Molecular Characterisation of Segment 3 Tilapia Lake Virus (TiLV) Isolated from Different Localities in Malaysia

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Abstract: Malaysia has declared TiLV positive in 2017 and following this OIE declaration, research and monitoring have been in place. This paper focuses on the molecular characterisation of the TiLV isolated from tilapia fish at different regions in Malaysia, during epidemiological studies and outbreak cases. The samples were collected between 2017 and 2019. The study aimed to identify if there are any molecular differences between the isolates, particularly on segment 3. Organs samples such as liver, kidney and spleen were pooled into viral transport media, processed, and inoculated into E-11 cell line. The inoculated cells were observed for any cytopathic effect (CPE) formation for at least 3 passages, before being harvested and confirmed with semi-nested RT-PCR. From 62 samples inoculated into cell culture, 33 samples (53.2%) were positive for CPE and 22 samples were selected and sent for sequencing. The sequences went through multiple nucleotide sequence alignments with the database in GenBank in the National Centre for Biotechnology Information (NCBI), and a phylogenetic tree was constructed by using MEGA 7 applying a maximum likelihood bootstrap analysis (1000 replication). The sequence similarities showed that segment 3 of all TiLV isolated from tilapia fish in this study had at least 98% similar to TiLV isolated from Thailand (accession number: KY381578.1).

Keywords: tilapia, TiLV, molecular characterisation, phylogenetic tree

Abstrak: Malaysia telah mengisytiharkan TiLV positif kepada OIE pada tahun 2017 dan setelah pengisytiharan OIE ini, penyelidikan dan pemantauan telah dilaksanakan. Artikel ini memfokuskan kepada pencirian molekul TiLV yang diasingkan dari ikan tilapia di beberapa kawasan di Malaysia, semasa epidemiologi dan wabak penyakit. Kajian ini bertujuan untuk mengenal pasti sama ada terdapat perbezaan molekul pada segmen 3 antara isolat TiLV yang dipencilkan. Sampel yang dikumpulkan adalah antara tahun 2017 dan 2019. Sampel organ seperti hati, ginjal dan limpa digabungkan dalam media pengangkutan virus, diproses dan diinokulasi ke dalam sel E-11. Sel-sel yang diinokulasi diperhatikan untuk sebarang pembentukan kesan sitopatik (CPE) untuk sekurang-kurangnya 3 *sub-passage*, sebelum dituai dan disahkan dengan *semi-nested* RT-PCR. Dari 62 sampel yang diinokulasi ke dalam kultur sel, 33 sampel (53.2%) positif untuk CPE dan 22 sampel dipilih untuk dibuat penjujukan cDNA. Hasil jujukan cDNA dibandingkan dengan pangkalan data di GenBank di Pusat Maklumat Nasional Bioteknologi (NCBI) dan pohon filogenetik dibentuk menggunakan perisisan MEGA 7 melalui analisis bootstrap kemungkinan maksima (1000 replikasi). Hasil kajian menunjukkan bahawa segmen 3 dari semua TiLV yang diasingkan dari ikan tilapia dalam kajian ini mempunyai sekurang-kurangnya 98% persamaan dengan TiLV yang diasingkan dari Thailand (nombor akses: KY381578.1).

Introduction

Tilapia is popular species for aquaculture due to its fast growers, efficient food converters, omnivorous diet, high-density aquaculture and relative disease resistance. It also serves as an important protein source, especially in developing countries (FAO 2016). Tilapia have been a major protein source for the poor countries, and the emergence of TiLV will have a huge impact on the industries locally and globally. The disease could affect US$ 7.5 billion a year in production to major producing countries including China, the Philippines, Thailand, Indonesia, Lao PDR and Bangladesh (Network of Aquaculture Centres in Asia-Pacific 2017).
Since 2009, substantial mortality of tilapia have occurred in Israel and Ecuador, and in 2014, a virus was identified as the cause of this death (Eyngor et al., 2014)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. Since then, TiLV outbreak has been reported to be present on three continents: Asia, Africa and America. Countries affected by TiLV include Colombia (Kembou Tsofack et al., 2017), Ecuador (Ferguson et al., 2014; Kembou Tsofack et al., 2017), Peru (Pulido et al., 2019), Israel (Eyngor et al., 2014), Egypt (Fathi et al., 2017), Uganda and Tanzania (Mugimba et al., 2018), Malaysia (Abdullah et al. 2018; Amal et al., 2018), Thailand (H. T. Dong et al., 2017; Surachetpong et al., 2017), Indonesia (Koesharyani et al., 2018), India (Behera et al. 2018), the Philippines (OIE 2017), Bangladesh (Chaput et al., 2020), United States, Mexico and Kenya (Aich et al., 2022).

TiLV virus resembles a virus in the family orthomyxoviridae, and is temporarily called tilapia lake virus (TiLV) (Eyngor et al., 2014)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. TiLV, officially known as Tilapia tilapinevirus is of the genus Tilapinevirus and family Amnoonviridae (Kuhn 2018; Siddell et al., 2019). The TiLV is an icosahedral virus and has 10-segment, negative-sense single-stranded RNA (-ssRNA) genome with total size of 10,323 kb and a diameter between 55 and 100 nm (Bacharach et al., 2016; del-Pozo et al., 2016; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The largest segment, segment 1, contains open reading frames with weak sequence homology to the PB1 subunit of influenza virus C. The other nine segments do not show homology to other viruses but have complementary sequences at terminals 5′ and 3’, respectively, in line with the existing genome organization in another orthomyxovirus (Bacharach et al., 2016)tolerance for high-density aquaculture, and relative disease resistance. Since 2009, tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and Ecuador. Here we report evidence implicating a novel orthomyxo-like virus in these outbreaks. The tilapia lake virus (TiLV. *In situ* hybridization shows replication and transcription of TiLV at the site of pathology in the liver and central nervous system with disease (Bacharach et al., 2016; Eyngor et al., 2014) tolerance for high-density aquaculture, and relative disease resistance. Since 2009, tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and Ecuador. Here we report evidence implicating a novel orthomyxo-like virus in these outbreaks. The tilapia lake virus (TiLV. Outbreaks and experimental studies showed that TiLV could be transmitted horizontally and vertically. Horizontal transmission of the virus has been confirmed through cohabitation of infected fish with clinically healthy fish (Eyngor et al., 2014; Liamnimitr et al., 2018). Vertical transmission affects mainly early developmental stages of tilapia, ie. fertilized eggs, yolk-sac larvae, fry and fingerlings (Ha Thanh Dong et al., 2015; Tattiyapong, Dachavichitlead and Surachetpong 2017; Yamkasem et al., 2019).

TiLV causes symptoms like redness on the skin, inflammation of the organs especially eyes and brain, while liver damage causes other organs failure. Examination of body tissues showed signs

of oedema, haemorrhage to leptomeninges and congestion of organs such as the kidneys, liver and spleen (Eyngor et al., 2014; Ferguson et al., 2014; Tattiyapong, Dachavichitlead and Surachetpong 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The major change is the formation of giant cells in the liver, called syncytial hepatitis (Ferguson et al., 2014). Other changes including gill pallor, exophthalmia, body discoloration (darkening), and scale protrusion and loss (Eyngor et al., 2014; Ferguson et al., 2014; Tattiyapong, Dachavichitlead and Surachetpong 2017)a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV. The disease can lead to high mortality, especially at juvenile stage. TiLV affected farms cultivating Nile Tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and hybrid tilapia (*O. niloticus* x *O. aureus*) may experience up to 90% mortality (H. T. Dong et al., 2017; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017). The virus is reported to only attack tilapia, but other fish that are raised together often have no sign of illness or death (Eyngor et al., 2014).

The majority of the wild tilapia, followed by barbs (Barbonymus schwanenfeldii and Barbonymus gonionotus) and other fish species like catfish and peacock bass (Cichla sp.) but at a very low rate, died in a man-made lake in the northern part of Malaysia in June 2017 (Abdullah et al., 2018). The body, operculum, ventral, and lateral fins of the tilapia were all crimson in colour, but the eyes were normal. The brains of half the fish that were dissected were fluid, squishy, or hemorrhagic. With the exception of one fish's pale liver and a somewhat congested kidney, river barb were in good physical condition. All of the tilapia samples tested positive for TiLV but negative for VNN and iridovirus, according to the PCR data. Surprisingly, the results for TiLV in river barbs that appeared healthy were also positive. Sequences from both tilapia and river barb are 96% similar to Segment 3 TiLV isolate Til42011 from Israel with Accession No. KU751816.1 after the amplicons received for sequencing were blasted in the GenBank. Understanding the genetic variants of the virus, carriers (fish that are subclinically infected), and vulnerability of other fish species to the virus are therefore necessary. In this investigation, the TiLV isolated from tilapia fish in Malaysian waters was genetically characterised.

Materials and methods

Fish samples and inoculation

Pools of liver, kidney and spleen of tilapia (*Oreochromis* spp) from Sarawak (15 fish), Perak (36 fish) and Perlis (291 fish) in 10 mL HBSS (Gibco, Thermo Scientific, Massachusetts, USA) transport medium containing 2% fetal calf serum (Gibco) and 100 µgmL−1 gentamycin-sulphate (Gibco) were processed according to methods described in Abdullah et al., (2017). 1ml of the supernatant then inoculated into E-11 cell lines (Iwamoto et al., 2000). The culture was maintained in Leibovitz's-15 (L-15, Gibco) medium supplemented with 2% foetal calf serum (FCS, PAA Laboratories) and 100 µgmL−1 gentamycin-sulphate (Gibco) and incubated at 25°C. The cells were observed daily for any cytopathic effects (CPE) formation for at least 3 passages (4-7 days/passage), before harvested. The CPE was confirmed with semi-nested reverse transcriptase–polymerase chain reaction technique (RT-PCR). 22 positive samples from the cell culture supernatant from Sarawak, Perak and Perlis were selected.

Nucleic acid extraction, RT-PCR and sequencing analysis

Total nucleic acid was extracted from the supernatant using Viral Gene-spin™ Viral DNA/RNA Extraction Kit (Intron Biotechnology, Gyeonggi-do, South Korea). Positive control for TiLV was

obtained from Dr. Thanh Ha Dong, Centex Shrimp, Mahidol University, Thailand. A set of new primers (forward: 5' TGGGCACAAGGCATCCTAC 3' and reverse 5' CACGTGCGTACTCGTTCAGT 3') targeting segment 3 of TiLV were designed using National Centre for Biotechnology Information (NCBI) software. Semi-nested RT-PCR assay for TiLV detection using total RNA extracted from the fish internal organ (liver, kidney and spleen) and cell culture supernatant as templates was performed following protocol described earlier by Dong et al., (2017). Briefly, RT-PCR was performed with reverse transcription at 45°C for 30 minutes, polymerase activation at 95°C for 60s followed by 30 cycles of denaturation at 94°C for 10s, annealing at 60°C for 10s, extension at 72°C for 30s and final extension at 72°C for 5 min, using MyTaq™ One Step RT-PCR kit (Bioline, UK) on T100 thermal cycler (BioRad, USA). The PCR products were analysed by electrophoresis in 1.5% agarose gel and stained with RedSafe™ (iNtRON, Korea). The results were viewed with bio-imaging system (Syngene, Cambridge, UK) and expected amplicon of 245bp was successfully amplified by single round amplifications.

Positive PCR products were purified using innuPREP DNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol. 245bp of purified products were sent for sequencing and the nucleotide sequencing of virus were compared with the known sequences in the GenBank database Nucleotide Basic Local Alignment Search Tool (BLAST) program. Molecular phylogenetic tree analysis was generated by Maximum Likelihood method of the MEGA 7 software (Koichiro Tamura et al. 2012)the practical application of these methods is challenging because of the exorbitant calculation times required by current methods for contemporary data sizes, the difficulty in correctly modeling the rate heterogeneity in highly diverse taxonomic groups, and the lack of reliable clock calibrations and their uncertainty distributions for most groups of species. Here, we present a method that estimates relative times of divergences for all branching points (nodes. Nucleotide sequence of gene encoding for viral coat protein of red spotted grouper nervous necrosis virus (RGNNV) was used as an outgroup for the comparison between the genus. The construction of phylogenetic trees was conducted by matching the nucleotides sequence of the isolates from Sarawak (A, B, C), Perak (TB, TC, TE, TL) and Perlis (TiTT 125 – 127) with four nucleotide sequences of segment 3 from other countries i.e., Thailand (Tattiyapong, Dachavichitlead and Surachetpong 2017), India (Behera et al., 2018), Israel (Eyngor et al., 2014) and Ecuador (Bacharach et al., 2016).

The evolutionary history and analysis were inferred using the Maximum Likelihood method based on the Tamura-Nei model (K Tamura and Nei 1993). The tree with the highest log likelihood (-1017.2769) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbou-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Branch lengths are expressed in terms of the number of substitutions per site, and the tree is depicted to scale. 15 nucleotide sequences were subject to the analysis. There were no positions left that had blanks or missing data. The final dataset included 241 positions in total. Software called MEGA 7 was used to assess evolutionary data (Kumar, Stecher, and Tamura 2016).

The evolutionary history and analysis were somewhat inferred with an approach of utilizing the Maximum Likelihood method based on the Tamura-Nei model (K Tamura and Nei 1993). The tree that presents the highest log likelihood (-1017.2769) is shown. Initial tree(s) for the heuristic search were achieved automatically by applying Neighbor-Join and BionNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) method, which then subsequently led to choosing topology with superior log likelihood value.

Results and Discussion

Virus inoculation

Inoculation of supernatant from positive isolate of TiLV in E-11 cell lines resulted in the CPE after three to five days post inoculation (pi) (Fig 1). There are 33 out of 62 samples (53.2%) inoculated into cell culture were positive for CPE. Figure 1 (B), the cell line shows visible cytopathic effect (CPE) at day 3 post infection with cytoplasmic vacuoles and plaque formation compared with control, Fig 1 (A). The cell monolayer disintegrates after nine to ten days of inoculation, suggestive the viability of the virus, compared with control cells which are still intact.

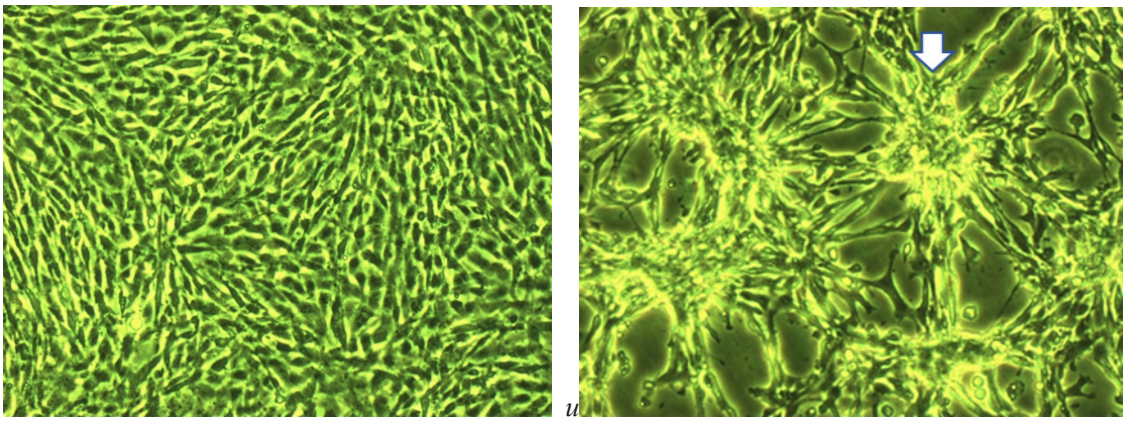

Figure 1. Uninfected E-11 cells, (A) and (B) cytopathic effect (CPE) formation in E-11 cells infected with TiLV at day 3 pi (white arrow).

RT-PCR and sequencing analysis

All positive samples gave a single amplicon of 245bp indicating light infection of TiLV (Fig 2). The sequencing results for segment 3 of TiLV were aligned with the database in GenBank using NCBI nucleotide BLAST (blastn), showed that all TiLV isolated from respective localities had closest kinship with TiLV isolate from Thailand (accession number: KY381578.1) i.e., 98% similarity (Fig. 3). However, this sequence is too small for the author to draw much out of it.

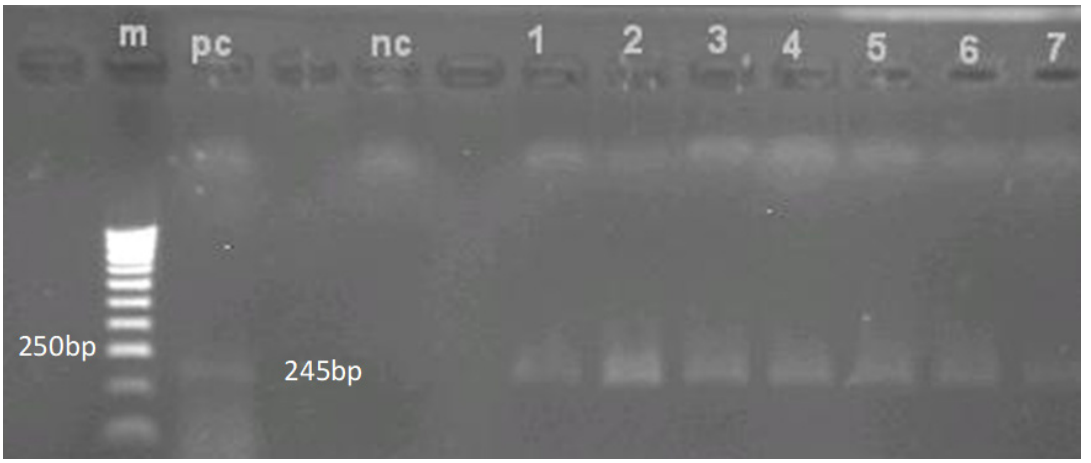

Figure 2. Agarose gel image of TiLV detection using semi-nested RT-PCR assay with RNA template from Sarawak isolates. All samples produced 245 bp amplicon. Lane M = DNA marker (100bp DNA ladder, Hyperladder MyTaq, Korea); pc = positive control; nc = negative control, lane 1-7 TiLV infected tilapia.

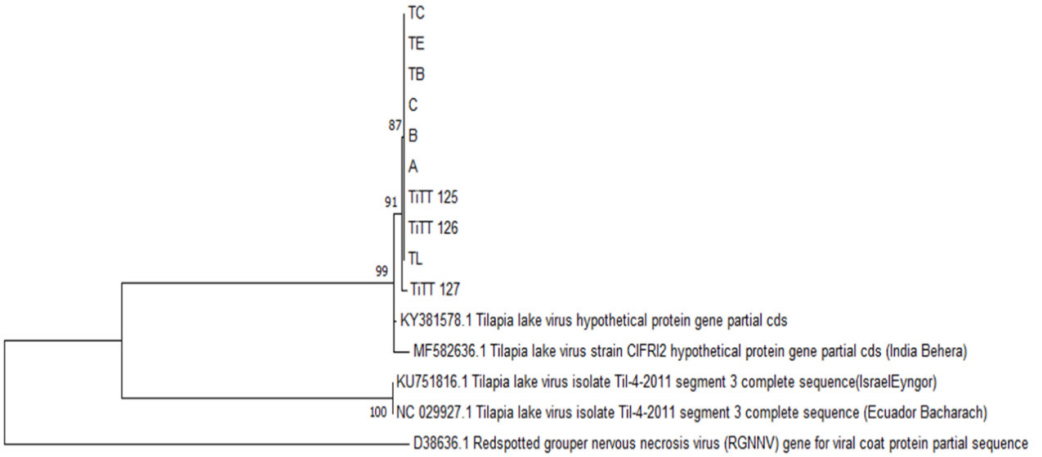

Figure 3. Phylogenetic tree showing the relationship between TiLV strain from Sarawak (A, B, C), Perak (TB, TC, TE, TL) and Perlis (TiTT 125 – 127) aligned with other published segment 3 of TiLV sequences deposited in GenBank. The tree was determined using neighbour-joining methods.

Among the 10 segments of TiLV, only Segment 1 has been extensively studied. Based on the phylogenetic tree, TiLV sequences separated into three clades namely Israeli – 2011, Israeli – 2012 and Thai clade which closely related to monophyletic Israeli – 2012 clade (Taengphu et al., 2020). Nucleotide sequences similarities of the viral isolates obtained from different countries shows 97.2% – 99% similarities between the Ecuador and Israel strains, 95.38% – 95.68% for Thailand – Peru strains, 96.32% – 97.71% in Thailand – Israel strain and 96.89% – 97.13% in Israel – Peru strains based on the genome sequence analysis (Pulido et al., 2019). Latest genomic study of TiLV strains reveal that the Ecuador strain has 95.77% similarities with TiLV strains isolated from Thailand and 97.24% similarities with strain from Peru (Aich et al., 2022). Isolates from Peru and Thailand was identified to be closely related with Israel strain although the genetic variation from both of the country shows genetic distant between themselves that suggest the infection of TilV in Thailand and Peru might have caused by the strains from Israel (Aich et al., 2022). Furthermore, TiLV from Thailand shares high sequences similarity with TiLV from Israel, Chinese Taipei, Ecuador, Egypt, India, Indonesia and Philippines (Jansen, Dong and Mohan 2019). Recently, phylogenetic analysis of the TiLV from United States showed that their isolates are most closely related to Thailand TiLV strains (Ahasan et al., 2020)we present the complete coding sequences of two tilapia lake virus (TiLV. Phylogenetic analysis of TiLV isolated from fish in Lake Victoria also showed homology with outbreaks in Israel and Thailand (Mugimba et al., 2018)and of the 442 samples examined from 191 fish, 28 were positive for TiLV by PCR . In terms of tissue distribution, the head kidney (7.69%, N = 65. This suggesting that the virus spreads across continents and tilapia trading around the world came from the same source (FAO, 2016).

Phylogenetic analysis is able to track how pathogens have dispersed across the globe, and inferences about the movement of TiLV have been made based on phylogenetic analysis of short sequences from a single segment (Nicholson et al., 2017; Surachetpong et al., 2017). Whether this is appropriate for TiLV, with its segmented, negative-sense RNA genome that may be prone to re-assortment, does not appear to have been tested, though previous studies with limited sequence data noted an absence of re-assortment in this virus (Nicholson et al., 2017; Pulido et al., 2019). However, recent study conducted by Chaput et al., (2020) using TruSeq RNA library preparation following the standard manufacturer's protocol (Illumina Inc., San Diego CA, USA) which further analysed

using Quartet Tree Analysis (Suzuki, 2010), shows the nucleotide sequence of TiLV can and do undergo reassortment process which may contribute to different evolutionary records outcomes and inaccurate estimation of the virus evolutionary history based on the multiple region genomic analysis. Recent study showed that phylogeny of TiLV isolates depends on the segment sequence; in particular, segments 5 and 6 appear to have undergone a relatively recent re-assortment event (Chaput et al., 2020). The findings illustrate the need to exercise caution when using phylogenetic analysis to infer geographic origin and track the movement of TiLV.

Conclusion

Future research should look at and demonstrate the capacity of local isolates to inflict diseases and virulence in susceptible fish, since these findings were predicated on nucleic acid detection by PCR. The results present in this article is preliminary, and only focus on a short frame of one gene of TiLV i.e., segment 3. More studies need to be done, and we recommend using whole genomes wherever possible or at least segment 5 and 6 which appear to have undergone a re-assortment.

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