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Detection and Molecular Characterization of *Tobacco mild green mosaic virus* Isolates Infecting Peppers in Turkey

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ABSTRACT

In this study, *Tobacco mild green mosaic virus* (TMGMV) infection was investigated in pepper crops from two regions having high economical importance in Turkey. A total of 397 leaf and fruit samples showing mosaic, yellowing, mottling, and pitting symptoms were collected to test by double antibody sandwich ELISA (DAS-ELISA) using polyclonal antiserum. DAS-ELISA results indicated that 97 out of all tested samples (24.4%) were found to be infected with TMGMV. Samples resulting positive for TMGMV infection were used to amplify the virus coat protein (CP) gene sequences with specific primers by RT-PCR for further molecular characterization of the virus isolates. A comparison of the CP sequences of the virus isolates revealed that the identity of nucleotides ranged between 97.2 and 100%, and the amino acid similarity ranged between 96.8 and 100% among themselves. To the best of our knowledge, this is the first report of TMGMV infection in *Capsicum annuum* in Turkey.

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Türkiye'de Biberde Enfeksiyon Oluşturan *Tobacco mild green mosaic virus* İzolatlarının Belirlenmesi ve Moleküler Karakterizasyonu

ÖZET

Bu çalışmada, Türkiye'de biber yetiştiriciliğinin ekonomik olarak önemli olduğu iki bölgede *Tobacco mild green mosaic virus* (TMGMV) enfeksiyonu incelenmiştir. Mozaik, sararma, beneklenme ve çukurlaşma simptomu gösteren toplam 397 yaprak ve meyve örneği toplanmış ve double antibody sandwich ELISA (DAS-ELISA) ile poliklonal antiserum kullanılarak testlenmiştir. DAS-ELISA testinde testlenen örnekler içerisinde 97 örneğin (%24.4) TMGMV enfeksiyonuna sahip olduğu belirlenmiştir. TMGMV ile enfekteli pozitif örnekler, gen spesifik primerler kullanılarak RT-PCR yöntemiyle virüs kılıf protein (KP) geninin çoğaltılması ve izolatların moleküler karakterizasyonu için kullanılmıştır. KP nükleotid dizilerinin karşılaştırması sonucunda, izolatların kendi aralarında nükleotid benzerliğinin %97.2-100, amino asit benzerliğinin ise %96.8-100 arasında değiştiği belirlenmiştir. Bildiğimiz kadarıyla, bu Türkiye'de TMGMV'nin enfeksiyonunun *Capsicum annuum*'daki ilk raporudur.

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INTRODUCTION

Pepper (*Capsicum annuum* L.) is the second most cultivated vegetable crop (2.636.905 tonnes) in Turkey (Faostat, 2020) where pepper is cultivated both in open field and greenhouse. Turkey is the world's third-largest producer of green pepper, so pepper plays important role in global trade. Viral diseases are significant constraint in pepper cultivation affecting the yield and quality of pepper fruits (Green and Kim, 1994). In the last 30 years, the

number and prevalence of infecting virus species in pepper growing areas in tropical and subtropical regions have increased remarkably. To date, more than 70 virus species have been reported infecting pepper throughout the world (Pernezny et al., 2003; Kenyon et al., 2014). Among these, about 20 virus species belonging to 15 different taxonomic groups cause economic damage to pepper cultivation in the Mediterranean basin (Moury and Verdin, 2012). The most important of these viruses are seed-borne tobamoviruses, insect-transmitted potyviruses, cucumoviruses, and tospoviruses (Moury and Verdin, 2012). Tobamoviruses are highly contagious and stable group of viruses causing significant yield losses in commercial pepper crops (Alonso et al., 1989; Smith and Dombrovsky, 2020). They are found all over the world and can be spread through infected seeds, infected soil, and mechanical contact between plants, but not transmitted by vectors (Broadbent, 1965; Lanter et al., 1982; Pares et al., 1996). There are 37 members within the *Tobamovirus* genus, and out of 8 are known to infect peppers (Kenyon et al., 2014; Fidan et al., 2021). Among them, *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), and *Pepper mild mottle virus* (PMMoV) are the most prevalent viruses in pepper production in Turkey (Ozaslan et al., 2006; Şevik, 2011; Şimsek et al., 2015).

Tobacco mild green mosaic virus (TMGMV) belongs to the genus *Tobamovirus,* and its genome consists of a positive-sense single-stranded RNA. Its genomic organization is similar to that of TMV and ToMV. The genomic RNA of TMGMV is 6355 nt long that encodes four proteins, namely; the 126K and 183K replicases, 28.5K protein homologous to TMV and ToMV the movement proteins (MP), and 17.5K coat protein (CP) (Solis and Garcia-Arenal, 1990). TMGMV has been recently isolated from tobacco plants in Turkey (Karanfil et al., 2020). In the current study, the occurrence of TMGMV was investigated in pepper plants in Turkey, and molecular experiments were carried out to have a preliminary result for the characterization of its isolates based on the coat protein gene. To our knowledge, this is the first report of natural infection by TMGMV in pepper crops.

MATERIALS and METHOD

Survey and sample collection

Surveys were conducted in commercial greenhouses and open fields in the Eastern Mediterranean (Mersin, Adana, Kahramanmaraş, Hatay), and Southeastern (Gaziantep, Kilis) regions of Turkey, where pepper production is economically important, during 2017-2020 growing seasons. A total of 397 leaf and fruit samples showing vein clearing, mosaic and discoloration of young leaves, and fruit distortion symptoms (Figure 1) were collected during surveys.

DAS-ELISA

All samples were ground in four volumes of 0.03 M phosphate buffer (pH 7.0) (wt/vol) supplemented with 0.2% diethyldithiocarbamate in extraction bags. The extracts were tested in DAS-ELISA (Clark and Adams, 1977) for the presence TMV, ToMV, *Paprika mild mottle virus* (PaMMV), PMMoV, and TMGMV using polyclonal antiserum (Loewe Biochemica GmbH, Germany). Absorbance at 405 nm was determined using a plate reader (Seac Sirio S). The samples were considered as positive if their average absorbance value were equal to or higher than twice that of the negative control.

Mechanical transmission of TMGMV

The sap from pepper source carrying TMGMV infection only was mechanically inoculated onto *Nicotiana tabacum* cv. Samsun plants at 2-4 leaf stage by rubbing infected leaf extract prepared in four volumes of 0.03 M phosphate buffer (pH 7.0) containing 0.2% (wt/vol) diethyldithiocarbamate, active charcoal at 20 mg/ml, and carborundum at 20 mg/ml (Moury et al., 2004). Negative control plants were prepared with mock inoculation and carborundum. Each plant species had three replicates. Inoculated plants were kept in a growth chamber with 16 h photoperiod and constant temperature of 25°C, and the symptom expression was monitored for 3 week-post inoculation.

Total RNA extraction and reverse transcription-polymerase reaction (RT-PCR)

Total RNA was isolated from TMGMV-infected *Nicotiana tabacum* cv. Samsun plants according to Chomczynski and Sacchi (1987). RNAs were reverse-transcribed with the specific reverse primer using M-MLV reverse transcriptase (Promega) for the synthesis of the first-strand cDNA. PCR amplification was carried out with the primer combination (TMGMVspec/Tob-Uni1) (Letschert et al., 2002) specific to the complete coat protein (CP) region. PCR amplicons were directly sequenced by the Sanger method with both primers in two directions (Medsantek, Turkey).

Nucleotide sequences and phylogenetic analysis

Among the TMGMV isolates, 19 were sequenced, and have been deposited in the GenBank database with the accession numbers OK182752- OK182770 (Table 1). Multiple nucleotide alignments were conducted using CLUSTALX 1.8 (Thompson et al., 1994). Phylogenetic tree was constructed with Maximum likelihood method using MEGA 7 software (Kumar et al., 2016) with 1000 bootstrap replicates to assess the robustness of the nodes.

Table 1. The accesion numbers of *Tobacco mild green mosaic virus* (TMGMV) isolates obtained in this study
Çizelge 1. Çalışmada elde edilen Tobacco mild green mosaic virus (TMGMV) izolatlarının erişim numaraları

Accession no	Isolate	Location	Year
OK182752	TMGMV-Adn26	Adana	2017
OK182753	TMGMV-Adn32	Adana	2017
OK182754	TMGMV-Adn16	Adana	2017
OK182755	TMGMV-Adn40	Adana	2018
OK182756	TMGMV-Adn45	Adana	2018
OK182757	TMGMV-Adn21	Adana	2017
OK182758	TMGMV-Adn53	Adana	2018
OK182759	TMGMV-Mrs61	Mersin	2018
OK182760	TMGMV-Mrs80	Mersin	2018
OK182761	TMGMV-Mrs84	Mersin	2018
OK182762	TMGMV-Mrs86	Mersin	2018
OK182763	TMGMV-Mrs116	Mersin	2018
OK182764	TMGMV-Mrs49	Mersin	2018
OK182765	TMGMV-Mrs62	Mersin	2018
OK182766	TMGMV-Gza125	Gaziantep	2020
OK182767	TMGMV-Kls280	Kilis	2020
OK182768	TMGMV-Khr180	Kahramanmaraş	2018
OK182769	TMGMV-Khr3	Kahramanmaraş	2020
OK182770	TMGMV-Khr261	Kahramanmaraş	2020

RESULTS and DISCUSSION

Symptom observation and TMGMV incidence

Infected plants showing severe symptoms consisting of chlorosis, curling, necrotic lesions, and mottling were occasionally found in the field (Figure 1). The plants were small and stunted, compared with healthy plants. Under open-field conditions, symptoms were recognized at an early stage of plant development; however, under greenhouse conditions, symptoms were observed at the advanced growth stage. Tobacco plants mechanically inoculated with each isolate showed mild green and chlorotic mosaic symptoms by 3–5 days post-inoculation (Figure 2).

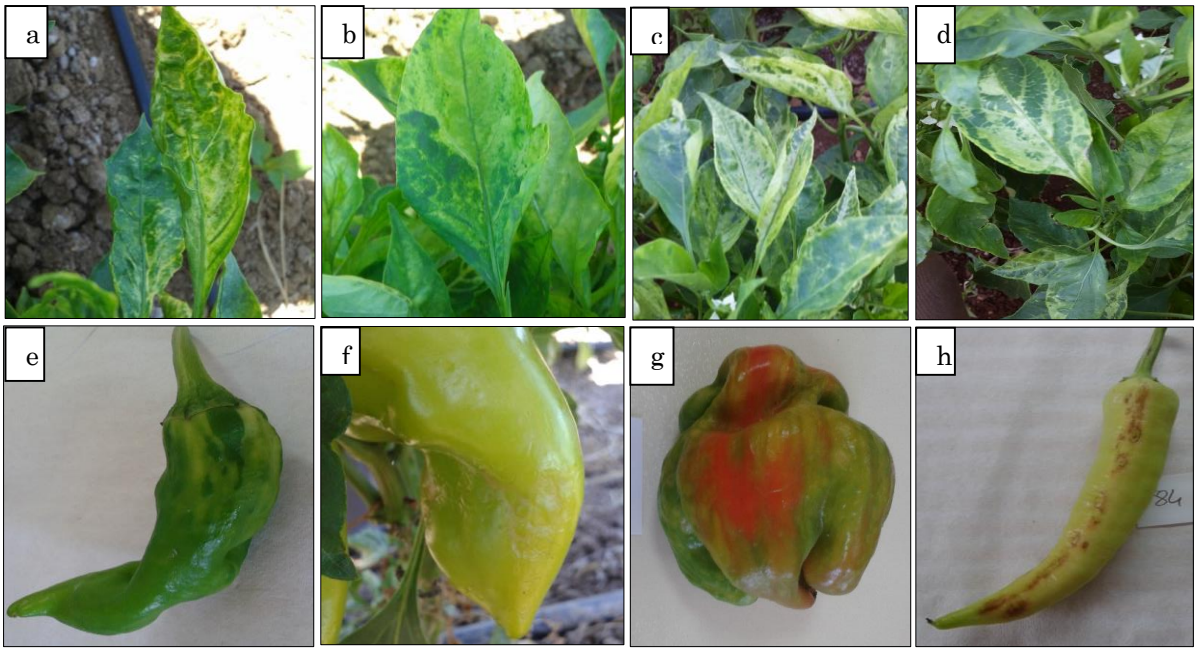

Figure 1. Field symptoms of TMGMV-infected pepper leaves and fruits. (a-d: mosaic and yellowing on pepper leaves, e: mosaic, f: pitting, g: mottle, and h: necrosis on pepper fruits)
Şekil 1. TMGMV ile enfekteli biber yapraklarının ve meyvelerinin simptomları. (a-d: yapraklarda mozaik ve sararma, meyvelerde e: mozaik, f: çukurlaşma, g: beneklenme, h: nekroz

Figure 2. Symptoms consisted of mild green and mosaic on *N. tabacum* cv. Samsun leaves
Şekil 2. N. tabacum cv. Samsun yapraklarında oluşan açık yeşil ve mozaik simptomları

DAS-ELISA results revealed that 97 samples out of 397 tested (24.4%) were infected with TMGMV. TMGMV was found in 8 samples from 49 collected in Adana, 14 samples from 81 collected in Gaziantep, 24 samples from 62 collected in Hatay, 15 samples from 112 collected in Kahramanmaraş, 26 samples from 77 collected in Kilis, and 10 samples from 16 collected in Mersin (Table 2).

Table 2. The presence of viruses in pepper samples collected during surveys according to DAS-ELISA results
Çizelge 2. DAS-ELISA sonucuna göre surveylerde toplanan biber örneklerinde virüslerin bulunma durumu

Location	Number of tested plants	Number of TMGMV infected plants (infection rate %)	Number of mixed infected plants			
			TMGMV+ TMV	TMGMV+ ToMV	TMGMV+P aMMV	TMGMV+P MMoV
Adana	49	8 (16.3%)	5	1	-	6
Mersin	16	10 (62.5 %)	4	3	-	8
Hatay	62	24 (38.7 %)	5	7	-	10
Kahramanmaraş	112	15 (13.4 %)	3	2	2	7
Gaziantep	81	14 (17.3 %)	9	4	-	11
Kilis	77	26 (33.8 %)	5	8	5	16
Total	**397**	**97 (24.4 %)**				

Multiple sequence comparisons and phylogenetic relationships

The nucleotide sequences of the CP gene derived from the 19 TMGMV isolates were subjected to sequence alignment and phylogenetic analysis. The DNA fragment (480 bp) containing the full length of CP was used to determine sequence identity at both the nucleotide and amino acid levels. The Turkish TMGMV isolates shared nucleotide identity of 97.2 to 100% with each other, and 96.6 to 100% with the sequences registered in GenBank (Figure 3a) while the amino acid similarities were between 96.8-100% and 95.5-100% (Figure 3b), respectively. The phylogenetic analysis showed that TMGMV-Adn26, TMGMV-Khr3, TMGMV-Adn16, TMGMV-Mrs49, and TMGMV-Adn45 isolates were closely related to the pepper isolates from Spain (FN594860.1). The isolates (TMGMV-Mrs80, TMGMV-Mrs84, TMGMV-Gza125) had the highest nucleotide identity (100%) with the pepper isolates from China (MF139550.1, JX534224.2) and Vietnam (MW012408.1). It should be noted that TMGMV-Adn40 isolate clustered with Turkish tobacco isolate (MK944273.1) was distantly related to the other pepper isolates (Figure 3c).

In recent years, increasing epidemics of tobamoviruses have started to be reported in commercial pepper fields in Turkey (Çağlar et al., 2013; Buzkan and Arpacı, 2017). The mixed infections of TMGMV with TMV, ToMV, (PaMMV), and PMMoV were detected during this survey (Table 2). Due to their high stability, tobamoviruses remain infectious in contaminated plant debris, compost, soil, and irrigation water. As mentioned by Salamon and Kaszta (2000), pollen grains also play an imported role in the distribution of tobamoviruses throughout the world. In terms of the importance of pepper growing in Turkey, there is a high risk of devastating consequences both in greenhouses and in open fields. However, the infected seeds and plantlets could provide an inoculum source for the initiation of disease epidemics in the crops. Especially, the use of population seeds (taking seeds from the fruits of the previous year) in open pepper cultivation in Turkey plays a very effective role in the spread of these seed-borne viruses. To prevent introductions of tobamoviruses, seeds can be disinfected by using 2% NaOH (Salamon and Kaszta, 2000) or with 10% trisodium phosphate (Na_3PO_4) for 2.5 hours at room temperature (Rast and Stijger, 1987; Jarret et al., 2008). Virus-contaminated disposable materials should be destroyed by burning out or deep burial. Because of the potential for this virus to cause significant losses in other susceptible plants, the hosts and distribution of the agent should be carefully monitored. Host resistance, phytosanitary and cultural control measures should be combined for the management of virus diseases.

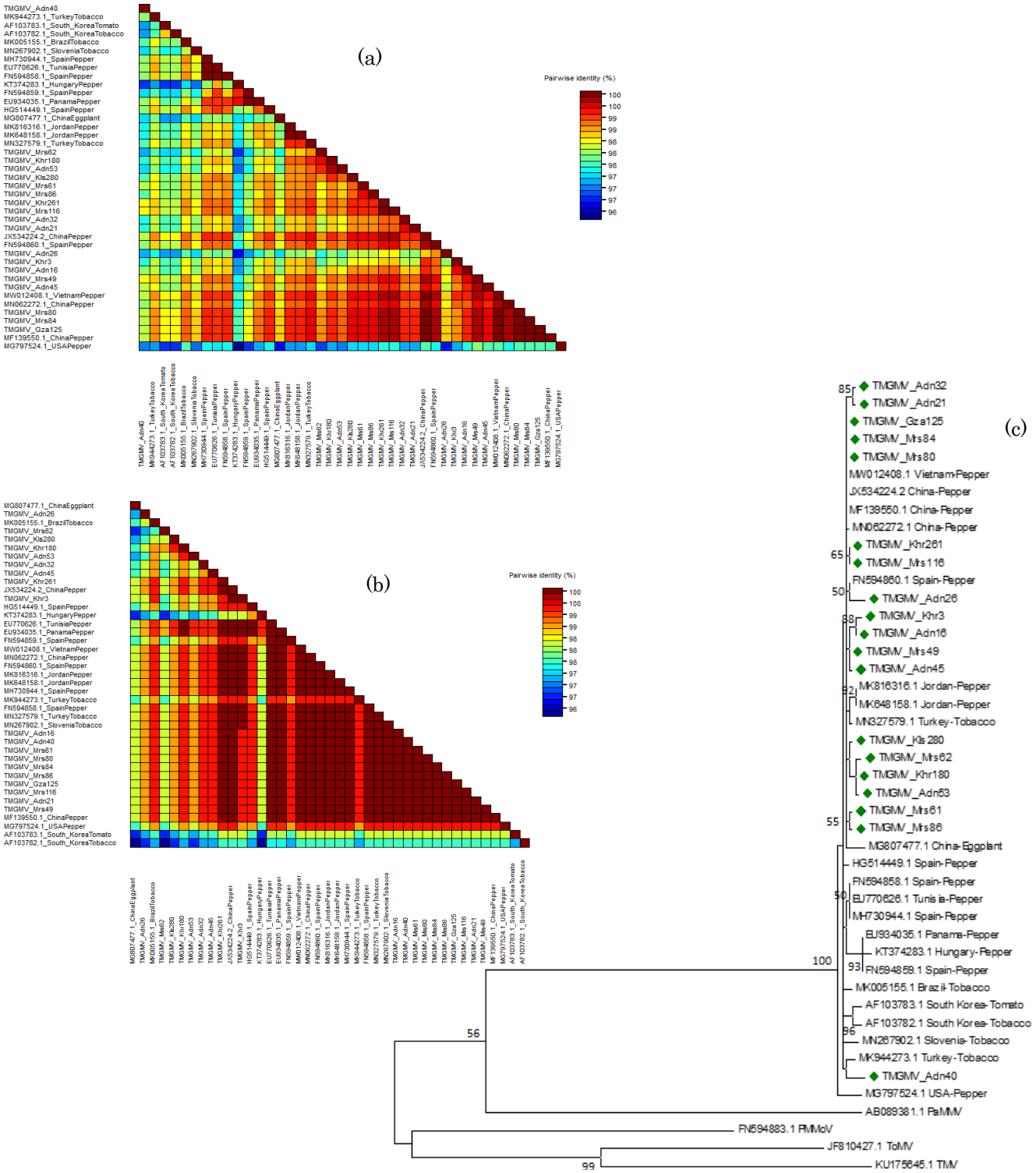

Figure 3. Nucleotide (a) and amino acid (b) sequence identity matrix generated using Sequence Demarcation Tool (SDT). Phylogenetic analysis of TMGMV isolates (c). The KU175645.1 (TMV), AB089381.1 (PaMMV), FN594883.1 (PMMoV), JF810427.1 (ToMV) pepper isolates were used as an outgroup. TMGMV isolates obtained in this study are labeled with symbol ( ).

Şekil 3. Sequence Demarcation Tool (SDT) kullanılarak oluşturulan nükleotid (a) ve amino asit (b) dizin benzerliği matriksi. TMGMV izolatlarının filogenetik analizi (c). KU175645.1 (TMV), AB089381.1 (PaMMV), FN594883.1 (PMMoV), JF810427.1 (ToMV) biber izolatları dış grup olarak kullanılmıştır. Bu çalışmada elde edilen TMGMV izolatları ( ) sembolü ile işaretlenmiştir.

CONCLUSION

In this study, the DAS ELISA test results showed that the TMGMV had a remarkable prevalence in open-field and greenhouse pepper-growing areas in the Eastern Mediterranean region (Mersin, Adana, Kahramanmaraş, Hatay) and the Southeastern region of Turkey (Gaziantep, Kilis). It has been determined that nucleotide sequences of TMGMV isolates showed high sequence identity with each other and pepper isolates from different countries, except for TMGMV-Adn40 isolate.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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