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Genotyping and Phylogenetic Analysis of Clinically Affected Puppies with Canine Parvovirus with Monitoring of Hematological and Biochemical Changes

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ABSTRACT

Canine parvovirus-2 (CPV-2) has been the most prevalent causative agent of enteritis in puppies throughout Egypt and all around the globe. Following its first identification in 1978, *CPV-2* became swiftly supplanted with three antigen subtypes: *CPV-2a, CPV-2b,* and *CPV-2c*. The three subtypes of *CPV-2* have been reported in Egypt. Therefore, this study aimed to monitor clinical findings, laboratory changes in puppies suffered from *CPV*, and its molecular characterization using polymerase chain reaction (PCR) and DNA sequencing to further understand the subtypes of *CPV-2* that are present in dogs in Ismailia, Egypt. The clinical signs of infected puppies were vomiting, dehydration, and foul-smelling bloody diarrhea. Microcytic hypochromic anemia and thrombocytopenia were evident in association with leucopenia, neutropenia, and lymphopenia. In addition, hyponatremia, hypokalemia, hypochloremia, hypocalcemia, and hypophosphatemia were also recorded in diseased puppies. All 47 tested fecal samples were positive to *CPV-2* when examined by PCR and gel electrophoresis. The sequence analysis of *CPV-2* showed that the most common subtype is *CPV-2a* that is followed by *CPV-2b* as well as *CPV-2c* with percentages of 71.4%, 14.3%, and 14.3%, respectively. In conclusion, *CPV-2a* was the predominant subtype isolated from the examined puppies and was responsible for the characteristic clinical signs and laboratory changes in diseased dogs. Moreover, epidemiological investigations are needed to determine the prevalence of *CPV* serotypes in Egypt.

Keywords: CPV-2, diarrhea, multiple sequence alignment, PCR, puppies.

INTRODUCTION

Canine parvovirus type 2 (CPV-2) is among the most significant infectious viral infections affecting the dog populations, particularly young pups, producing vomiting, myocarditis, severe hemorrhaging enteritis, and immunosuppression (Ahmed et al., 2018). It is a single-stranded DNA virus, non-enveloped icosahedral with a genome size of roughly 5200 nucleotides and a diameter of 26 nm (Milne et al., 2010). *CPV-2* is a member of the *Parvoviridae* family, the *Parvovirinae* subfamily, the genus *Protoparvovirus*, and the species carnivores *protoparvovirus 1* (Cotmore et al., 2019). Both *canine coronavirus (CCoV)* and *canine parvovirus (CPV)* are the most frequent viral gastrointestinal infections in dogs in the world (Decaro and Buonavoglia, 2012). *CPV-2* is a *feline parvovirus (FPV)* or an *FPV-like virus* that infects wild carnivores and has a wide host range (Cotmore et al., 2019; Stucker et al., 2012).There are at least 6 amino acids changes between *FPV* and *CPV-2* (Chang et al., 1992). The original *CPV-2* was replaced by *CPV-2* variations. In 1979, the first *CPV-2* variation, known as *CPV-2a*, appeared. The single substitution of Gly for Asp at VP2 residue 300 (Organtini et al., 2015). It was discovered in the United States in 1984, as well as Italy in 2000 that *CPV-2b* or *CPV-2c* has been found in the country at that time. The VP2 protein's amino acid 426 was shown to be the

source of antigenic variation among such three variants (Asn in *CPV-2a*, Asp in *CPV-2b*, and Glu in *CPV-2c*) (Miranda and Thompson, 2016). It had both clinical and pathological symptoms. It was proven in 2012 that *CPV-2b* was present in Vero cells after PCR, sequencing, and polymerase chain reaction (PCR). While in 2014, this virus has been shown to be clustered between genotypes 2b or 2c (AL-Hosary, 2018; Yanni, 2012). Generic characterization was used in 2018 to identify genotypes 2a or 2b, with specific attention paid to numerous alterations within genotype 2b (AL-Hosary, 2018). As the number of *CPV* genotyping or research publications in Egypt is limited, this study will help us to understand clearly the genotypes spreading in Egypt.

The current study has been designed to elucidate the clinical findings, biochemical and hematological changes in naturally infected puppies with *CPV*, and molecular characterization of emergent types of *CPV* in order to comprehend the variety and development of canine species parvovirus strains in Ismailia, Egypt from 2019 to 2020.

MATERIALS AND METHODS

Ethical approval

The current study was authorized by the Scientific Research Ethics Committee at Faculty of Veterinary Medicine, Suez Canal University, Egypt with approval number 2021051.

Study period and location

The current study was carried out in pet animal clinic in Ismailia province, Egypt between January 2019 and May 2020.

Animals and clinical examination

A total number of 57 puppies of aged between 2 to 6 months were included in the study. Puppies were classified into 2 groups; the first control group (G1, n= 10) was clinically apparent healthy. The second group (G2, n= 47) suffered from bloody diarrhea and vomiting. The clinical examinations were carried out according to the procedure cited by Côté *et al.* (Côté et al., 2015). A total number of 47 different breed of non-vaccinated puppies (32 male and 15 female) aged between two and six months with average body weight ranged from 5-15 kg were included in the study. Concerning breeds, (17/47) German shepherd, (8/47) Rottweiler, (5/47) Husky and (5/47) Griffon and (3/47) Pit bull, (3/47) Golden, (3/47) Canecorso and (3/47) Dalmatian.

Sampling and procedures

A total number of 47 fecal samples were collected from the diseased dog for molecular characterization of *CPV*. Besides, a total of 114 blood samples were collected from the cephalic vein during the study (two samples / dog), 20 samples from G1 and 94 samples from G2. The first whole blood samples were collected in sterile vacutainer tubes containing EDTA (for hematological analysis). While the second blood samples were collected in a plain tube to separate sera. Clear non-hemolyzed sera samples were pipetted into Eppendorf tubes and stored at −20°C for later biochemical analysis (Coles, 1986).

Qualitative identification of CPV antigens in the faces of dogs

All fecal swabs collected were analyzed for *CPV* Ag using the Rapid *CPV/canine coronavirus [CCV]* Ag Test Kit (Cat. No. RC1105DD, Bionote, Hwasung, Republic of Korea) as directed by the manufacturer.

PCR and molecular characterization of CPV

Genomic DNA of *CPV* was extracted from the previously prepared feces as described by Schunck *et al.* (Schunck et al., 1995). Reference *CPV* strain was used as a positive control. DNA was extracted using Biotake Mini Kit (Biotake Company, Beijing, China) according to the protocol of the manufacturer. The extraction kits contain lysis buffer, column tube, washing buffer, and nuclease-free water. The PCR reactions were carried out in a 50-µl volume containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Kusatsu, Japan), 1 µl of each primer, and 5 µl of DNA template. Double distilled water was applied to complete the amount of the reaction to 50 µl. The used primers sequences (Metabion International AG, Metabion, Germany) were chosen to amplify the partial VP2 region, using full genomes of CPV. The PCR cycling conditions are illustrated in detail in Table (1). PCR products were electrophoresed using 1 % agarose gel and visualized using Gel documentation system (Electrophoresis, Major science, Taoyuan City, Taiwan).

Table (1) Primers sequences, target genes, amplicon sizes, and polymerase chain reaction cycling conditions.

Target gene	Primers sequence	Amplicon size (bp)	Amplification (30cycle)		
			Denaturation	Annealing	Extension
VP2	VP2-F AGCAGATGGTGATCC AAGAT	529bp	56^{0}C 45 sec	94^{0}C 30 sec	72^{0}C 1min
VP2	VP2-R TGGATTCCAAGTATG AGAGG	529 bp	56^{0}C 45 sec	94^{0}C 30 sec	72^{0}C 1min

VP2 gene sequencing & sequence analysis

The amplicons were treated and purified using a Qiagen purification kit according to manufacturer's instruction. Sequencing of VP2 has been generated in one direction using the forward PCR primer. VP2 sequences were received as an ABI file. All of the sequences have been matched to the double complementary sequence, which was then analyzed. In all cases, the sequences became homozygous, and there was no indication of the dual and otherwise mixed infections. Blast searching for the highly similar sequence was downloaded from the gene bank. Consensus sequences were generated that use the Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA, USA) and the Application Biosystems 3130 genomic analyzer equipment (ABI, USA). These alignments included sequences available in gene bank for *CPV-2* strains. Utilizing MODELTEST, we were able to determine the best suited evolution model (Milne et al., 2010). When constructing bootstrapped maximum likelihood trees, the final model of nucleotide substitution has been employed (Guindon and Gascuel, 2003). Amino acid-rooted trees have been drawn utilizing MEGA X, using CPV-2strains distributed worldwide.

Hematological analysis

Total erythrocyte count (RBCs), hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), total leukocyte count (TLC), differential leukocyte count, and platelet count (PLT) were estimated according to the methods adopted by Feldman *et al.* (Feldman et al., 1976).

Biochemical analysis

Sodium, potassium, chloride, calcium, phosphorus, and magnesium concentrations were measured with commercial kits. (Spectrum Co., Cairo, Egypt).

Statistical analysis

The data have been statistically analyzed using an independent T-test with the IBM SPSS software computer program (version 20, USA). The obtained data were shown as mean ± the standard error of the mean (SEM). Significance has been designated at a value of probability <0.05.

RESULTS

Clinical manifestations of CPV-2

The most prominent recorded clinical signs included anorexia, lethargy, depression, vomiting, dehydration, and foul-smelling bloody diarrhea. There was a rise in body temperature (greater than or equal to 39.5 ºC) which progressively turned to subnormal with the progression of vomiting and diarrhea.

Rapid strip test

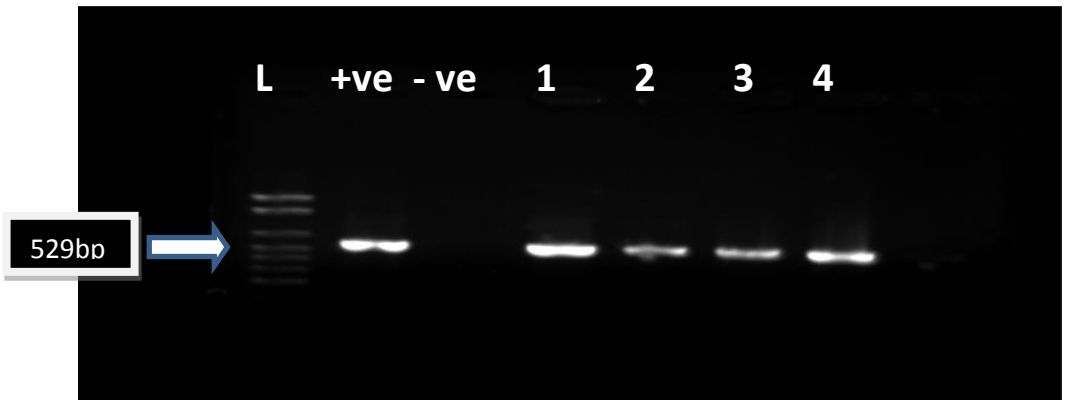
A total of 43 (91.5 %) of the 47 fecal swabs from suspected puppies showed positive Rapid *CPV/CCV* Ag Test.

Conventional PCR detection of CPV

A total of 47 fecal samples have been investigated and examined through PCR and gel electrophoresis for detection of *CPV2* DNA using a specific set of forward and reverse primers for VP-2 capsid protein (Figure 1). All 47 (100 %) fecal samples gave predicted bands in agarose gel at 529 bp to *CPV2.*

Figure (1): Agarose gel 1% showing the polymerase chain reaction amplification products of an expected fragment of 529 bp (lanes 1-4 samples). Lane L represents a 100 bp DNA ladder

(GeneRuler™, Fermentas). Lane Neg represents the negative control. Lane Pos represents the positive control.



Phylogenetic analysis of VP2 and Genotyping of CPV-2

Seven DNA extracts have been amplified in a conventional PCR-based method using a specific set of forward and reverse primers to VP-2 of *CPV*. Amplified 529 bp segments of the VP-2 gene were generated, purified, and sequenced and the sequence was trimmed and cleaned to get the 500 bp length of the VP-2 gene. This segment contains the sequence of the strategic amino acid residue at position 426 which was the main key for *CPV-2* classification into different subtypes (subtype *CPV-2a*, *CPV-2b*, and *CPV-2c*).

The sequence analysis of VP-2 showed that *CPV-2a* was the most common subtype with *CPV-2b* and *CPV-2c* with percentages of 71.4%, 14.3%, and 14.3%, respectively (Table 2).

Table (2): Characterization of CPV2 variants by sequencing:

Fecal samples	Type by sequencing	Percentage %
CPV2aB12, CPV2aB22, CPV2aC15, CPV2aA12, CPV2aA11	2a	71.4%
CPV2bB12	2b	14.3%
CPV2cC2	2c	14.3%

The phylogeny was based on the partial sequences of the VP2 gene (500 bp) of the variations as well as other global *CPV-2* variants (Gene Bank references are included in the phylogenetic tree) as shown in Figure (2).

Hematological analysis

Hemogram of diseased dogs revealed a highly significant (P≤ 0.05) decrease in the mean values of hemoglobin, PCV, RBCs MCV, MCH, and MCHC of dogs suffered from CPV compared with the control group. Blood indices indicate the presence of microcytic hypochromic anemia in dogs suffering from CPV. Significant (P≤ 0.05) thrombocytopenia at was recorded in dogs suffered from CPV compared with the control group (Table 3).

Leucogram revealed a significant (P≤ 0.05) decrease in the mean values of total leukocytes, neutrophils, lymphocytes, monocytes, eosinophil count. While the basophils showed non-significant changes in diseased dogs compared with the control group (Table 3).

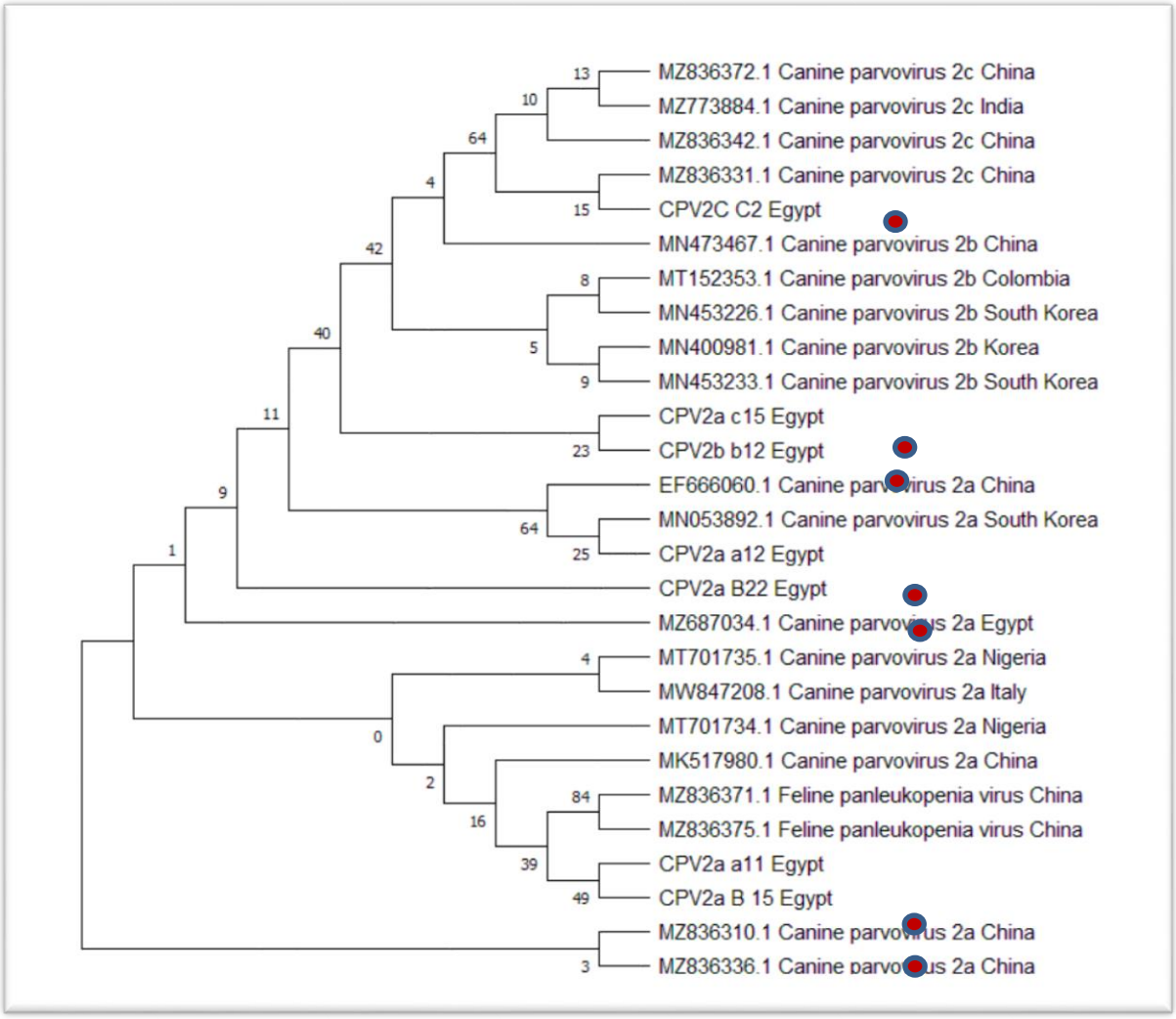


Figure (2). Nucleotide and amino acid phylogenetic tree of CPV2 variants. The phylogeny was carried out on the partial sequences of the VP2 gene (500 bp) of the variants in addition to other worldwide CPV2 variants (GeneBank references are included in the phylogenetic tree).

Table (3): The mean values ± S.E of hematological findings in affected dogs with CPV compared with the control group.

Groups / Parameters	Control (10)	Diseased dogs (47)	P-value
HB (g/dl)	15.16 ± 0.47	6.87 ± 0.17	0.001**
PCV %	431.92 ± 1.23	24.00 ± 1.08	0.002**
RBCs (x106/ml)	± 0.084.58	3.00 ±0.11	0.036**
MCV (fl)	123.6 ± 6.12	80.19±3.81	0.001**
MCH (pg)	37.74 ± 2.11	22.97±0.69	0.003**
MCHC %	35.8 ± 0.90	28.75±0.98	0.001**
Platelet (m/mm3)	387 ± 25.17	306.25±8.0	0.045*
WBCs (x103/ml)	8.58±1.48	3.06±0.44	
Neutrophils %	4.46±0.77	1.66±0.24	
Lymphocyte %	3.27±0.54	1.13±0.16	
Monocyte %	0.66±0.12	0.19±0.03	
Eosinophil %	0.20± 0.06	0.09±0.01	
Basophil %	0.0±0.0	0.3±0.15	0.160 NS

NS: Non-significant (P > 0.05), *: Significant (P ≤ 0.05). **: Highly significant (P < 0.01).

Biochemical analysis

The mean values of sodium, potassium, chloride, phosphorous, and calcium showed significant ($P \leq 0.05$) decreases in diseased dogs compared with the control group. Meanwhile, a non-significant ($P > 0.05$) change in the mean values of magnesium was recorded between groups (Table 4).

Table (4): The mean values ± S.E of serum level biochemical parameters in dogs suffered from CPV compared with the control group.

Parameters \ Groups	Control (10)	Diseased dogs (47)	P-value
Sodium (mmol/l)	144.52 ± 0.66	135.7 ± 1.9	0.032*
Potasium (mmol/l)	5.02 ± 0.11	3.37 ± 0.14	0.019*
Chloride (mmol/l)	99.00 ± 0.71	64.00 ± 8.73	0.001**
Calcium (mmol/l)	1.03 ± 0.01	0.44 ± 0.04	0.047*
Phosphorous (mmol/l)	5.67 ± 0.42	3.73 ± 0.13	0.003**
Magnesium (mmol/l)	2.86 ± 0.36	2.77 ± 0.24	0.160 NS

NS: Non-significant ($P > 0.05$), *: Significant ($P \leq 0.05$). **: Highly significant ($P < 0.01$).

DISCUSSION

CPV infection is one of the most important destructive enteric viral diseases of young puppies that leads to high morbidity and mortality rates in unvaccinated puppies as well as untreated infected dogs (Feldman et al., 1976).

The clinical findings in our study came in agreement with those reported by Zaghawa *et al.* (Zaghawa and Abualkhier, 2019). Bloody diarrhea may be due to the destruction of the epithelium of the intestinal crypts and the resulting villous atrophy (Bastan et al., 2013). Dehydration was also one of the characteristic clinical sequelae, which may be results from loss of a large quantity of fluid from vomiting and diarrhea as recorded by Greene *et al.* (Greene and Carmichael, 2006).

Microcytic hypochromic anemia and thrombocytopenia in *CPV*-infected dogs might be attributed to losses of blood in the stool or the direct effect of parvovirus on the bone marrow precursor cells resulted in decreased erythropoiesis and platelets production as explained by Dash *et al.* (Dash et al., 2020). In addition, the recorded leucopenia, neutropenia, and lymphopenia in diseased dogs ascribed to depletion of the cell lines (granulocytes and megakaryocytes) due to the effect of the virus on the bone marrow during the acute phase of the disease (Terzungwe et al., 2018).

The pronounced hyponatremia, hypokalemia, hypochloraemia, and hypocalcemia in diseased dogs might be attributed to severe losses through vomiting and/or secretion of intestinal fluid during diarrhea resulting in intestinal villous atrophy (Bhat et al., 2015).

Fast *CPV/CCV* Ag Test Kit was used to test a total of 47 fecal swabs of suspected cases of puppies (2-6 months), and 43 (91.5%) samples were confirmed positive. In the field, this is the most often utilized diagnostic technique since it is applicable to be performed by both veterinarians and owners. Since it requires so much viral antigen to create a discernible band, this test's sensitivity can't be expected to be as great as it might be. High specificity and poor sensitivity are confirmed by this test (Soliman et al., 2018).

The 100% of all 47 fecal samples examined by PCR and gel electrophoresis for detection of *CPV-2* DNA that gave predicted bands in agar gel at 529 bp to *CPV-2* (Figure 1), showed that molecular-based tools (PCR) were highly sensitive than immunochromatographic method (rapid test) (Soliman et al., 2018).

VP2 gene fragments were amplified from seven DNA sources. BLASTP found that sequence analysis on all chosen samples matched known

CPV-2 sequences, which was encouraging. The seven *CPV-2* genomes that were identified have been named as *CPV-2ab12*, *CPV-2ab22*, *CPV-2aC15*, *CPV-2Aa12*, *CPV-2aA11*, *CPV-2Bb12*, and *CPV-2cC2*.

The development of the *CPV* virus was linked to particular alterations in amino acids in the capsid area, which is highly antigenic and is the location of binding of neutralizing antibodies that was the basis of serotyping (Miranda and Thompson, 2016; Mochizuki et al., 1996). In this work, seven sequences were compared to each other in terms of nucleotides and deduced amino acid sequences (from *CPV-2* positive) and 20 sequences retrieved from the Gene Bank evidenced three non-synonymous mutations. As such from this study, five of the samples are *CPV-2a*; one sample is *CPV-2b* whilst only one is *CPV-2c*. Therefore, this research provided a clear picture of the *CPV-2* subtypes that are prevalent in Egyptian dogs (Battilani et al., 2006).

In conclusion, *CPV-2* is considered as one of the most serious viral diseases that leads to sever biochemical and hematological alterations in clinically affected puppies. Besides, three subtypes of *CPV-2* are present in Egyptian dogs. Maintaining a close eye on the complete length of VP2 in both clinical samples and vaccinations is critical to identifying the mutations that might lead to vaccine failure and the processes that drive *CPV-2* evolution.

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AUTHOR CONTRIBUTIONS

KEE performed molecular characterization for *CPV* virus through PCR and sequence of some samples and drafted the manuscript. AEM performed the clinical examination of diseased cases, collected and analyzed samples, did the statistical analysis, and reviewed the manuscript. All authors wrote the original draft, read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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