	99.2	99.6	99.59	99.6	99.6	99.6	99.6	99.6	91.9	93.3	93.8	89.1	92.76	
:AdV-UM-67-NAGPUR-2023	97.5	98.7	98	98.4	99.6	98.8	99.2	99.2	100	100	99.6	100	100	99.6	99.6	99.6	99.6	100	92.71	93.81	94.3	89.57	93.42
:AdV-KO-55-NAGPUR-2023	97.5	98.7	98	98.4	99.6	98.8	99.2	99.2	100	100	99.6	100	100	99.6	99.6	99.6	99.6	100	92.71	93.81	94.3	89.57	93.42
NOL22095.1_1-247	97.5	98.7	98.4	98.8	99.6	99.2	99.2	99.6	99.6	99.6	100	100	100	100	100	100	100	100	92.31	93.81	94.3	89.57	93.42
JIO87089.1_4-249	97.5	98.7	98.8	99.2	99.6	99.6	99.6	99.6	100	100	100	100	100	100	100	100	100	100	92.68	93.81	94.3	89.57	93.42
JIO87104.1_2-247	97.5	98.7	98.8	99.2	99.6	99.6	99.6	99.6	100	100	100	100	100	100	100	100	100	100	92.68	93.81	94.3	89.57	93.42
CKI16504.1_29-285	97.5	98.7	98.4	98.8	99.6	99.2	99.2	99.6	99.6	99.6	100	100	100	100	100	100	100	100	92.31	93.81	94.3	89.57	93.42
MNA21506.1_50-296	97.5	98.7	98.4	98.8	99.6	99.2	99.2	99.6	99.6	99.6	100	100	100	100	100	100	100	100	92.31	93.81	94.3	89.57	93.42
VDP09033.1_53-299	97.5	98.7	98.4	98.8	99.6	99.2	99.2	99.6	99.6	99.6	100	100	100	100	100	100	100	100	92.31	93.81	94.3	89.57	93.42
EQ91175.1_41-287	97.5	98.7	98.4	98.8	99.6	99.2	99.2	99.6	99.6	99.6	100	100	100	100	100	100	100	100	92.31	93.81	94.3	89.57	93.42
NKIA9924.1_53-298	97.5	98.7	98.8	99.2	99.6	99.6	99.6	99.6	100	100	100	100	100	100	100	100	100	100	92.68	93.81	94.3	89.57	93.42
:AdV-U-57-NAGPUR-2023	91	92.3	92.7	91.5	92.1	91.9	91.9	91.9	92.7	92.7	92.3	92.68	92.7	92.3	92.3	92.3	92.7	100	93.81	94.3	87.26	93.42	
:AdV-KH-22-NAGPUR-2023	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	100	96	92.78	93.42	
:AdV-NC-48-NAGPUR-2023	94.3	94.3	94.3	94.3	94.3	94.3	94.3	94.3	94.3	94.3	94.3	94.33	94.3	94.3	94.3	94.3	94.3	94.3	94.33	98.97	100	92.27	93.42
:AdV-MO-84-NAGPUR-2023	89.6	89.2	89.6	89.6	89.6	89.6	89.1	89.6	89.6	89.6	89.6	89.57	89.6	89.6	89.6	89.6	89.6	89.6	87.26	92.78	92.3	100	93.42
:AdV-GO-19-NAGPUR-2023	93.4	93.4	93.4	93.4	93.4	93.4	92.8	92.8	93.4	93.4	93.4	93.42	93.4	93.4	93.4	93.4	93.4	93.4	93.42	93.42	93.4	93.42	100

The more intense colour indicates high similarity while as less intense colour indicates lower similarity.

Fig 4: Amino acid per cent identity matrix of partial hexon sequences of FAdV-11 isolates.

2019). Indian poultry industry thrives on the importation of chickens, eggs, feed, poultry machinery, etc., from several countries where FAdV outbreaks have been recorded previously. The close clustering of FAdV strains from this study to the strains reported from Pakistan and China could be due to the aforementioned factors.

Amino acid identity matrix

In the present study, the deduced amino acids showed 87.26-100% sequence identity with other between isolates. The amino acid sequences of FAdV-PI-29-2023, FAdV-KA-35-2023, FAdV-WA-72-2023, FAdV-MT-02-2023, FAdV-SO-86-2023, FAdV-UM-67-2023, FAdV-KO-55-2023 and FAdV-BA-06-2023 showed higher homology (96.3-100%) among themselves and with other global sequences in comparison to FAdV-U-57-2023, FAdV-KH-22-2023, FAdV-NC-48-2023, FAdV-MO-84-2023 and FAdV-GO-19-2023 which showed less similarity (87.26-94.3%) (Fig 4). The amino acid sequences showed higher homology with to FAdV-11 isolates. Similar results by (Xie *et al.*, 2022) reported amino acid sequence identity of 89.5-98.6% among hexon gene sequences of FAdVs.

CONCLUSION

This study offers molecular insights into the prevalence of fowl adenovirus with serotypes 11 and 8b of species FAdV D and E actively circulating, linking them to the ongoing epidemics of inclusion body hepatitis in commercial broiler chickens of Nagpur. Regular screening and monitoring of FAdVs utilizing molecular approaches such as PCR and sequencing are necessary for the detection of circulating serotypes and further assisting in formulating improved immunization strategies to curb the propagation of the disease. Farm biosecurity measures are also necessary to reduce the incidence of FAdV infections in broiler chickens.

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Conflict of interest

The authors declare that they have no conflict of interest.

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