



Molecular Diagnosis of Major Emerging Tomato Infecting Viruses and Their Vector Population Dynamics

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Abstract

Tomato is an important vegetable crop in Bangladesh, growing year-round in both kitchen gardens and large-scale commercial farming. Many diseases affect the solanaceous vegetable crop, particularly viral diseases. Using modern molecular methods and vector population dynamics, we attempted to detect major emerging viral infections of tomato. A survey was conducted in different locations of Bangladesh, namely Rajshahi, Jamalpur, Jessore, and Joydebpur to assess viral infections in tomato and associated insect vectors. Various forms of viral disease symptoms were observed to infect tomato in the examined area, including mosaic, yellowing, mottling, leaf curling, and bud necrosis, with a incidence ranging from 20 to 100%. Among the different viral diseases, leaf curl was the most common, accounting for 100% of the cases. Field resistance to viral infections was investigated among the BARI released tomato varieties, and all of the tomato types tested were infected with the leaf curl virus. In addition, the examined area had a moderate to high prevalence of chlorosis and purple vein disease. The viruses identified by DAC-ELISA, PCR, and RT-PCR were tomato leaf curl virus (ToLCV) and groundnut bud necrosis virus (GBNV).

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Introduction

Tomato (*Solanum lycopersicum*) is a high value solanaceous vegetable crop cultivated in both winter and summer seasons in Bangladesh. Viral diseases are a severe threat to sustainable tomato cultivation in Bangladesh. At least 22 viruses infecting tomato are known whose outbreaks have led to significant yield and quality losses of tomato in Bangladesh (Akhter *et al.*, 2019). Among the viral diseases of tomato, leaf curl disease is the most important disease of tomato, which is prevalent throughout the country and causes serious crop loss (Akanda *et al.*, 1991; Alam *et al.*, 1994; Akanda *et al.*, 1999).

During the last two decades, leaf curl disease has emerged as a devastating disease-causing economic loss of up to 100% in many tropical and sub-tropical regions including Bangladesh (Lukyanenko, 1991; Verma and Malathi, 2003). Leaf curl disease of tomato caused by different species of Begomoviruses transmitted by whitefly (*Bemisia tabaci*). Different types of symptoms are developed in tomato plants due to begomovirus infection. The disease symptoms range from mild leaf curl to yellow leaf curl and severe curling and stunting. The infection at the early stage of plant growth resulted in severe curling, stunting and complete loss of yield (Maruthi *et al.*, 2005).

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Tospoviruses are an emerging threat to tomato cultivation in the Indian subcontinent including Bangladesh (Mandal *et al.*, 2012). Groundnut bud necrosis virus (GBNV) causing leaf mottling and necrotic veins, short internodes, necrosis of terminal buds, and concentric rings on fruits of tomato has emerged as a significant problem and most of the popularly grown tomato cultivars are susceptible (Farooq and Akanda, 2007; Akhter *et al.*, 2012). On the other hand, recent reports from India, Pakistan, and China have identified species of white fly-transmitted crinivirus, tomato infectious chlorosis virus (TICV), and tomato chlorosis virus (ToCV), which pose a global threat to tomato production today. In Bangladesh, various types of viral diseases affect tomato, but systematic studies on these diseases and their causal viruses are unidentified. Whitefly-transmitted viral diseases are emerging problems in tomato in Bangladesh and other countries. Severe disease symptoms like yellowing and chlorotic spots on foliage are frequently observed in several tomato throughout the country but the causal agent of those diseases has not yet been identified. The early and accurate diagnosis of plant viruses is an essential component of sustainable crop production. The identification of new and emerging plant viruses and their vector is a time demanding research for sustainable tomato production in Bangladesh. Under this study, a clear picture of emerging tomato infecting viruses and their vectors may come out which will directly help to develop a durable integrated control strategy against the viruses.

Materials and Methods

The survey was conducted in four locations, namely Godagari, Rajshahi; the experimental field of the Regional Agriculture Research Station (RARS) in Jamalpur and Jessore; and BARI headquarters in Joydebpur, Gazipur, to assess the viral diseases of tomato. The viral diseases like samples were collected and brought to the Plant Virology Lab,

Plant Pathology Division, BARI for further analysis. The viral disease-like symptoms were categorized using visual observation, and the percent disease incidence was measured using the following formula.

Diagnosis of Tomato Infecting Viruses and Their Insect Vectors

Serological Diagnosis of Tomato Infecting Viruses

Direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) (Clark and Bar-Joseph, 1984) was performed to detect the association of viruses from the viral disease-like symptoms in tomato. The assay was performed in 96-well polystyrene microtitre plates (Costar, Sigma, USA). Briefly, 96-well plates were coated with diseased leaf extracts diluted 1:4 (w/v) in coating buffer containing 15 mM sodium carbonate, 35 mM sodium bicarbonate, 2% polyvinylpyrrolidone (PVP-40) with pH 9.6 and incubated at 37 °C for 1 hour (h). After 3 subsequent washings with PBS-T buffer containing 136 mM NaCl, 1.4 mM KH₂PO₄, 2.6 mM KCl, 8 mM Na₂HPO₄, 0.05% Tween-20, with adjusted pH 7.4, these plates were further blocked with 2% bovine serum albumin (BSA) for 1 h at 37 °C. After 3 repeated washings with PBS-T, specific antiserum against the targeted viruses was diluted (1:1000) was loaded to the wells of ELISA plate and incubated at 37 °C for 1 h followed by 3 washing with PBS-T. Goat anti-rabbit IgG-AP conjugate (Sigma-Aldrich, St. Louis, USA) at a dilution of 1:30,000 in PBS-TPO) was added and incubated at 37 °C for 1 h. Finally, the plates were washed 3 times with PBS-T and para-nitrophenyl phosphate (pNPP) substrate (at 0.5 mg/mL pNPP dissolved in 9.7% diethanolamine buffer, pH 9.6) was added. The OD values @ 405 nm were measured by ELISA reader after 1 h of substrate incubation at 37 °C. Tomato leaf samples showing absorbance (OD at 405) values more than 2 times than that of healthy control were considered as infected with the viruses. The primary antibody used for the DAC-ELISA are listed in Table 1.

$$\text{Percent Disease Incidence} = \frac{\text{The number of diseased plants counted}}{\text{Total number of plants counted}} \times 100$$

Table 1. List of the antibody and targeted virus

Antibody used	Target virus/s
Cucumber mosaic virus (Polyclonal)	<i>Cucumber mosaic virus</i>
Pathoscreen (Polyclonal)	<i>Potyvirus group</i>
Geminivirus	<i>Tomato leaf curl virus</i>
GBNV (Polyclonal)	<i>Ground nut bud necrosis virus</i>
ToCV	<i>Tomato Chlorosis virus</i>

Molecular Diagnosis of The Tomato Infecting Viruses Using PCR & RT-PCR

Polymerase Chain Reaction

The ELISA positive symptomatic samples were further subjected to polymerase chain reaction (PCR). For this purpose, plant total genomic DNA was isolated using Promega Wizard® DNA Extraction kit. The isolated DNA was quantified using Nanodrop (50~100) ng of total DNA were used as a template. The geminivirus universal primers were used for PCR amplification. The 25 µL total reaction mixture containing 2.5 µL dream taq DNA buffer, 2.5 µL 2mM dNTPs, 0.75 µL reverse primer and 0.75 µL forward primer, 0.25 µL dream Taq DNA polymerase and ultra-pure water up to 25 µL. The primers use for the amplification of begomovirus (Tomato leaf curl virus- Primer A: 5'-TAATAnACCKGWKGVCCSC-3' Primer B: 5'-TGGACYTTRCAWGGBCCTTCACA-3' (Deng *et al.*, 1994) and Curtovirus (Beet curly top virus) BCTV-Svr (Cfh-c197 5'-CTTGTGGGGA CCACAATGATCTAC-3') and BCTV-PeCT (BV3-c1484 5'-GGATTGGGTACTGGAGCGTCAA-3') (Chen *et al.*, 2010).

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

The ELISA-positive samples were further subjected to the specific detection of viruses by reverse transcription-ppolymerase chain reaction (RT-PCR) using species-specific primers. The primers have been designed by retrieving sequence from NCBI. The list of the primers and their sequence has been listed below. Total RNA was extracted from the symptomatic and healthy (control) tomato leaf samples (~100 mg) using SV total RNA isolation kit

(Promega, USA) according to the manufacturer's instruction. Extracted RNA was used as template for RT-PCR along with the positive and negative control. The first strand cDNA was synthesized using Verso cDNA synthesis kit (Thermo Scientific, USA). The 20 µL cDNA reaction mixture contained 200–500 ng template RNA (1-5 µL), 1 µL (100 pg) reverse primer, 2 µL 10 mM dNTP, 4 µL 5× cDNA synthesis buffer, 1 µL RT Enhancer, 1 µL Verso Enzyme Mix Nuclease free water up to 20 µL. The reaction mixture was incubated at 42 °C for 60 minutes (min) and 95°C for 2 min. The 50 µL PCR reaction mixture comprised of 4 µL of cDNA template, 5 µL 10X Dream Taq buffer, 2.5 µL dNTP Mix (0.2 mM each), 1.5 µL each of reverse and 2 forward primers, 1.0 µL of Dream Taq DNA polymerase and Nuclease free water up to 50 µL. The PCR was conducted in a thermal cycler (DNA Engine, Biorad, Germany) with the following temperature conditions: 2 min hot start at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 seconds (s), annealing for 1 min at 52 °C (annealing temperature vary depending on the primers of the targeted virus and synthesis at 72 °C for 1 min, and a cycle of final extension at 72 °C for 10 min. The PCR products were analyzed in 1% agarose gel in Tris-acetate EDTA buffer containing ethidium bromide (Sambrook and Russell, 2001).

Table 2. List of *Oligonucleotides sequences used in this study*

Name of the primer	Seq (5'→3')
Solanaceae-CriniR	GTGTTBGAYAACCAWGTGTT
TICV-F	AAGAATGGACCTACCCAG
TICV-R	CTGTTCTCTCCCCTTTCC
ToCV-F	GCACCCTGATTGGTTCTAAAC
TOSPO-R' (Degenerate)	CTTCTTWGARTGTHCACCATARTCATC
GBNV-F'	ATGGTTGAAAAGAGCAAGAATGATGC

Vector Virus Relationship in Different Tomato Varieties

To assess the interactions on different tomato genotypes, virus and insect vector, Bangladesh

Agricultural Research Institute (BARI) released 13 tomato varieties were planted. The design of the experiment was RCBD with 3 replications. The disease incidence and insect vector population were measured regularly.

Results and Discussion

Tomato is an important vegetable crop in Bangladesh, and a survey was conducted in four locations, namely Godagari upazila at Rajshahi district, RARS Jessore and Jamalpur, and BARI headquarters, Gazipur, to identify the viral species infecting tomato in Bangladesh. The tomato plants were found to suffer from many viral diseases, including mosaic, yellowing, mottling, leaf curl, and bud necrosis (Table 3).

The serological (DAC-ELISA) and molecular results suggested that leaf curl was the predominant virus in the surveyed area and the incidence of GBNV, ToCV and BCTV was medium to low. The tomato leaf samples in Godagari, RARS Jamalpur and Jessore, and BARI, Gazipur showed typical symptoms of tospovirus and reacted positively against polyclonal antibody of tospovirus. The ELISA positive samples were further analyzed through RT-PCR and revealed

that the virus has been amplified using specific primers of GBNV (Figure 1). Tomato spotted wilt virus (TWSV) and Capsicum chlorosis virus (CaCV) tomato infecting tospoviruses were negative in the RT-PCR results. The TSWV is previously reported in tomato in Bangladesh (Farooq and Akanda, 2007) but CaCV still not reported in Bangladesh. The CaCV is also reported in tomato (Huang *et al.*, 2010). The GBNV, orthotospovirus incidence is more in 60 to 80% in Godagari and RARS Jamalpur, and 40 to 50% in other locations and positive in the RT-PCR (Figure1).

Table 3. Viral disease symptoms of tomato in different selected areas

Name of the crop	*Viral disease like symptoms (%)			
	Bud Necrosis	Leaf curl	Chlorosis	Purple vein
Godagari, Rajshahi	60–80	80–100	20–30	10–20
RARS, Jamalpur	40–50	80–100	60–80	10–20
RARS, Jessore	60–80	80–100	80–100	10–20
BARI, Gazipur	40–50	80–100	60–80	15–20

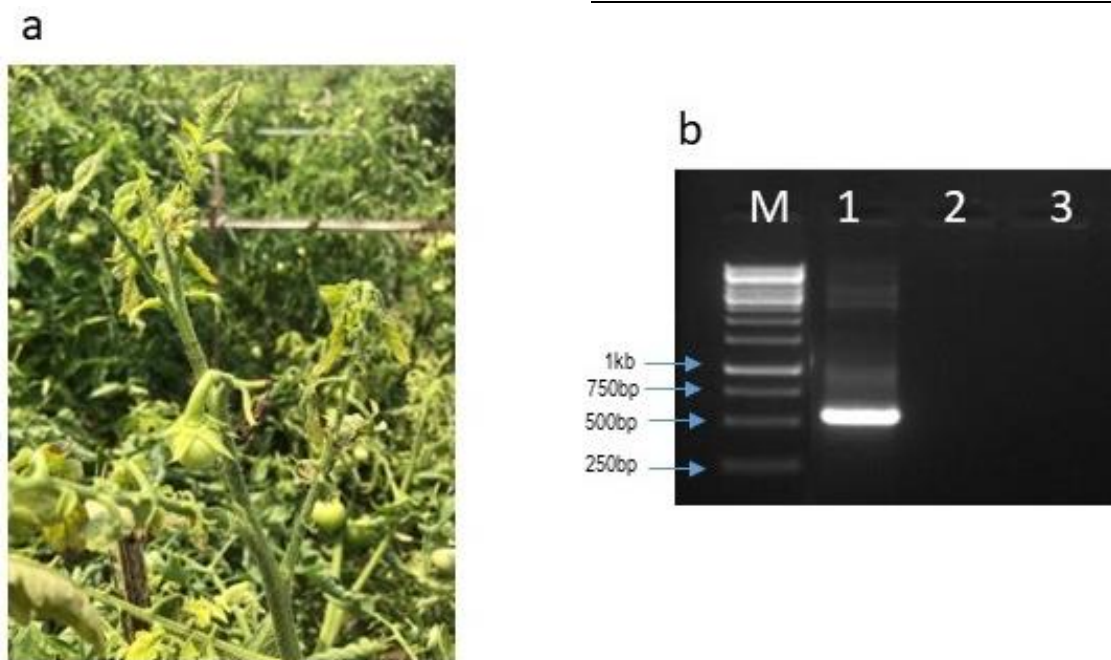


Figure 1. Diagnosis of groundnut bud necrosis virus (GBNV) in tomato. Tomato twig showing bud necrosis disease (1a) and gel electrophoresis of RT-PCR (1b). Lane 1 = GBNV; Lane 2 = TSWV, and Lane = 3 CaCV. M = 1 kb DNA marker.

Leaf curl viral illness incidence ranged between 80 and 100% in all four locations, and the presence of the virus was confirmed by serological and molecular testing in PCR with Geminivirus-specific degenerate primers (Figure 2).

Leaf curl disease caused by many leaf curl virus species transmitted by whitefly is a severe threat to tomato cultivation in around the globe including Bangladesh. Biotypic variants of white flies and frequent recombination in the viral genome leads to the new virus emergence. Similar to leaf curl disease chlorosis disease also appeared as a high incidence

80–100% at RARS Jessore and 60–80% in Jamalpur and BARI Gazipur respectively. The chlorosis disease incidence in low in Godagari 20–30% (Table 3 and Figure 3a). Tomato chlorosis virus is transmitted by whitefly a crinivirus genus is a severe threat to tomato and other vegetables production globally (Olivé and Castillo, 2019). Another tomato viral disease observed in the surveyed location was the purple vein disease. This was diagnosed as curtovirus under the Geminiviridae family with low incidence of 10–20% (Table 3 and Figure 3b).

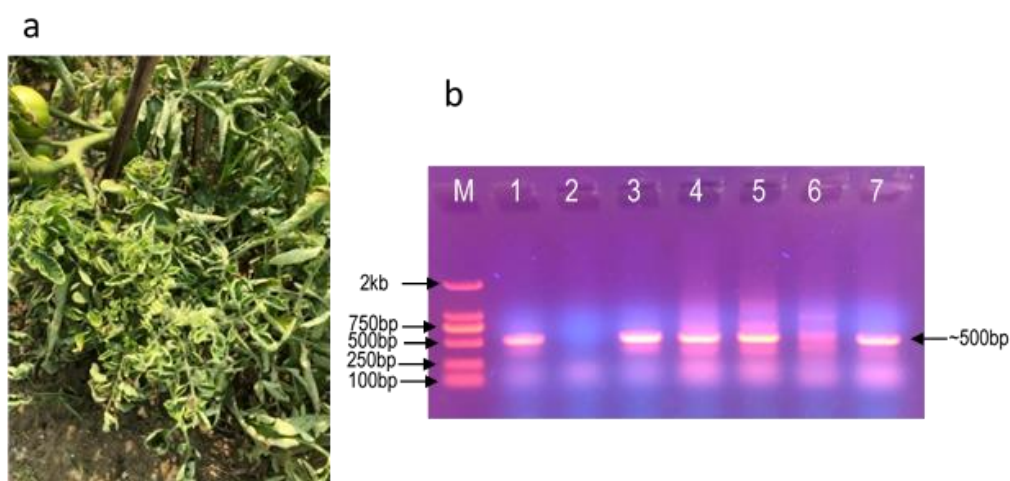


Figure 2. Diagnosis of leaf curl virus in tomato. Symptomatic tomato plants showing leaf curl disease (2a). Gel electrophoresis of PCR (2b). Lane 1 = positive control, lane 2 = healthy tomato leaf as a negative control, and lane 3 to 7 = tomato leaves showing leaf curl symptoms. The arrow head indicates the amplification at desired size by the degenerate primers and M is the 100 bp DNA marker.

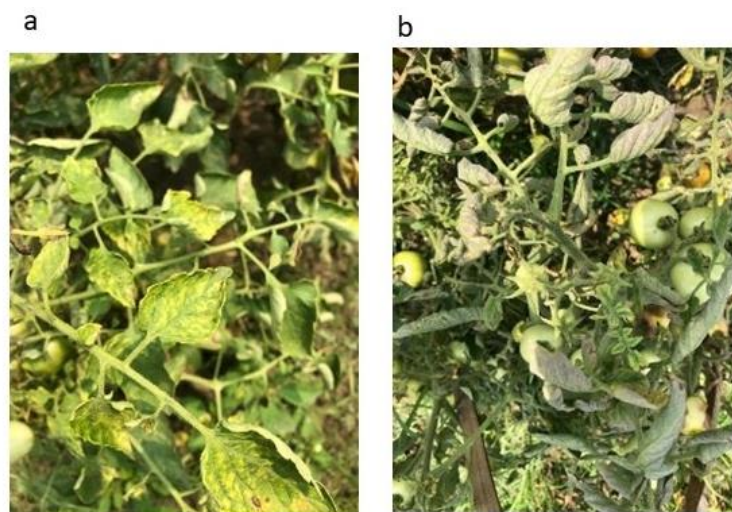


Figure 3. Tomato chlorosis and curly top disease. Tomato plant show chlorosis symptoms (3a) and tomato plants with Curly top virus (CTV) infection frequently show increased vein purpling (3b).

White fly-transmitted geminiviruses are a major constraint to sustainable crop production, causing 80–100% leaf curl symptoms in investigated locations. Pearson's correlation coefficient analysis was used to examine virus-vector interactions in all 13 BARI-released tomato cultivars.

The correlation plot demonstrates a somewhat positive association (about 38%) between disease incidence and white fly population (Figure 4). However, cluster analysis yielded more thorough data (Figure 5), indicating that all treatments were

grouped into seven separate sub-groups (Sub. Gr. 1 to 7) and two major groups (Gr. 1 and 2) (Figure 5).

While BARI Hybrid Tomato 8 and BARI Tomato 2 (Ratan) have demonstrated a comparable level of vulnerability to viral infection, the T6 (BARI Tomato 7) variety has the highest disease incidence, followed by T5 (BARI Hybrid Tomato 6) and T10 (BARI Tomato 9). Consequently, there is a significant variation in the disease incidence among these tomato cultivars.

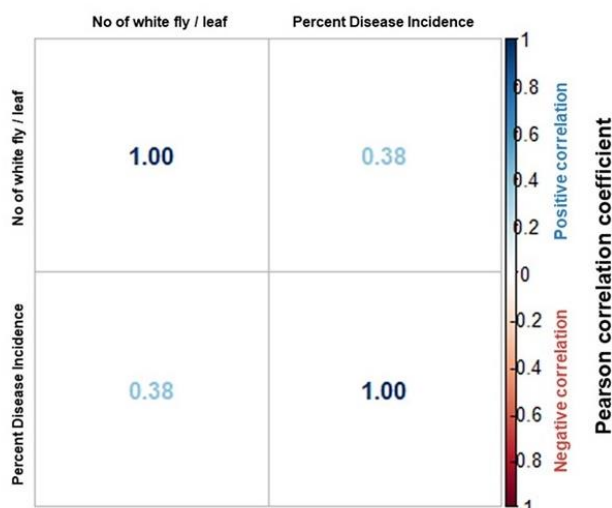


Figure 4. The correlation plot shows the percentage of leaf curl disease incidence and the number of white fly.

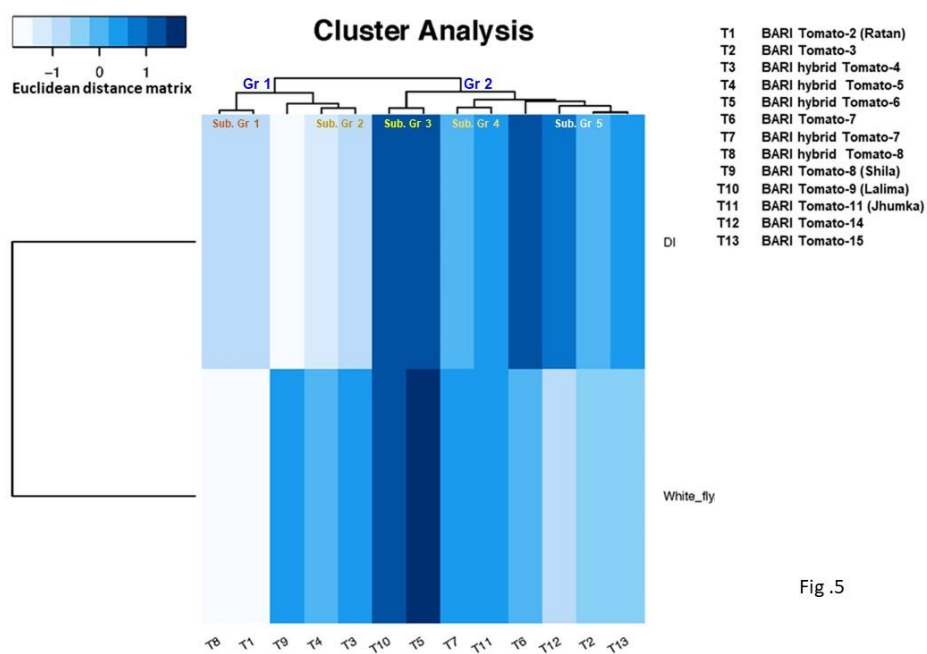


Fig .5

Figure 5. Cluster analysis shows the leaf diseases incidence and whitefly population dynamics.

Conclusions

The results revealed that leaf curl, bud necrosis, chlorosis, and purple vein disease are the most common viral diseases of tomato in the four examined locations, with disease incidences ranging from 80 to 100%. The major insect vector that transmits the tomato leaf curl virus is *Bemisia tabaci*. However, more research is needed to understand the genetic variety of the Bangladeshi tomato-infecting viruses, as well as the molecular cross-talk between tomato types and white fly interactions.

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Declaration

The authors declare no conflicts of interest.

Author's Contributions

MSA conceptualized and designed the study; MSA, MSR, and RI collected the data and the samples; MSA carried out the tests and analyzed the results; MSA provided reagents, laboratory supplies, and analysis tools. All authors contributed to writing, revising, and approving the final manuscript.

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