

**ISOLATION AND MOLECULAR CHARACTERIZATION OF LUMPY SKIN DISEASE
VIRUS IN EGYPT DURING 2017- 2018****Ahmed Hodhod¹, Emad Elgendy², Mervat I. Abd El-Moniem³ and Madiha S. Ibrahim^{2*}**¹Animal Health Research Institute (AHRI), Virology Department, Damanhour, Egypt.²Department of Microbiology, Faculty of Veterinary Medicine, Damanhour University, Egypt.³Animal Health Research Institute (AHRI), Virology Department, Dokki, Egypt.***Corresponding Author: Madiha S. Ibrahim**

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ABSTRACT

Lumpy skin disease is not associated with high mortalities, but the economic losses accompanying its eruption are higher in cattle trade. Lumpy skin disease virus is a member of genus *Capripoxvirus* (CaPV), family *Poxviridae* and is the cause of lumpy skin disease. In our study, we isolated and molecularly characterized Lumpy skin disease virus circulating in Egypt from April 2017 to September 2018. A total number of 295 samples including skin biopsies (243), whole blood samples (50) and tick groups (2) were tested by real-time PCR. The results showed that 91.3%, 76% and 100% of skin biopsies, whole blood and tick samples were positive, respectively. Thirty positive samples were isolated on embryonated chicken eggs chorioallantoic membranes. The results showed that 24 samples (80%) displayed characteristic pock lesions. The results were confirmed by conventional PCR and all 24 samples were confirmed as lumpy skin disease virus. Two samples were sequenced and phylogenetic analysis showed high similarity between the isolated lumpy skin disease virus and sheep and goat poxviruses. Furthermore, the tick samples and skin biopsies showed higher viral titration indicating their usefulness for viral detection in suspected cases. Moreover, the real-time PCR is one of the rapid diagnostic tools that can be used for viral detection in endemic areas with high specificity and sensitivity compared to other routinely used tools.

KEYWORDS: Lumpy skin disease virus, real-time PCR, Isolation, CAM, conventional PCR, Sequencing.**INTRODUCTION**

Lumpy skin disease (LSD) is one of the most serious poxvirus diseases of livestock with high morbidity (up to 90%) and low mortality (less than 10% and in some outbreaks 20-75%).^[1,2] The economic losses of LSD come from decreased milk production and severe skin damage.^[3] The disease also causes a temporary or permanent infertility in bulls and death due to secondary bacterial infections.^[4]

Lumpy skin disease is a pox disease of cattle characterized by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, edema of the skin and sometimes death.^[4] LSD was detected for the first time in Zambia in 1929 and was termed pseudo-urticaria.^[5] LSD is caused by Neethling virus, which is the prototype strain of LSD.^[6] LSD virus (LSDV) together with sheep pox and goat pox viruses form the genus *Capripoxvirus* within the subfamily *Chordopoxvirinae* of the family *Poxviridae*.^[7] LSDV cannot be distinguished from sheep and goat pox by neutralization test or other serological tests.^[8] It had been documented that LSDV strains have sequence homology more than 98% with Kenyan strain (O 240/KSGP) of sheep and goat poxvirus (SGPV).^[9] The

virus spread from Zambia to Botswana in 1943.^[10] In 1957, it appeared in Kenya, associated with an outbreak of sheep pox.^[11] The first appearance of LSD outside Africa was reported in Kuwait in 1986.^[12] After that the disease appeared in the United Arab Emirates and Republic of Yemen.^[13] LSDV infection had been reported in Saudi Arabia in 1992^[14] and then in 2015 and 2016.^[15] The disease was reported in Israel, Bahrain, Oman and the West Bank.^[16,17] Subsequently, the disease appeared in Iraq, Iran, Azerbaijan, Cyprus, Greece and Armenia in 2014 and 2015.^[18,19]

In Egypt, LSDV was isolated for the first time from cattle in 1988 during two disease outbreaks in Suez and Ismailia governorates.^[20] Later on, LSD had been recorded in different Egyptian governorates like Banisuef, Beheira, Ismailia and New Valley in 2006.^[21] LSDV infection was detected in both cattle and water buffaloes in Kalubiyah governorate where the prevalence rate and clinical signs were less in buffaloes than in cattle.^[22] The disease was associated with high prevalence of insect vectors which facilitate the transmission of LSDV. Animals are mostly exposed to insect vectors due to the unregulated breeding system and poor animal husbandry in farms.^[23]

The natural host for LSD is cattle and it is more severe in young animals.^[23] Infections had been reported also in Asian water buffalo (*Bubalus bubalis*) in Egypt.^[22,25] Wild ruminants like Giraffe, Arabian Oryx and Impala are also affected.^[26]

The principle route of transmission for LSDV is mechanically by arthropod vector.^[27,28,29] In the absence of insects, transmission by direct contact is not effective.^[3] Mechanical transmission of LSDV from infected to susceptible cattle has been documented by female *Aedes aegypti* mosquitoes.^[29] Mosquitoes can transmit the virus to healthy cattle over a period of 2 to 6 days after infective feeding, so it had been suggested that mosquito species are basic vectors of LSDV.^[29] High climatic changes and new marketing tools for animals and animal products had made capripoxviruses worldwide threats.^[2] LSD outbreaks tend to be sporadic, depending upon animal movements, immune status, wind and rainfall patterns affecting vector populations. The recurrence of LSD outbreaks in Egypt and Israel in 2006 after an absence of 17 years might be due to the previous factors together.^[30]

Due to the recurrent occurrence of LSD in Egypt and Middle East and high economic losses resulting from its outbreaks, the aim of this work was to isolate and characterize field isolates of LSDV during 2017- 2018. Furthermore, the isolates were phylogenetically analyzed to stand on the latest changes in viral genome and its molecular characteristics in order to improve the control and prevention strategy of LSD.

MATERIAL AND METHODS

Samples

Totally, 295 samples were collected from cattle showing cutaneous nodules of different sizes on different parts of the body (head, neck, abdomen and legs), anorexia, fever and enlarged superficial lymph nodes. Particularly, 243 samples were skin biopsies, 50 samples were blood samples from only feverish animals and 2 samples from tick groups at the site of infection. The samples were prepared as described by OIE, 2010.^[4] The samples were collected from different Egyptian governorates including; Beheira, Gharbia, Giza, Sharkia, Kafrelsheikh, Kalubia, and Fayoum. The sample distribution is shown in Table (1).

Table (1): Number of collected skin biopsies, blood and tick samples from different locations from April 2017 to September 2018.

Governorate	Skin biopsy	Blood	Ticks	Total
Beheira	53	8	2	63
Gharbia	50	0	0	50
Giza	39	9	0	48
Sharkia	29	14	0	43
KafrElsheikh	31	5	0	36
Kalubia	26	8	0	34
Fayoum	15	6	0	21
Total	243	50	2	295

Viral DNA extraction

The viral DNA was extracted using **Magna pure Total Nucleic Acid Isolation kit (Roche, Germany)** on a MagNA Pure compact LC Instrument (**Roche, Germany**) according to the manufacturer's instructions.

Real-time PCR

All collected samples were tested by real-time PCR using the specific capripox primers (F 5' GGCGATGTCCATTCCTG 3', R 5' AGCATTTTCATTTCCGTGAGGA 3') and fluorogenic TaqMan Probe (5' CAATGGGTAAAAGATTTCTA 3') as described previously by **Balinsky et al., 2008.**^[31] The reaction was performed on LightCycler[®] 2.0 PCR instrument according to the manufacturer's instructions (**Roche Diagnostics, Germany**) and the thermocycling conditions were adjusted according to the protocol described by **Balinsky et al., 2008.**^[31]

Viral isolation

Thirty representative positive samples by real-time PCR were inoculated on chorio-allantoic membrane (CAM) of 9 days old embryonated chicken eggs (ECE) according to the method described by **Burleson et al.**^[32] The

harvested CAMs were examined for pathological changes after 6-7 days of incubation at 33 °C.

Conventional PCR

The nucleic acid was extracted from the CAMs that showed pock lesions according to the manufacturer's instructions (**QIAamp DNA mini kit**). Conventional PCR was performed for amplification of LSDV envelope protein gene (P32)^[23] and ORF 103 (Open Reading Frame) gene^[33] by using Emerald Amp GT PCR mastermix kit (**Takara**) according to manufacturer's instructions. The primers used are shown in **Table (2)**, and the cycling conditions are described in **Table (3)**.

Table (2): Oligonucleotide primers sequences of P32 and ORF 103 genes.

Gene	Sequence	Amplified product
P32	5'-TTTCCTGATTTTTCTTACTAT-3'	192 bp
	5'-AAATTATATACGTAAATAAC-3'	
ORF 103	5'-ATGTCTGATAAAAAATTATCTCG-3'	570 bp
	5'-ATCCATACCATCGTCGATAG-3'	

Table (3): Cycling conditions of conventional PCR.

Gene	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No of cycles	
P32	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	35	72°C 7 min.
ORF 103	94°C 5 min.	94°C 30 sec.	52°C 45 sec.	72°C 45 sec.	35	72°C 10 min.

The produced PCR products were electrophoresed in 1.5% agarose gel containing 1 µg/ml ethidium bromide in Tris-acetate buffer as described by Sambrook and Russell, 2006.^[34] The DNA bands were visualized using ultraviolet transilluminator at 192pb and 570bp for P32 and ORF 103 genes, respectively.

Sequencing and phylogenetic analysis

The PCR products were sequenced for ORF 103 gene by Applied Biosystems automated DNA Sequencer (ABI, 3130, USA) using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA Cat. No 4336817). BLAST[®] analysis^[35] was initially performed to establish sequence identity to GenBank accessions. Finally, phylogenetic analysis was performed using Phylogeny.fr software.^[36,37]

RESULTS

Molecular detection and identification of LSD virus from collected samples by real-time PCR

All collected 295 samples were rapidly tested with real-time PCR. The results showed that 222 out of 243 skin biopsies (91.3%), 38 out of 50 blood samples (76%) and both collected tick group samples (100%) were positive. These results exhibited that 262 out of 295 samples (88.8%) were positive for LSD virus detection as shown in Table (4). The results showed that LSD virus was detected in Beheira, Kalubia, Kafrelsheikh, Sharkia, Gharbia, Giza and Fayoum with 95.2%, 94.1%, 91.7%, 88.4%, 86%, 85.4% and 71.4%, respectively as shown in Table (4). Furthermore, the results of real-time PCR showed that the CT (cycle threshold) value of 72 out of 262 positive samples, 102 out of 262 and 88 out of 262 were <15-20, 21-30 and > 30, respectively.

Table (4): Number and percent of different positive samples from different Egyptian governorates.

Governorate	Skin biopsy	Blood	Ticks	Total
Beheira	51/53 (96.2%)	7/8 (87.5%)	2/2 (100%)	60/63 (95.2%)
Kalubia	24/26 (92.3%)	8/8 (100%)	0/0 (0%)	32/34 (94.1%)
Kafr Elsheikh	29/31(93.5%)	4/5 (80%)	0/0 (0%)	33/36 (91.7%)
Sharkia	29/29 (100%)	9/14 (64.3%)	0/0 (0%)	38/43 (88.4%)
Gharbia	43/50 (86%)	0/0 (0%)	0/0 (0%)	43/50 (86%)
Giza	35/39 (89.7%)	6/9 (66.7%)	0/0 (0%)	41/48 (85.4%)
Fayoum	11/15 (73.3%)	4/6 (66.7%)	0/0 (0%)	15/21 (71.4%)
Total	222/243 (91.3%)	38/50 (76%)	2/2 (100%)	262/295 (88.8%)

Isolation of LSD virus on CAM of embryonated chicken eggs

The results revealed that 20 out of 25 skin biopsies, 2 out of 3 blood samples and 2 out of 2 tick samples showed characteristic pin-point pock lesions arranged in streaks after 6-7 days (Figure 1 A and B).

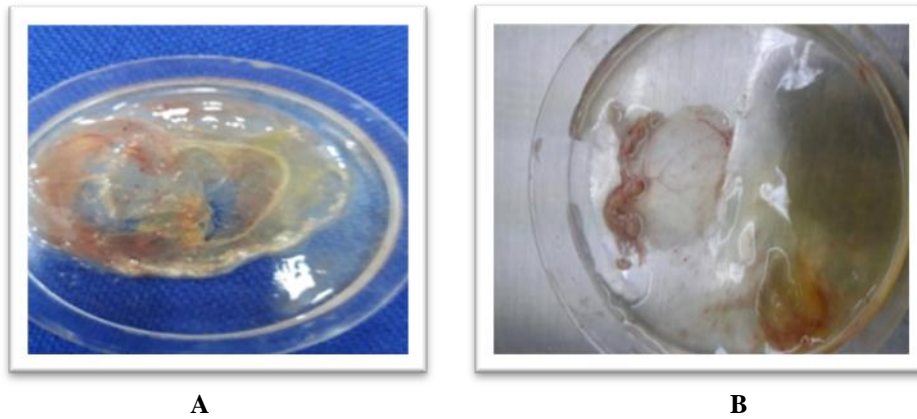


Fig (1): Pock lesions in the form of pin-point white foci on the CAM arranged in streaks as shown in A and B.

Polymerase Chain Reaction (PCR) for the confirmation of LSDV isolation on CAM

All positive CAM samples (24 samples) were tested by conventional PCR. The results showed that all the 24

samples (100%) were positive by conventional PCR. The expected PCR product size was at 192 bp and 570 bp regarding to P32 and ORF 103 gene primers, respectively as shown in **Fig (2, A and B)**.

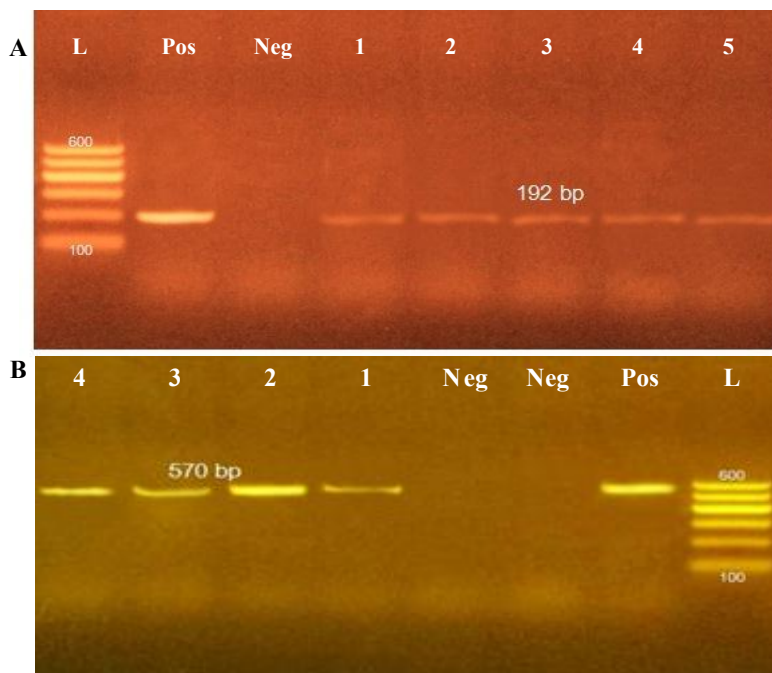


Figure (2): Agarose gel electrophoresis of the amplified (A) P32 gene detected at 192 bp, and the amplified (B) ORF 103 gene detected at 570 bp. L: DNA ladder, Pos: positive control, Neg: negative control, 1, 2, 3, 4, 5 are positive samples.

Sequencing and phylogenetic analysis

Two out of 24 PCR positive samples from Beheira and Sharkia were sequenced and analyzed for ORF 103 gene. The results showed that the two isolates shared similarity with sheep and goat poxviruses as shown in **Fig (3)**. The samples were closely related to Goat poxvirus isolate GTPV/28-08/Palampur/2012 and Sheep poxvirus isolate SPPPV/30-02/Ahmedabad/2008.

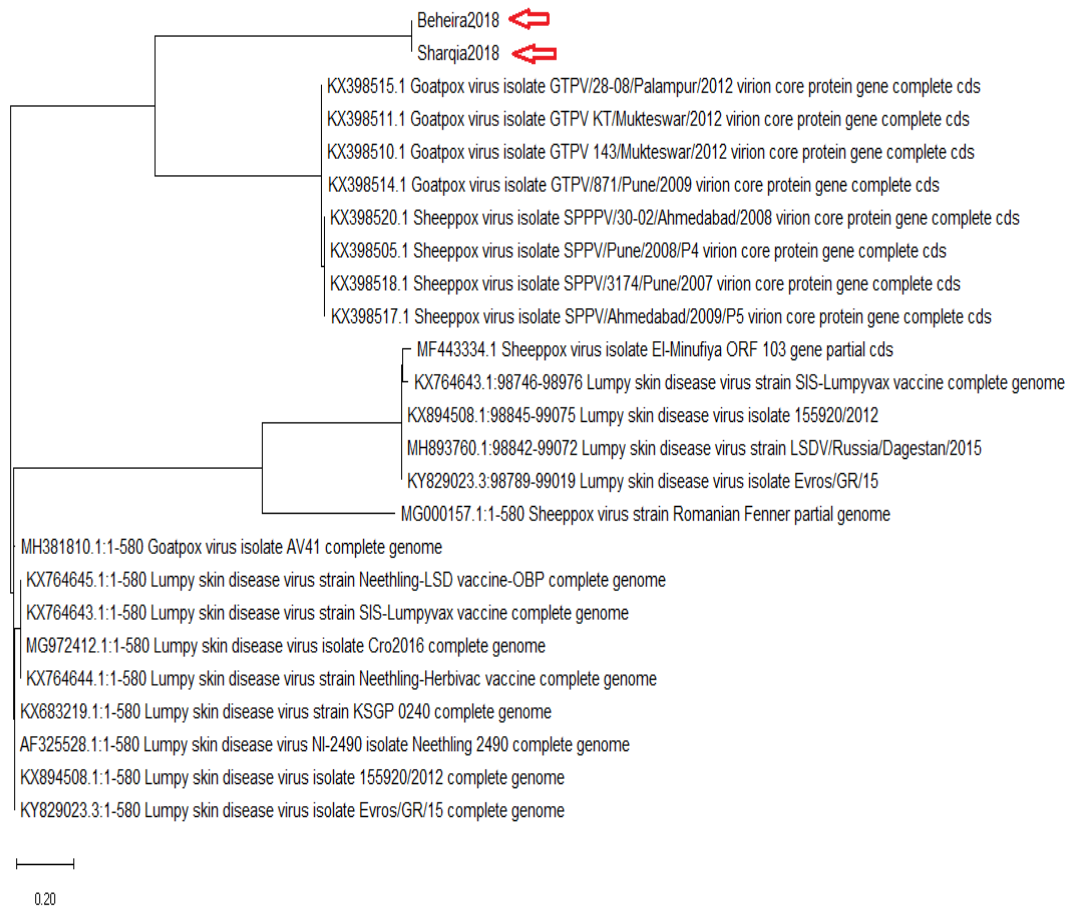


Fig (3): Phylogenetic analysis of LSD virus ORF 103 gene showing high similarity between the isolated LSDV and SGPV.

DISCUSSION

Although LSD is not associated with high mortalities (1-3%), the economic losses accompanying LSD eruption is higher. It results in great economic losses due to decreased feed intake, milk production and weight conversion beside abortion, infertility, and damaged hides.^[38] The annual financial cost included the average production losses, due to morbidity and mortality arising from milk loss, beef loss, traction power loss, and treatment and vaccination costs at herd level.^[24] The disease is currently endemic in most African countries and spread out of Africa into the Middle East region including Egypt.^[39]

In this study, we investigated sudden appearance of scattered skin nodules on cattle in different Egyptian governorates from April 2017 to September 2018. On clinical examination, animals showed high temperature (> 40°C), small and large nodules on the neck, back, abdomen and legs beside enlarged superficial lymph nodes and edema of legs in some animals. Although sheep pox vaccines have been used in Egypt to immunize cattle against LSD, many cases of the disease have been noticed.^[40,41,42]

In Egypt, LSD erupts at intervals which may be attributed to many factors such as; refusal of some owners to vaccinate their animals, unsuccessful vaccination and uncontrolled animal movement together with the high stability of LSDV in the environment and presence of insect vectors.^[23,39] Previous findings showed that rainy seasons were predisposing for the increase of insect population, which subsequently enhances the occurrence of LSD outbreaks.^[24,43] The important determinants for transmission of LSDV are the presence of blood sucking arthropod vectors, which predispose to generalized infection and increase the opportunity for further transmission of the virus by arthropod vectors.^[28]

Therefore, Rapid diagnosis of LSDV by PCR would facilitate rapid application of control measures and allows rapid removal of positive cases.^[23] Real-time PCR is faster, easier and safer than conventional PCR, which requires the use of ethidium bromide (carcinogenic) and ultraviolet rays during the test. These advantages give a great priority to real-time PCR over conventional assay to be used in diagnosis of LSDV especially in endemic areas like Egypt. On the other hand, virus isolation in tissue culture takes longer time^[44] beside the probability

of bacterial and fungal contamination.^[45] Also, the use of immunofluorescent assays to detect LSDV may cause nonspecific fluorescence leading to false diagnosis^[46] and cannot discriminate members of capripoxvirus.^[21]

Here, we used the real-time PCR assay for detecting viral DNA in skin biopsies, blood samples and tick groups present at the site of infection. It was reported that CaPV real-time PCR assay is comparable to or exceeds the virus isolation for preclinical detection of sheep poxvirus (SPPV) in sheep.^[31] The real-time PCR assay also is a useful tool for early detection and control of infections by other CaPV viruses, including goat poxvirus (GTPV) and LSDV. This assay can differentiate between CaPV and other viruses causing vesicular disease in ruminants and has a high sensitivity in clinical samples. The CaPV real-time PCR assay represents a significant improvement over other established methods of CaPV detection due to its speed, simplicity, and ability to be carried out in laboratories without the need for tissue culture facilities.^[31]

The real-time PCR results showed a proper efficacy in detecting viral DNA, especially in skin biopsies, which contain more viral particles as mentioned before.^[47,48,49] In our study, most of the positive samples with real-time PCR (174 out of 262) had low CT values (11-30) which means high nucleic acid (DNA) concentration in these samples (viral load) and may reflect high severity of animal infections. These samples were skin biopsies and samples collected from ticks. Real-time PCR could detect viral DNA in tissue samples (91.3%) which is considered the predilection site for LSDV, more than in blood samples (76%) which agree with **Babiuk et al., 2008**^[2] and **Awad et al., 2010**^[50] who mentioned that LSDV was present at high levels in skin nodules and at low levels in the blood of clinically affected cattle.

LSDV can be routinely isolated on embryonated chicken eggs (9-12 day old) through CAM inoculation providing maximum yield of LSDV.^[45] Here, we isolated 24 out of 30 representative positive samples by real-time PCR on the CAM, the results showed different degrees of pathological changes as hyperemia and thickening but the characteristic pock lesions were prominent after the third passage in agreement with **El-Nahas et al., 2011**^[22] and **Lamya et al., 2017**^[41] who isolated LSDV on CAM of ECE and reported the same characteristic lesions for LSDV.

CAM samples were confirmed by conventional PCR that has high sensitivity in detecting LSDV in tissue and blood samples as mentioned before by **Ireland and Binopal, 1998**.^[51] Here, conventional PCR identified viral DNA in all 24 isolated samples (100%) agreeing with **El-kholy et al., 2008**^[52] and **Amal et al., 2010**^[53] who detected LSD viral DNA in all samples by conventional PCR and recommended the use of PCR assay for rapid detection and identification of LSDV.

The nucleotide sequence of two field isolates from Beheira and Sharkia showed relative relation with other sheep and goat poxviruses obtained by blast analysis of nucleotide sequences in the Genbank, and this was confirmed by phylogenetic analysis. These results support the concept that all capripoxviruses originated from one ancestor lineage as they are genetically related.^[54] Our result agreed with **El-kholy et al., 2008**^[52] who found that the outbreak isolates in Egypt in 2006 were identical to LSDV and closely related to sheep and goat poxviruses.

CONCLUSION

LSDV is still circulating in cattle in Egypt and its recurrence despite the use of routine vaccination programs indicate that epidemics are possible. Our results support the use of real-time PCR as a fast and reliable tool for diagnosis of LSDV especially in endemic countries, and recommend establishing of novel strategies to control LSD in Egypt.

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