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Occurrence of *Tomato Brown Rugose Fruit Virus* in Lorestan Province Tomato Greenhouses: A First Report

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ABSTRACT

Tomato brown rugose fruit virus (ToBRFV) is a recently identified, highly contagious, and destructive *Tobamovirus* that primarily infects tomatoes. To investigate the presence of this virus, forty leaf samples were collected from plants exhibiting viral symptoms such as mosaic, leaf malformation, and blistering from the tomato greenhouses in Lorestan Province in 2024. Mechanical inoculation on *Nicotiana benthamiana* and *Solanum lycopersicum* was done, and symptoms appeared as a systemic mosaic on *N. benthamiana* seven days post-inoculation (dpi), while tomato plants exhibited mosaic, blistering, severe deformation, and shoestring-like symptoms, particularly in apical leaves, by 10dpi. Then, an RT-PCR assay was operated on the samples, and a 623-base pair fragment was successfully amplified utilizing ToBRFV-specific primers corresponding to the coat protein gene region. Sequence BLAST analysis confirmed that the Lorestan isolates exhibited a nucleotide sequence identity ranging from 99.32% to 99.83%, as did other isolates in GenBank. The phylogenetic tree generated using MEGA11 software indicated that ToBRFV isolates clustered into two distinct groups, with the isolates from Lorestan province classified as group II. Within group II, two Lorestan isolates clustered alongside three other Iranian isolates. The length of the branches in this group suggests that mutations are present in these isolates compared to group I. Isolates from other countries were classified into a distinct subgroup within the group I. The two-by-two comparison of nucleotide sequences using SDT v1.2 software demonstrated the Lorestan isolates exhibited the highest genetic similarity to the Iranian isolates ZAR-K-20 and Zar-Kp, with identities ranging from 99.30% to 99.50%. In contrast, the Lorestan isolates demonstrated the greatest genetic distance from the Peru isolate, which had an identity of 98.80%. This report represents the first documented ToBRFV detection instance in Lorestan Province tomato greenhouses.

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Introduction

Tomatoes are one of the biggest and most economically important species of vegetables because of their high nutritional value. They are scientifically known as *Solanum lycopersicum*, which belongs to the *Solanaceae* family. Iran ranks as the seventh-largest tomato producer in the world, with an annual output of approximately 5.8 million tons (Canton, 2021). This crop is significant due to its high nutritional value, which is attributed to its phytochemical

compounds, such as antioxidants (Ali *et al*., 2020). Like other agricultural products, tomatoes are susceptible to damage from a range of destructive pathogens, especially viruses (Jones and Naidu, 2019). *Tobamovirus* is an important viral disease group encompassing several destructive plant viruses (Fuji *et al*., 2022). In recent years, a new virus from this group has emerged as a significant threat due to its potential for severe damage and rapid transmission (Jones, 2021).

In 2016, indicative symptoms of a *Tobamovirus* that impacted crop marketability were widely observed in a greenhouse in Jordan. This situation prompted experts to employ molecular methods for pathogen identification, ultimately leading to the official designation of the virus as *Tomato brown rugose fruit virus* (ToBRFV) (Salem *et al*., 2016). In controlled environments, such as greenhouses, mechanical transmission is the primary mode of spread of this virus. Transmission often occurs via infected soil residues, water circulation, and contact with infected tools used by farmers and workers (Oladokun *et al*., 2019). Tomato seeds sourced from fruits infected with ToBRFV are entirely contaminated, although the virus is only detectable on the seeds' outer coating (Salem *et al*., 2022). The global trade of tomatoes has facilitated the rapid spread of this virus through contaminated fruits and seeds across numerous countries (Abrahamian *et al*., 2022) including Mexico (Cambrón-Crisantos *et al*., 2019), Germany (Menzel *et al*., 2019), Italy (Panno *et al*., 2019), Turkey (Fidan *et al*., 2019), the USA (Ling *et al*., 2019), the Netherlands (Van De Vossenberg *et al*., 2020), and Spain (Alfaro-Fernández *et al*., 2021).

The first signs of this virus were reported in the tomato greenhouses of Isfahan city, Iran, in 2021 (Ghorbani *et al*., 2021). ToBRFV was also identified in bell pepper greenhouses near Tehran in 2022 (Esmaeilzadeh and Koolivand, 2022b). Then, it rapidly spread to several tomato and pepper greenhouses across Iran, including regions such as Alborz, Ardabil, East Azerbaijan, Golestan, Markazi, Qazvin, Yazd, and Zanjan (Bananej *et al*., 2024).

Tomato plants infected with ToBRFV may display symptoms ranging from mild to severe mosaic patterns, anatomical abnormalities on the leaf surfaces, and vein clearing. This disease causes bumps, deformation, and irregular fruit ripening, accompanied by brown spots, leading to a reduction in quality and commercial value (González-Concha *et al*., 2021).

ToBRFV, a member of the *Virgaviridae* family, possesses a rod-shaped structure and contains a single-stranded positive RNA genome that encodes four open-reading frames (ORFs). ORF1 and ORF2 are directly translated from the genomic RNA and are involved in genome replication. In contrast, ORF3 and ORF4 encode the movement and coat proteins translated from subgenomic RNA (Salem *et al*., 2022; Hasanvand & Pakbaz, 2022). Given the numerous reports of ToBRFV in Iran and the neighboring provinces of Lorestan, this study used biological and molecular methods to investigate the occurrence of infection with this virus in Lorestan.

Materials and Methods

Samples collection

During visits to the tomato greenhouses and fields in Lorestan Province (specifically in Khoramabad, Aligoudarz, Poldokhtar, and Boroujerd cities, 10 samples from each city) in 2024, samples were collected from plants exhibiting viral symptoms such as mosaic, leaf malformation, shoestring in leaves (narrowing), reduced leaf blade size, rolling, blistering, and bubbling. Forty leaf samples were transported to the laboratory on the ice, and a portion of each sample was stored in a -80℃ freezer for future use.

Mechanical transmission

For biological assays, the seeds of *Nicotiana benthamiana* were planted and grown in a greenhouse until they reached the 3-4 leaf stage. Mechanical inoculation was performed by grinding the suspected samples in 0.1 M potassium phosphate buffer (pH 7.0) and applying carborundum (500 mesh) to the tobacco seedlings. Inoculation was also performed on healthy tomato seedlings (*S. lycopersicum*) at the 4- 5 leaf stage to maintain the virus. Following the rubbing process, the leaves of the inoculated plants were rinsed with distilled water. The inoculated plants were then placed in an insect-proof greenhouse, maintained at a temperature

range of 22-25ºC, with a photoperiod of 16 hours of light and 8 hours of darkness and relative humidity of 50-70%. During the experiment, yellow cards, netting, and insecticides were employed to prevent the transmission of other viruses to these plants. The presence or absence of the virus in the inoculated leaves was assessed seven days post-inoculation (dpi).

RNA extraction and RT-PCR

Total RNA was extracted from 100 mg of fresh symptomatic leaf tissue using the RNX Plus Kit (SinaClon Co.Iran) for all collected samples. The extracted RNA was then used to synthesize first-strand cDNA with the First Strand cDNA Synthesis Kit (SinaClon Co., Iran) and a reverse primer, following the manufacturer's instructions. To detect the presence of ToBRFV, reverse transcriptase polymerase chain reaction (Rt-PCR) was performed using specific primers ToBRFV-F-5722 (5'-CACAATCGCAACTCCATCGC-3') and ToBRFV-R-6344 (5'- GTGCCTACGGATGTG TATGA-3') to amplify a 623-bp fragment corresponding to the coat protein gene (Yan *et al*., 2021).

PCR amplification was performed in a final volume of 25 microliters using a 2x PCR Bio Taq Mix Red kit (PCR BioSystem Co., England). The thermal cycling program for amplifying the desired fragment consisted of an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 35 seconds, annealing at 56°C for 35 seconds, and elongation at 72°C for 35 seconds. A final extension step was carried out at 72°C for 10 minutes. Sterile distilled water was used instead of cDNA in the negative control sample. The PCR products were analyzed by 1% agarose gel electrophoresis alongside a 100bp standard molecular marker (Pishgam Co., Iran). They were visualized using DNA Safe stain solution (Pishgam Co., Iran) in the gel documentation.

Phylogenetic analysis

Two PCR product samples were selected as representatives and sent for purification and sequencing to Macrogen in South Korea. The resulting nucleotide sequences were compared with existing sequences in GenBank using the nucleotide blast tool (nBLAST) available on the National Center for Biotechnology Information (NCBI) website. After assessing the sequence quality and editing their ends with Chromas and BioEdit software, the finalized sequences were used to construct a phylogenetic tree. Several complete coat protein (CP) sequences from other ToBRFV isolates were taken from the NCBI database to determine the phylogenetic position of the virus isolates identified in Lorestan Province. For this analysis, multiple sequence alignment was conducted using the ClustalW tool in MEGA version 11 software, and the phylogenetic tree was generated using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates. Additionally, pairwise nucleotide comparisons were performed using Sequence Demarcation Tool version 1.2 (SDT v1.2) to calculate the nucleotide sequence identity matrix (Muhire *et al*., 2014).

Results and Discussion

Observation and mechanical transmission

Tomato plants that were naturally infected in the greenhouse exhibited characteristic symptoms, including leaf mosaic (Fig. 1c), blistering, reduced leaf blade size, and deformation (Fig. 1b and c). The severity and type of symptoms varied depending on the cultivar in the greenhouses. Variety 4129 (Seminis Vegetable Seeds, USA) was one of the cultivars affected by ToBRFV in the tomato greenhouses of Lorestan Province. The symptoms observed in this study were similar to those of viral infections identified on the leaves and fruits of imported tomato seeds from several cultivars, including Emperador RZF1 (Netherlands), Eshkol, and 4129 (USA), in the greenhouses of Isfahan province. These symptoms included severe mosaic patterns and deformations of the leaves, particularly affecting the younger foliage (Ghorbani *et al*., 2021). Additionally, similar symptoms, including chlorosis, severe mosaic patterns, and blistering, were observed in tomatoes cultivated in several greenhouses around Tehran, Iran. Mature tomato fruits also displayed yellow spots and a rugose texture (Esmaeilzadeh and Koolivand, 2022a).

After dpi, the inoculated plants demonstrated symptoms of ToBRFV. Systemic mosaic symptoms were observed in *N. benthamiana*

seven days following mechanical inoculation (Fig. 2f) and ultimately resulted in the decline of the plant after about two months. Following the findings of Yan *et al*. (2021), *N. benthamiana* was destroyed after exhibiting leaf yellowing and necrotic lesions. Furthermore, tomato plants showed mosaic symptoms (Fig. 2d and 2e), blistering and bubbling (Fig. 2b and 2c), severe deformation, and shoestring symptoms (Fig. 2a), especially in the apical leaves 10 dpi with ToBRFV. The ToBRFV isolate from Yazd caused mild mosaic symptoms in tomato plants, chlorotic local lesions, and systemic leaf distortion in *N. rustica* (Bananej *et al*., 2024). Mechanical inoculation with infected tomato sap also caused necrotic or chlorotic local lesions on *N. rustica* three dpi (Esmaeilzadeh *et al*., 2023).

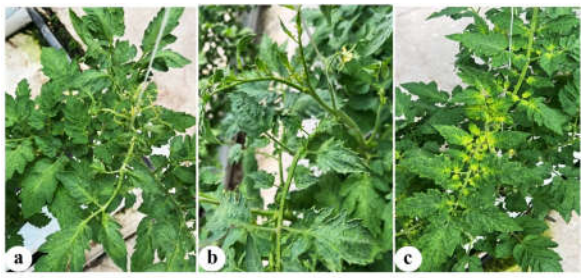

Fig. 1. Illustrates the natural infection of tomato plants by ToBRFV, which causes several symptoms in the greenhouse: a) shoestring and reduced leaf blades, b) blistering and leaf deformation, and c) leaf mosaic.

Rt-PCR results

An Rt-PCR assay utilizing ToBRFV-specific primers successfully amplified a 623-base pair fragment corresponding to the coat protein gene region, while no band was amplified in the negative control sample (Fig. 3). Nineteen out of forty tomato samples were found to be infected with ToBRFV.

Phylogenetic relationships

Two representative samples were selected for sequencing. The resulting sequences were analyzed on the NCBI site, where the nBLAST tool confirmed that the isolates belong to ToBRFV based on the nucleotide sequence of the coat protein gene. Sequence BLAST analysis confirmed that the Lorestan isolates exhibited a nucleotide sequence identity ranging from 99.32% to 99.83%, as did other isolates in GenBank. The sequences obtained have been deposited in GenBank under the accession numbers PQ760178 and PQ760179.

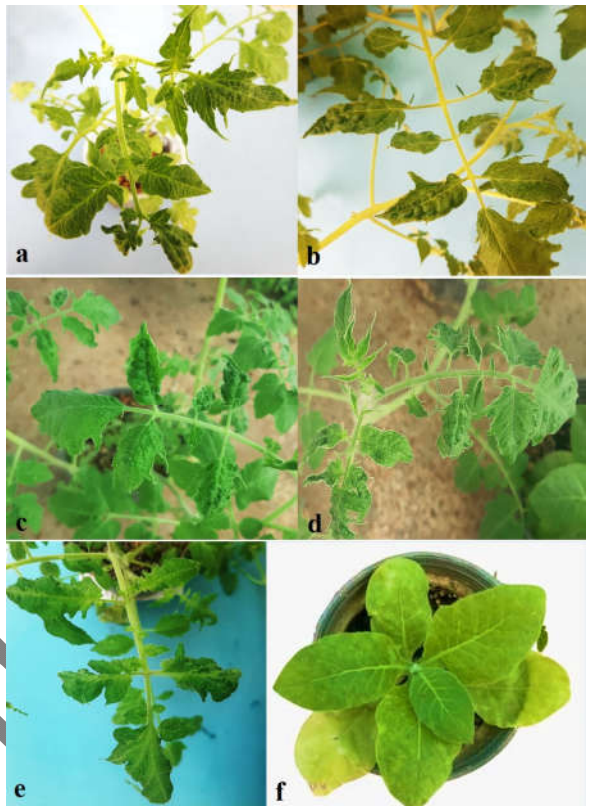

Fig. 2. The mechanical inoculation of suspected plants on indicator plants in the greenhouse: a) Symptoms observed on inoculated *S. lycopersicum* included mosaic patterns, deformation, and shoestring; b and c) Severe blistering and bubbling; d and e) Mosaic and severe deformation symptoms; f) Mosaic symptoms were noted on *N. benthamiana* at seven dpi.

This confirmation facilitates phylogenetic analysis in conjunction with other registered isolates in GenBank. To this end, several sequences of ToBRFV isolates were retrieved from GenBank, and the accession numbers, along with the characteristics of the sequences related to the coat protein genomic region utilized in this study, are presented in Table 1.

A multiple-sequence alignment was performed utilizing the ClustalW tool to analyze the relationships and phylogenetic position of the isolates from Lorestan province. This alignment included several coat protein sequences of ToBRFV isolates from different hosts and geographical regions, including Iran and other

countries (Table 1). Based on the coat protein gene, a phylogenetic tree was constructed.

The phylogenetic tree generated using MEGA11 software indicated that ToBRFV isolates clustered into two distinct groups, with the isolates from Lorestan province classified as group II (Fig. 4). Within group II, two Lorestan isolates clustered alongside three other Iranian isolates, namely ZAR-K-20, Zar-Kp, and TOM5. However, the Lorestan isolates were positioned on a separate branch. The length of the branches in this group suggests that mutations are present in these isolates compared to group I.

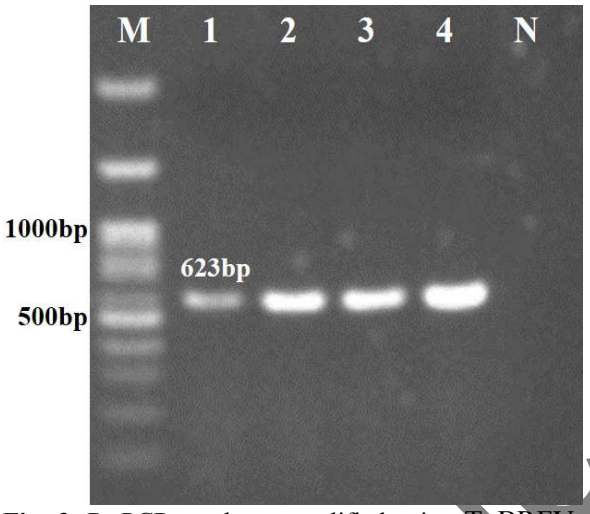

Fig. 3. Rt-PCR products amplified using ToBRFV-specific primer pairs. M= 100bp DNA ladder (Pishgam, Iran); 1-4= Amplification of a 623bp fragment in the tomato plants; N= Negative control.

Three other Iranian isolates, including ToBRFV-Ir, IR-Pep (isolated from Yazd province), and Pep-27, are categorized in group I, but they formed a separate subgroup distinct from those of other countries. Isolates from other countries, including Mexico, Turkey, Palestine area, China, Peru, Canada, the United States, Jordan, the Netherlands, Poland, Italy, Egypt, Palestine, Pakistan, Germany, Belgium, Switzerland, and the United Kingdom, were classified into a distinct subgroup within the group I. Mutations seem to have occurred less frequently in this group, with only the Peru isolate undergoing a mutation.

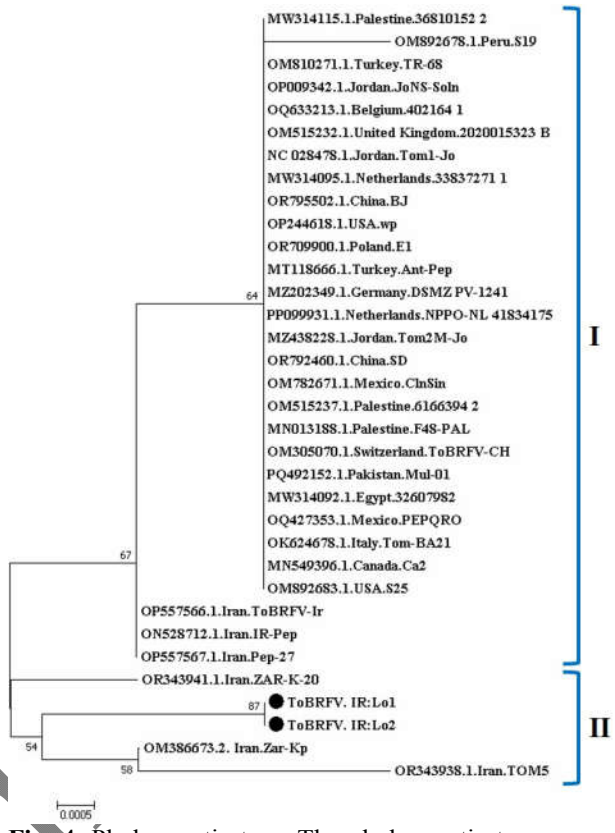

Fig. 4. Phylogenetic tree: The phylogenetic tree was constructed using MEGA11 software, employing the Neighbor-Joining method with 1000 bootstrap replications (Bt×1000) based on the coat protein genome region of ToBRFV isolates. The Lorestan isolates are indicated by a ● on the tree.

The two-by-two comparison of nucleotide sequences from the analyzed genomic region, performed using SDT v1.2 software, demonstrated that the sequences of the two viral isolates from Lorestan Province exhibit a high level of similarity, with a nucleotide identity percentage of 99.80% (Fig. 5). The result comparison matrix indicated that the Lorestan isolates showed the highest genetic similarity to the Iranian isolates ZAR-K-20 and Zar-Kp, with identities ranging from 99.30% to 99.50%. In contrast, the Lorestan isolates demonstrated the greatest genetic distance from the Peru isolate, which had an identity of 98.80%. The results from SDT software aligned with those from the phylogenetic tree analysis. The sequence analysis results were consistent with findings from prior studies conducted on other Iranian isolates. The nucleotide sequence of the amplified 842-base pair fragment corresponding

to the coat protein gene region of the viral isolate from infected tomatoes in Isfahan province, Iran (OK075081) demonstrated a 99.75% identity to the Jordan Tom1-Jo isolate (KT383474) (Ghorbani *et al*., 2021). The coat protein gene region of Iranian isolates from infected tomatoes in Tehran Province was also sequenced.

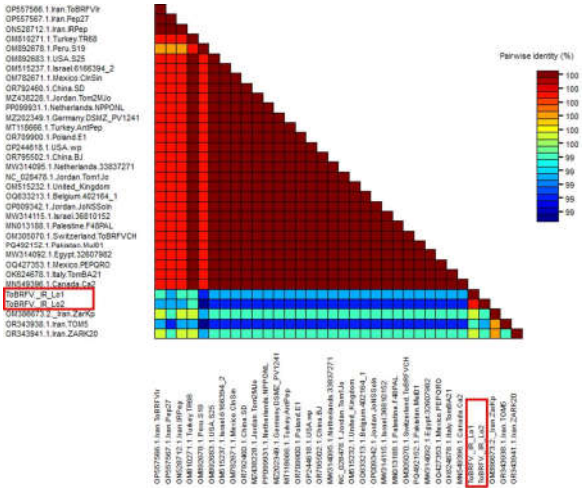

Fig. 5. Pairwise nucleotide identity matrix: A matrix was constructed using Lorestan isolates and 32 ToBRFV isolates, focusing on the coat protein region. This analysis was conducted with SDT v1.2 software.

The analysis indicated that these isolates clustered with those from Turkey, the Netherlands, and the United States, demonstrating a high nucleotide sequence identity of 99.3-99.6% with these groups. Furthermore, the analysis revealed a remarkable 99.8% nucleotide identity among the CP gene sequences of Iranian TBRFV isolates (Esmaeilzadeh and Koolivand 2022b). The complete genome sequence of the ToBRFV-Yazd isolates from Iran was determined to be 6,392 nucleotides in length, featuring four open reading frames that encode the characteristic proteins of *Tobamovirus*. The ToBRFV-Yazd isolate demonstrated 99.4% to 99.7% identity with other ToBRFV isolates and 81.8% with the *Tobacco mosaic virus* (TMV). Notably, the Yazd isolate clustered with two isolates from Jordan. It is important to note that no other Iranian isolates were included in these analyses (Bananej *et al*., 2024).

Overall, the analysis of 34 ToBRFV isolates in the present study, consistent with previous findings, confirmed a significant percentage of genetic identity among the isolates. The greatest genetic distance observed was with the isolate from Peru, which measured 98.80%. Comparisons of full genome nucleotide sequences and phylogenetic analyses of the ToBRFV isolates from various countries revealed a high degree of genetic uniformity among isolates. This finding indicates a relatively short evolutionary history. The isolates of this virus demonstrated low genetic diversity, with nucleotide sequence identity exceeding 99% across their complete genomes. Furthermore, the nucleotide sequence identity for each open reading frame (ORF), including 126 K, 183 K, MP, and CP among the 34 isolates studied, was 98%. For example, no recombination was observed in the virus isolates' ORF4/CP gene region. This similarity is unusual for a ssRNA$^+$ virus and may be attributed to the recent emergence of ToBRFV and its rapid spread, potentially facilitated by seed transmission. Globalization has significantly enhanced the exchange of seeds and plant materials worldwide briefly, enabling the swift dissemination of this virus across all five continents (Bananej *et al*., 2024). Tracking additional isolates from various regions around the world will likely enhance our understanding of the genetic diversity of this virus and clarify the origin and lineage of the global ToBRFV population (Çelik *et al*., 2022).

ToBRFV is a recently identified, highly contagious, and destructive *Tobamovirus* that primarily infects tomatoes. So far, the virus has been reported in at least 35 countries across five continents. Similar to other Tobamoviruses, such as TMV, ToBRFV is seed-borne and can spread rapidly through various forms of mechanical contact, with its virions exhibiting remarkable stability. Therefore, it is unsurprising that common prevention measures such as eliminating infected plants and debris, disinfecting seeds and contaminated tools and greenhouses, and practicing crop rotation may slow the spread of ToBRFV but cannot effectively eliminate the ToBRFV endemic. So, breeders and researchers must be prepared for a long-term battle against ToBRFV (Zhang *et al*., 2022).

Table 1. Accession numbers and characteristics of coat protein genomic region of ToBRFV isolates, as retrieved from GenBank

Accession Number	Isolate Name	Host	Country
OM386673.2	Zar-Kp	*Capsicum annuum*	Iran
OR343941	ZAR-K-20	*Capsicum annuum*	Iran
OP557566	ToBRFV-Ir	*Solanum lycopersicum*	Iran
ON528712.1	IR-Pep	*Capsicum annuum*	Iran
OP557567.1	Pep-27	*Capsicum annuum*	Iran
OR343938.1	TOM5	*Solanum lycopersicum*	Iran
OM515237.1	6166394_2	*Solanum lycopersicum*	Palestine area
MW314115.1	36810152_2	*Solanum lycopersicum*	Palestine area
OM782671.1	ClnSin	*Solanum lycopersicum*	Mexico
OQ427353.1	PEPQRO	*Capsicum annuum cv. Ocelote*	Mexico
OR792460.1	ToBRFV-SD	*Solanum lycopersicum*	China
OR795502	ToBRFV-BJ	*Solanum lycopersicum*	China
NC_028478	Tom1-Jo	*Solanum lycopersicum*	Jordan
MZ438228.1	Tom2M-Jo	*Solanum habrochaites*	Jordan
OP009342.1	JoNS-Soln	*Solanum nigrum*	Jordan
MN013188.1	F48-PAL	*Solanum lycopersicum*	Palestine
PP099931.1	NPPO-NL 41834175	*Solanum lycopersicum*	Netherlands
MW314095.1	33837271_1	*Solanum lycopersicum*	Netherlands
MZ202349.1	DSMZ PV-1241	*Solanum lycopersicum*	Germany
MT118666.1	Ant-Pep	*Capsicum annuum*	Turkey
OM810271.1	TR-68	*Solanum lycopersicum*	Turkey
OR709900	E1	*Solanum lycopersicum*	Poland
OM892683.1	S25	*Solanum lycopersicum*	USA
OP244618.1	Wp	*Solanum polyadenium Greenm*	USA
OM515232.1	2020015323_B	*Solanum lycopersicum*	United Kingdom
OQ633213.1	402164_1	*Solanum lycopersicum*	Belgium
OM305070.1	ToBRFV-CH	*Solanum lycopersicum*	Switzerland
MN549396.1	Ca2	*Solanum lycopersicum*	Canada
OK624678.1	Tom-BA21	*Solanum lycopersicum*	Italy
OM892678.1	S19	*Solanum lycopersicum*	Peru
MW314092.1.	32607982	*Solanum lycopersicum*	Egypt
PQ492152.1	Mul-01	*Solanum lycopersicum*	Pakistan

Conclusion

In this study, ToBRFV was reported for the first time in tomato greenhouses in Lorestan Province. The isolates from Lorestan, based on the coat protein genomic region, exhibited a closer phylogenetic relationship to Iranian isolates than those from other countries. Given the nature of the disease and its ease of transmission through mechanical contact, such as contaminated agricultural tools along with infected seeds and seedlings, the spread of the disease in the province's greenhouses appears to be more extensive and severe than initially anticipated. The utilization of imported seeds, such as seed 4129, by greenhouse owners has likely contributed to introducing this invasive virus into the province. Since no commercial tomato variety resistant to ToBRFV has been reported to date, integrated management strategies are recommended until a resistant variety is introduced. These strategies include eradicating infected plants, disinfecting seeds, and rotating with non-host crops.

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Conflicts of interest

The authors declare no conflict of interest.

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