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Construction and Rescue of a Rabies Virus with Duplicated Glycoprotein Gene

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ABSTRACT

Introduction: Rabies is almost always fatal but entirely preventable through proper vaccination. Inadequacy of costly high-quality cell culture vaccines is sometimes a bottleneck for expanded rabies control plans. Reverse genetics along with other molecular biology means are trying to improve the immunogenicity and yield of rabies vaccine products. **Methods:** An additional glycoprotein gene of the rabies virus PV strain was inserted between the glycoprotein and polymerase genes of the virus. The viral proteins were expressed at the T7BHK cell line to rescue the recombinant virus. **Results:** The recombinant virus containing two consecutive glycoprotein genes was rescued from T7BHK cells. The virus particles were functional and successfully infected the permissive BSR cell line. **Conclusion:** The new virus strain with an additive copy of the glycoprotein gene has a good potential to be utilized in different studies, including cell biology and immunological properties of the rabies virus. In this study, the recombinant rabies virus was successfully rescued from cell culture which would pave the way for further investigations on this virus.

Citation:

INTRODUCTION

The fatal encephalomyelitis of rabies is the most lethal infection by case fatality. Rabies occurs in more than 150 countries and territories worldwide causing tens of thousands of annual human deaths, almost half of them being children and adolescents [1]. Several variables contribute to rabies exposure and deaths, with education and awareness among the most significant causes [2, 3]. Dogs are the main source of rabies and are responsible for the transmission of the virus to humans in 99% of the cases. Although there is no cure for clinical rabies, it is 100% vaccine-preventable. Rabies control through vaccination in humans cannot control virus spread as a human is the dead-end rabies host. Dog vaccination, on the contrary, has proven to prevent rabies spread and cut its transmission to other animals and humans if it reached 70% coverage of dog populations. Vaccination of the owned dogs is of critical importance due to their vicinity to humans and higher risk of virus transmission in the case of infection [4-6] . High quality vaccines produced through cell culture technology are needed for mass dog vaccination campaigns. Those vaccines are generally expensive and not affordable in poor rabies endemic areas where they are most needed.

Complete virus inactivation makes the vaccines less advantageous compared to vaccines that contain live attenuated viruses, in terms of immune response induction and duration. Therefore, the inactivated rabies vaccines need to be administered regularly in order to provide effective and sufficient immunity, which compounds their accessibility [7, 8]. Although widely employed in wildlife rabies control operations, live attenuated rabies vaccines are not allowed for human use. Furthermore, abandoning the use of nerve tissue vaccines and its replacement by modern, concentrated, purified vaccines has strongly been recommended by the WHO since 1984 [9]. Modern rabies vaccines are highly priced due to their production complexity and need for multi-dose administration. Although it has been proven to be very cost-effective to administer postexposure prophylaxis (PEP) to victims of a suspected animal bite, these are considered as important factors for their insufficient use in underprivileged regions where they are most needed [10, 11].

Continuous research efforts are being made in order to reach more affordable vaccines while maintaining the proper quality, including vaccine safety and efficacy [12, 13]. The rabies vaccine price has, in certain aspects, directly related with the manufacturing costs. This in turn, is proportional to other factors such as the virus production yield and the vaccine immunizing potency. Certain modifications in rabies virus phenotype through reverse genetics technology have positively affected vaccine immunogenicity in experimental models.

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Certain studies have sought higher stimulation of the immune response through the insertion of immune-stimulatory factors between the viral genes. The rabies virus glycoprotein is the main inducer of the virus neutralizing antibodies. Therefore, certain other studies have intended to induce more protective anti-rabies antibodies and more potent experimental vaccines through duplication of the glycoprotein gene in the viral genome [14, 15].

According to previous studies [16-18], the rabies virus glycoprotein is related to the higher immunity against rabies virus, either due to overexpression of the glycoprotein or through addition of a second glycoprotein into the rabies virus genome. Based on PV-2061 vaccine strain, in the present study we aimed to construct a recombinant rabies virus carrying an additional glycoprotein gene in the intergenic region between the glycoprotein and the polymerase genes.

MATERIAL AND METHODS

Cell lines, Rabies Virus Strain, and Genetic Constructs

Rabies virus strain PV 2061, BHK and T7BHK (expressing the T7RNA Polymerase) cell lines were provided by the rabies vaccines unit of Pasteur Institute of Iran. The presence of T7 RNA Polymerase in T7BHK cell was shown using 4T7A assay vector which was kindly donated by Dr. M. Ghaderi [19]. The full genome construct of rabies virus PV-2061 strain as well as the polymerase complex coding construct including N, P and L were received from the rabies vaccines unit of Pasteur Institute of Iran [20, 21]. All the full genome and N, P and L expressing constructs were designed under the control of T7 promoter.

Priming the Single Cut Full Genome Plasmid

The restriction site of *Dra*III (Thermo, USA) was chosen using NEBcutter software [22] in order to clone an additional copy of the rabies glycoprotein gene in the full genome construct between G and L genes. A second undesired *Dra*III restriction site has existed in the full genome vector construct. Therefore, the mono digest vector was obtained through partial digestion of the full genome vector by means of adjusting the time and the enzyme concentration. Briefly, serial 1:2 dilutions of the enzyme were prepared and added to reaction mixtures. Reactions were stopped at 15 mins intervals and products were resolved by running in a 0.5% agarose gel in TAE buffer (Tris 40mM, Aceate 20mM, EDTA 1mM). The desired mono digest plasmid was recovered from the gel using the QIAEX II gel extraction kit (QIAGEN, USA).

Preparation and Cloning of the Second Glycoprotein Gene

The same glycoprotein gene was amplified from the full genome construct using PCR Mastermix (YTA, Iran) utilizing forward and reverse primers F1 and R1 (Table1). The 5'-CACGGGGTG-3' restriction sites out of two cutting sites for DraIII was impeded at 5' ends of both primers to be used in directional cloning. The PCR product carrying adenine nucleotide hangovers at 3' ends was initially cloned in GetClone™ PCR Cloning Vector II (SMOBIO, Taiwan). The insert fragment flanked with sticky ends was later recovered through digesting the GetClone vector by DraIII restriction enzyme. The fragment was inserted in the intergenic region between G and L genes to form a double glycoprotein (dG) genome. Ligation process was accomplished using T4 DNA Ligase (Thermo, USA) and the relevant buffer plus 5% PEG into the reaction.

Verification of the Glycoprotein Duplication

Verification of gene cloning was performed by colony-touch PCR and enzymatic digestion of the extracted plasmids using *Dra*III. Consequently, two sets of specific primers were designed to verify the cloning. The F2R2 primer pair (Table 1) was designed for the PV and the dG genomes in different PCR product lengths. On the other hand, an F3-R3 primer pair (Table 1) was designed to detect successful insertion of the second G gene through relevant PCR product (Fig 1). The nucleotide sequence of recombinant dG full genome was verified bases on the Sanger sequencing method (Pishgam Biotech Co., Iran). The results were aligned against the reference sequences obtained from GenBank, using Mega Software (version 4).

Table1. The sequence of the primers used in this study. F1R1 primers were used for amplification of the G gene, F2R2 for sequencing the recombinant construct, and F3R3 for verification of cloning.

Forward primer	Reverse primer	Function
ACACACGGGGTGAGACTCAAGGAAAGATGGTT	ACACACCCCGTGATGGAGTTCAAGGAGGACT	Amplify the glycoprotein gene (**F1R1**)
CTGACTGCCTTGATGTTGAT	CTCAGCCCTCTATTTCCTA	Sequencing the recombinant construct and virus, Verification of cloning (**F2R2**)
AGTCCTCCTTGAACTCCAT	AACCATCTTTCCTTGAGTCT	Verification of cloning (**F3R3**)

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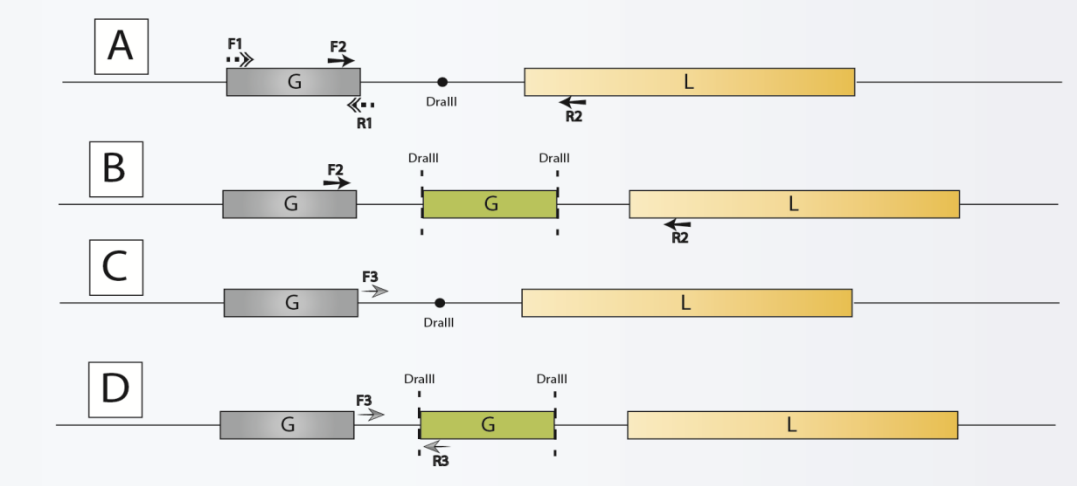

Fig. 1. Design of the cloning and verification primers. **(A)** The glycoprotein gene was primarily amplified from full genome sequence using F1 and R1 primers. **(B)** Two set of primers, namely F2 and R2, were designed to amplify an 1100bp band when the template is the original full genome of rabies virus and another 2700bp band in case of glycoprotein gene duplication in the genome. **(C)** The F3 and R3 primers were designed for another cloning verification PCR. This PCR could not amplify any band from the original PV full genome; **(D)** however, the PCR of the recombinant dG genome could conclude a 694 bp fragment.

Expression of RABV Proteins from cDNA Clone

In order to express the plasmids encoding the virus proteins responsible for rabies virus construction, the T7RNA Polymerase expression in T7BHK cell was primarily confirmed using 4T7A vector [19]. For this mean, 4T7A plasmid was transfected to T7BHK cells using Lipofectamine 2000 (Thermo Fisher Scientific, USA), with different ratios of plasmid and lipofectamine. The best ratio was 1:2 of plasmid and lipofectamine. It revealed the accurate function of T7 RNA polymerase in the T7BHK cell line. In the next step, the full genome construct, along with plasmid constructs encoding N, P and L genes were transfected into T7BHK cell line, using Lipofectamine 2000, according to the instructions of the manufacturer. Briefly, 15 µl lipofectamine with 7.5 µg of DNA were mixed and left for 20 min at room temperature before exposure of the cells to the mixture. The same protocol was used to rescue both the original PV strain and the modified dG virus. The presence of rescued viruses in the cells were assessed by fluorescent antibody test (FAT) using rabies anti-nucleocapsid conjugated antibody (Bio-Rad, USA), according to standard protocols [23]. Briefly, the cells infected with rabies virus were fixed with acetone for 1 h at -20˚C, then dried and incubated for an additional hour with polyclonal FITC-conjugated anti-ribonucleoprotein antibody.

Rabies Virus Rrescue from the Cloned Genes

The protein expression in the transfected cells was considered as an indication for the virus formation. Based on that, after 3 successive passages in T7BHK cells, the rescued viruses were transferred to a monolayer of BHK cells through regular inoculation to assess the functionality and further amplification of the viruses. After 3 virus passages on BHK cells, the final supernatant was collected, and the acquired virus was stored for further investigations. The genomic RNA was extracted from the rescued dG virus using a single-step RNA extraction method [24] and the reverse transcription was carried out using a cDNA synthesis kit (YTA, Iran). The additive glycoprotein of dG virus was amplified using the F2R2 primer set and any possible mutation was checked by nucleotide sequencing (Pishgam, Iran).

RESULTS

The Cloning and Verification of the Construct

The linear form of the full genome construct was obtained by partial digestion of the plasmid through serial dilutions of the *Dra*III enzyme (1:2 to 1:16) (Fig. 2). The PCR amplified glycoprotein was successfully cloned in the GetClone™ cloning vector II (Fig. 3) and subsequently inserted in the linearized full genome. The desired bacterial colonies were selected using after detection by colony PCR method and digestion of the extracted recombinant plasmid by *Dra*III (Fig. 4). The recombinant dG genome construct was further confirmed by sequencing. It showed a successful cloning and precise sequence of the second G in *Dra*III site at the G-L intergenic location.

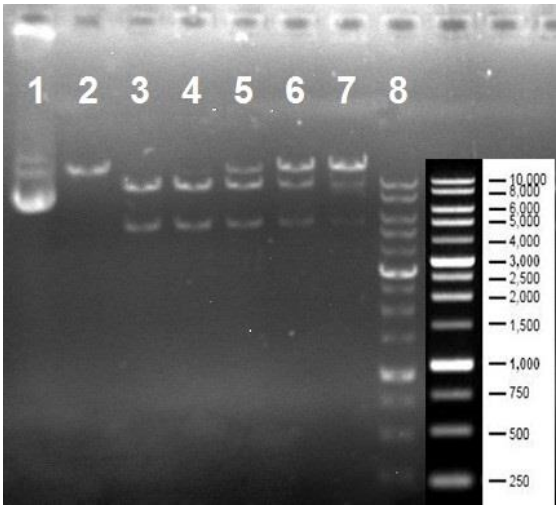

Fig. 2. Partial digestion of full genome construct. Lane1 undigested full genome, Lane 2 mono digested full genome with *Snab*I, Lane 3 to lane 7 dilutions (1:2 to 1:16) of *Dra*II used for full genome digestion, lane 8 1 kb Ladder.

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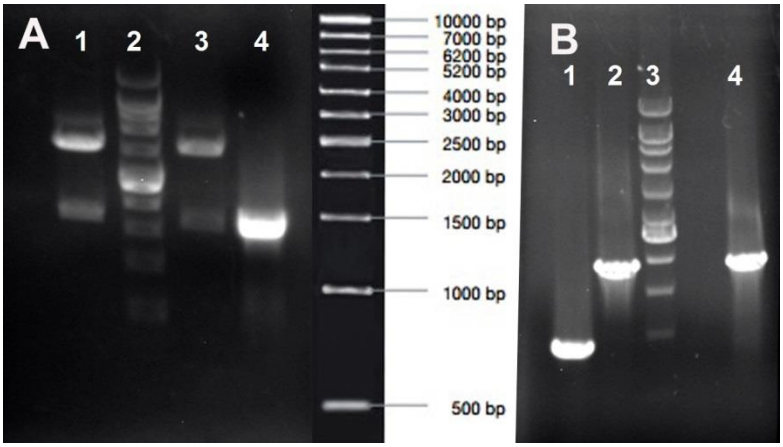

Fig. 3. The PCR amplified glycoprotein initially cloned in GetClone™ PCR Cloning Vector II. **(A)** Digesting the amplified G gene cloned in recombinant pGET vector, using *Dra*III enzyme. Both Lane 1 and lane 3 show proper cloning, Lane2: 1 kb ladder, Lane 4: PCR amplified G gene. **(B)** Colony-Touch PCR on G gene cloned in pGET vector using F1R1 primer set. Lane1: negative colony, Lane 2 and lane 4: positive colonies, Lane 3: 1kb ladder.

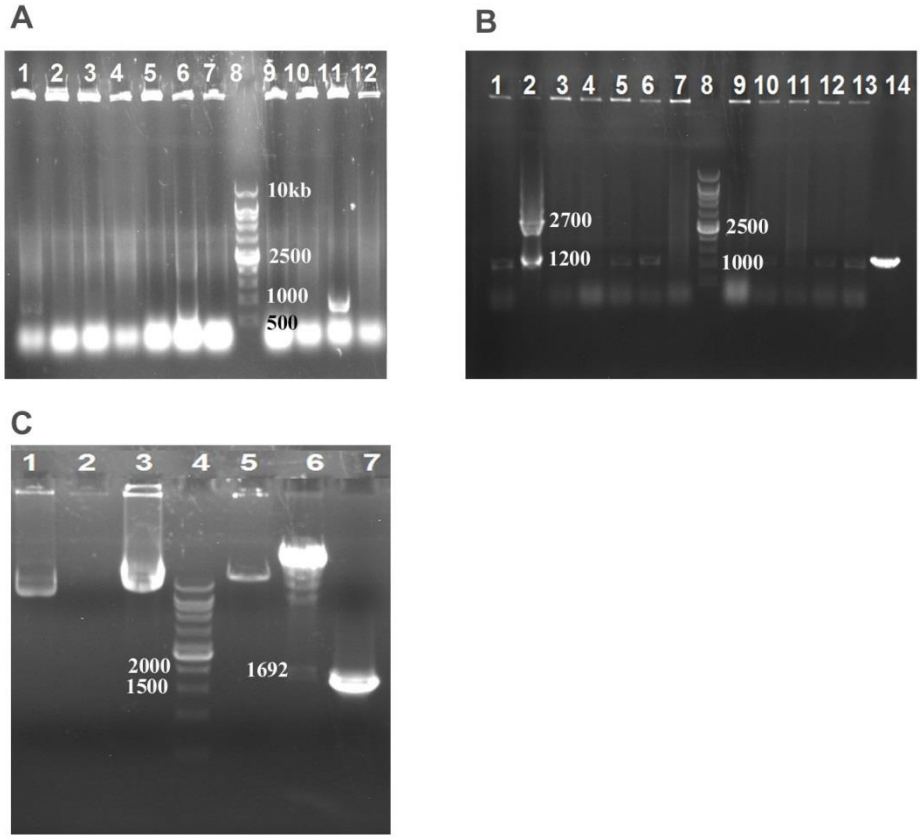

Fig.4. (A) Verification of cloning by colony PCR method using F3R3 primers. The templates for each lane were the colonies acquired from the ligation reaction. Only lane 1 and lane 11 verified the cloning of second G gene**. (B)** Verification of cloning by colony PCR method using F2R2 primers. Only lane2 shown a band on 2700bp which confirms the cloning of second G gene. Lane8: ladder, lane14: Negative control (linear full genome construct). **(C)** Verification of cloning by digesting with *Dra*III. Lane1: PV full genome construct, lane3: recombinant dG construct, lane4: 1kb ladder, lane5: PV full genome construct linearized using *Sna*bI, lane6: recombinant dG construct digested with *Dra*III, lane7: PCR amplified G gene**.**

Rescuing the Rabies Viruses

In order to rescue both the PV and the recombinant dG viruses, any of the relevant full genome constructs were transfected to T7BHK cells along with vectors expressing N, P, and L proteins of the virus necessary for polymerase complex formation. Our results showed that both rescued viruses (PV and dG), were functional and could ordinarily infect the BSR cells after 3 virus passages on T7BHK cells. Normal inoculation of BSR cells showed that the immunofluorescence staining was due to the virus infection and not limited to N, and P proteins, expressed by the transfected vectors. As shown in Fig. 5, the BSR cells infected by the rescued recombinant dG

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and the rescued PV strains of the rabies virus. Verifying the selected recombinant clones by different methods, including colony PCR, restriction digestion as well as DNA sequencing showed that the additional glycoprotein was correctly inserted in the genome with no point mutation.

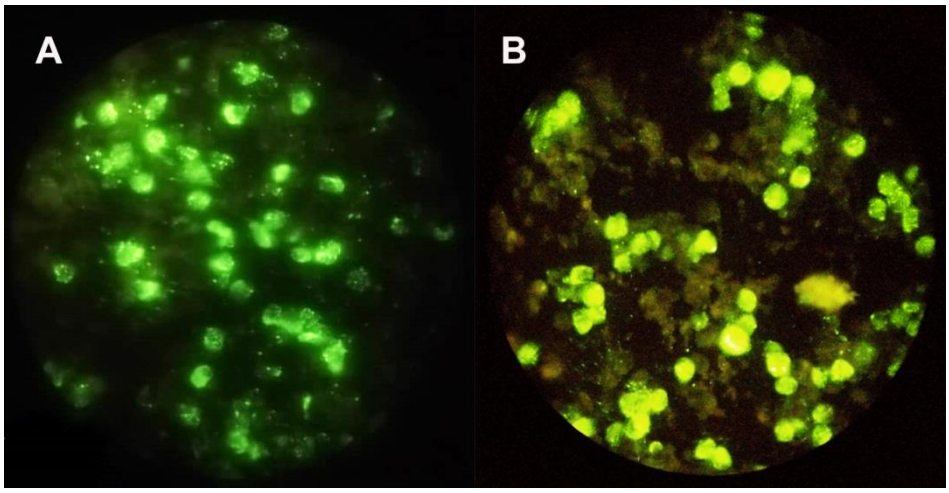

Fig. 5. BSR cells infected by the rescued recombinant dG and PV viruses through standard virus inoculation protocols. **(A)** BSR cells infected with rescued recombinant dG virus. **(B)** BSR cells infected with the rescued PV strain virus.

DISCUSSION

The present study demonstrated that a recombinant rabies virus with an additional glycoprotein (Gp) gene was successfully constructed and rescued. The second Gp was inserted between the glycoprotein and the polymerase genes. The recombinant virus demonstrated the ability to infect cells permissive to the rabies virus. Rabies virus is a pathogen infecting the nervous system in all mammals, with 59'000 annual human deaths, mostly in unprivileged regions of the globe [25, 26]. Implementation of standard vaccination protocols utilizing high-quality vaccines could vastly control rabies. However, those vaccines are not always easily affordable where they are most needed.[27]. Therefore, scientists seek new means to develop efficient and affordable rabies vaccines, based on virus-like particles, epitope-based, nucleic acid-based, simian adenovirus, and chimeric rabies [12, 28-30]. Accordingly, certain studies have shown that the glycoprotein gene duplication in the rabies virus could confer better protection in dogs against a challenge by rabies virus, compared to a commercial vaccine [17, 31]. Two different locations of the rabies virus genome have been compared to insert an additional glycoprotein, with NPGMGL and NPMGGL rabies virus gene orders. In a study by Navid et al., it has been found that insertion of an additional G between the G and L residues conferred higher virus titer and immunogenicity to the recombinant virus [18]. In the present study, the same region was used for the glycoprotein insertion, and the latter gene order was obtained. The G-L intergenic region has also has been shown to possess the capacity to incorporate other genes in the virus genome [32]. Rabies virus Gp is a type 1 membrane glycoprotein with a crucial role in attachment, infectivity, virus budding, and inducing the virus-neutralizing antibodies and immunogenicity against the virus [33, 16]. Particular protein and non-protein receptors are reported to be involved in the virus uptake, with the nicotinic acetylcholine receptors (nAChR) as the main receptors for rabies virus Gp [34, 33]. The rescued recombinant virus in the present study showed in vitro properties comparable to those of parental virus, concerning the cell infection and growth. Normal infectivity of the recombinant virus suggested no attachment hindrance due to duplication of the Gp. Therefore, while the relevant vaccine could raise better immune protection, we expected it to have a similar neurotropism index and virulence in laboratory animals. It has been reported that the overexpression of the rabies Gp and its accumulation in cells have been associated with higher rates of apoptosis [35, 36]. Rabies virus Gp has a pivotal role in inducing apoptosis and antiviral immunity [37, 38]. In a study Faber et al. have reported a recombinant strain of rabies virus CVS in which the pseudogene was replaced with an extra Gp gene and higher expression of the Gp, increased immunogenicity, higher induction of VNAs, and higher induction of apoptosis in host cells that subsequently resulted in decreased titer of the virus, are documented [16]. However, in the current study, the PV strain of the rabies virus was used which has distinct properties compared to the CVS. Therefore in this study, one might expect different antiviral immunity or apoptosis behavior associated with the recombinant virus.

In conclusion, a PV strain of the rabies virus possessing a duplicated Gp gene was constructed and rescued in the current study which could serve as a platform to study the rabies virus's cell biology. It could also be used to study the immunological properties, either in terms of rabies pathogenesis or the vaccine production. Detailed in vitro and in vivo characterizations would be needed in order to verify whether the dGp virus can strengthen the immunogenic properties of the vaccine strain prior to use in the animal studies. Moreover, since there have been many reports on higher virus titer and higher apoptosis rate conferred by duplicating the glycoprotein of the rabies virus, the repetitive series of virus titration would be informative for the future studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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