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Original Article

Detection of Canine Parvovirus Type 2 by Designing Multiple Methods and Genetic Characterization in Iran

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Abstract

Canine parvovirus infection is the most highly infectious in dogs younger than six months. Our study aimed to design and optimize an In-house PCR Assay for Rapid Detection of parvovirus type 2 and compares it with REAL-TIME PCR and LAMP Assay and phylogenetic analysis. The virulence gene selected for the categories was *vp2* for CPV-2. PCR products were cloned in pTZ57R/T plasmid for preparation of positive control. Determination of the specificity of primers was done with the negative control virus genomes, and the limit of detection was determined for the Homemade PCR, REAL-TIME PCR, LAMP, and to perform a phylogenetic study using partial *vp2* gene sequences. Added analysis of PCR products using agarose gel electrophoresis for the *vp2* gene showed 485bp, and GAPDH 900 bp bands, respective amplification using negative control genomes as template was negative. The least detectable copy number for *the vp2* gene in a 25 µl PCR reaction equals 19 copies by homemade PCR, LAMP, and REAL-TIME PCR 25 and 21 copies, respectively. The phylogenetic analysis for the five field sequences formed three distinct clusters. The in-house PCR has advantages such as high specificity, sensitivity, and the ability to detect major CPV-2 pathogens. This assay may replace the previous laboratory methods and work as an essential supplement to the more time-consuming assays. Phylogenetic analysis is necessary for epidemiological studies to control and prevent disease.

Keywords: Polymerase chain Reaction, CPV, Molecular diagnostic, *vp2*, Phylogenetic Analysis

1. Introduction

Canine *Parvovirus* (CPV) is one of the most common infectious diseases in dogs younger than six months (1). The first reported new infection mortality was observed in puppies in the late 1970s. Without treatment for CPV infection, the mortality rate can be as high as 91%; with aggressive treatment, the mortality rate drops from 4% to 48%. Understanding the pathophysiology of CPV infection is essential for enabling veterinary technicians to provide prompt treatment and adequate patient care. CPV-2 was an etiological agent of severe hemorrhagic gastroenteritis in dogs and spread rapidly worldwide (2, 3). CPV-2(Parvoviridae family, Parvovirinae sub-family, parvovirus genus) is a 5.2 kb small and non-enveloped single-stranded DNA genome with two promoters; this virus expresses two structural (vp_1 and vp_2) and two non-Structural (ns_1 and ns_2) proteins through alternative splicing of viral mRNAs (4). Based on a few substitutions in amino acid sequence generates new genotypes (CPV-2a, CPV-2b and CPV-2c). In 1979 and 1984, CPV-2a and CPV-2b new genetic variants were characterized (5). This variant distinguishes CPV-2a (426 Asn) and CPV-2b (426) Asp by amino acid in the capsid protein gene. CPV-2c ,

a new genotype in, 2001 firstly described in Italy (6). Diagnosis of CPV-2 infections is critical, especially in dogs, and prevents transmission to susceptible contact animals (6, 7). Diagnosis based on a clinical sign is not definitive since several other pathogenic organisms can cause diarrhea in dogs. Therefore, a clinical diagnosis of CPV-2 infection should always be confirmed with laboratory tests (8). Various laboratory methods have been developed to detect CPV-2 in the feces of infected dogs, for example, electron microscopy (EM), ELISA, immunochromatographic tests (IC), hemagglutination (HA) tests, Viral isolation (VI), hemagglutination inhibition (HI) tests, Conventional Polymerase chain reaction (PCR), Real-Time PCR, and isothermal method (such as LAMP) (9). Molecular techniques are the methods of choice for CPV-2 diagnosis because they are based on detecting DNA, which is highly sensitive. In the present study, we aimed to develop a molecular detection kit based on PCR assay for specific identification of CPV genus based on *the* vp_2 conserved part (10).

On the other hand, SYBER Green REAL-TIME PCR is known for its simultaneous detection and DNA quantification. Simultaneous analysis of its amplifies during amplification significantly shortened the rotation time by agar gel electrophoresis after amplification. Despite having comparable performance, it depends on the REAL-TIME PCR device, which is often expensive and requires regular maintenance (11). The lamp method is fast, Simple, and cost-effective. The measurements were primarily validations in the laboratory. Our study aimed to design and optimize an In-house PCR Assay for Rapid Detection of parvovirus type 2 and compare it with REAL-TIME PCR and LAMP Assay. It was preferred to compare various methods because serology methods are often used in Iran and may not detect new variants. We selected marker genes exhibiting the highest degree of homology among the accepted sequences found in databases. Another aim of this study was the phylogenetic study and genetic relation of CPV2 in Tehran city.

2. Materials and Methods

The viral genome of CPV-2 as positive control and genome of some other negative control were received from the institute pasture Iran. The target gene was vp_2, and Used *GAPDH* for internal control.

2.1. Primer Design

For the vp_2 gene, 25 sequences get from NCBI (https://www.ncbi.nlm.nih.gov). The targets gene primer was designed by the Gene Runner (version 6.5.52) and CLC sequence viewer software (version 8.0) (CLC bio, Aarhus, Denmark co.), for Specific analysis of primers used Primer–Blast and Designed Primers amplified 485 bp for vp_2 gene was synthesized by Sinaclon Bioscience Co (Sinaclon, Iran) 200 bp for SYBER Green REAL-TIME PCR and LAMP primer using Primer Explorer V5 software, Eiken- specific primers were designed (http://primerexplorer.jp/e/) (11).

2.2. PCR Amplification

PCR reaction was ready for diagnosis of this gene in a volume of 25 µl. This reaction mixture consisted of 1X Buffer and dNTPs. /2mM, $MgCl_2$ 2 mM, 100 ng viral genome, 10 pmol forward 5'-GAAACCAACCATACCAACTCC - 3' and reverses primers 5'- CCCGTCCTGCTGCAATAG -3' and 1-unit *Taq* DNA polymerase enzyme. PCR program was done for 33 cycles in conditions of the initial denaturation at 94℃ for 4 minutes, Second denaturation at 94℃ for 40 seconds, an annealing temperature of 52℃ for 40 seconds, elongation time of 72℃ for 45 seconds and final elongation 72℃ for 5 minutes (12).

2.3. LAMP Reaction

The reaction was performed in a final volume of 25µl containing 40 pmol of internal primers (FIP and BIP), (FIP: CGTCCTGCTGCAATAGGTGTT-CCATATTATTCTTTTGAGGCGT, BIP: GGAGCGCAAACAGATGAAAATC-TTTTTGACCATGTTGTCTACC) 5pmol external primers (F3 and B3)(F3: GCTGAGGTTGGTTATAGTGC , B3: GGTGTTTCTCCTGTTGTGG), 10pmol loop primer

(GCAGCAGATGGTGATCCAAG)1.4mM of-deoxy nucleotide triphosphatase (dNTPs), 1M betaine, 1x ThermoPol Reaction Buffer, 8mM MgSo4, 8 units of *Bst* DNA polymerase enzyme (New England Biolabs, Ipswich, MA, USA) and 2µl of the DNA (13). Also, a 25 Micromolar concentration of fluorescent Calcein compound (Dojindo Molecular Technologies, Inc., Tokyo, Japan) was added to the mix that indicated the reaction. The mixture was incubated at 65 ° C for 1 hour and finally for 5 min at 80 ° on - Real-time turbidimeter (LA-320C; Ceramics, Kyoto, Japan), and turbidity in the reaction mixture was checked every 6 seconds at a wavelength of 650nm (13).

2.4. SYBER Green REAL-TIEM PCR

The REAL-TIME PCR reaction was ready for diagnosis of this gene in a volume of 20 µl. 10 µl 2x PCR master mixture with SYBER Green, 10 pmol forward 5'- CGTGGTGTAACTCAAATGGG - 3' and reverses primers 5'- GGTGTTTCTCCTGTTGTGGTAG -3', 6µl deionized water, and 2µl DNA (220ng/µl). PCR program was done for 33 cycles in conditions of the initial denaturation at 94℃ for 10 minutes, the Second denaturation at 94℃ for 15 seconds, an annealing temperature of 56℃ for 25 seconds, elongation time of 72℃ for 20 seconds.

2.5. Specificity Determination

The PCR reaction was done by controlling negative genomes for assessing the primer specification by *Rotavirus*, *Norovirus*, *Sapovirus*, *Salivirus and Astrovirus* Genome.

2.6. Cloning and Positive Control Preparation

The PCR products for the *vp2* gene were cloned for positive control. Purifying products using the Sinaclon purification kit (Sinaclon, Iran), ligation reaction between *pTZ57R*/T plasmid, and purifying the *vp2* gene were done separately according to work instructions of the Sinaclon PCR cloning kit (Sinaclon, Iran). After preparing competent *E. coli JM107*, the cells were transformed and cultured onto Luria-Bertani medium (Merck) containing X-gal(40g/ml), IPTG (38.4g/ml), ampicillin (100g/ml), tetracycline (50g/ml) and incubate overnight at 37°C. The selected colonies received each fragment and confirmed. For final confirmation of the insert receiving clones, enzymatic digestion was done after plasmid extraction using a Sinaclon plasmid mini-Extraction kit (Sinaclon, Iran). The confirmed recombinant plasmids were named *pTZ57R*/T*vp2*, representing plasmids containing the *vp2* gene, respectively (11).

2.7. Sensitivity and Limit of Detection (LOD)

To determine the reaction sensitivity, the minimum copy number of the target gene to show a visible bond in the PCR was calculated. For this reason, 10- fold serial dilutions (10^{-1}-10^{-8}) of the *pTZ57R/T-vp2* plasmids with specific concentrations were prepared. After PCR on the serial dilutions of plasmids, the previous dilution that showed a visible band was determined as the method's limit of detection (LOD). Finally, the DNA concentrations of the last dilutions were calculated and converted as the copy number of the respective gene (13). We used *the GAPDH* gene to confirm DNA Extraction and reaction done as Internal Positive Control. PCR reaction was ready for diagnosis of this gene in a volume of 25 µl. This reaction mixture consisted of forward 5'- GAAGGTCGGAGTCAACGGA - 3' and reverses primers 5'- GGCCATGAGGTCCACCAC -3' and 1-unit *Taq* DNA polymerase enzyme and PCR Mixture. PCR program was the same with the *vp2* gene. This homemade setup method studied fifty fecal samples from Tehran veterinary laboratory.

2.8. DNA Sequencing and Phylogeny

The products of PCR were purified from the PCR Product by Sinaclon purification kit (Sinaclon Co., Iran) and PCR purified template sequencing. Sequencing data editing, assembling and analyses were made using CLC Sequence Viewer 6.8.1 homology analysis of 485 bp region to detect reference sequences and alignment of multiple sequences using Mega X version 10.0.05. The phylogenetic tree was constructed with the neighbour-joining method (13).

3. Results

3.1. PCR Reaction and Cloning

Amplified fragments of the *vp2* with lengths of 485 bp respectively were determined on the agarose gel (Figure 1A). The PCR reaction related to the *vp2* gene did not show any band using genomes of control negative; it showed the PCR specificity in this study. Digestion reaction with *kpnI* enzyme was performed as a confirming reaction for cloning.

Results of the enzymatic digestions confirmed the suitable cloning of the target gene and the creation of the *pTZ57R/T-vp2* recombinant plasmid. In sensitivity determination assays, the last dilutions of the *pTZ57R/T-vp2*, a recombinant plasmid that showed the clear band on agarose gel, were 10^{-7}, respectively. The copy number of the last detectable for *vp2* equals 19 copies (Figure 1B).

3.2. Internal Positive Control

We used *the GAPDH* gene for Confirmation of DNA Extraction, and the reaction was done as IPC; in positive control observed *vp2* and *GAPDH* bands on agarose Gel (Figure 2A). Based on unknown Samples for Test Set Up and confirmation of CPV-2 diagnosis, in this study, we collected 80 unknown samples, and 50 were positive using this homemade method (Figure 2B). Blast in NCBI was used to determine the sequence homology and similarity of the *vp2*. Results showed that nucleotide homogeneity among these isolates with the *vp2* gene was 100% (Figure 2).

3-3 Phylogenetic Analysis

NCBI BLAST was used to determine the sequence similarity and homology of the *vp2* sequence. Homology results indicate it was between 96 to 100 %. The MW248917- MW248918- MW248919- MW248920- MW248921sequences are deposited in the Gene Bank with the accession numbers: MW248920 high similarity to Canine parvovirus type 2c, MW248917 to type 2b, MW248921, MW248919 to type 2a and MW2489918 to canine parvovirus (Table 1 and Figure 3) (14).

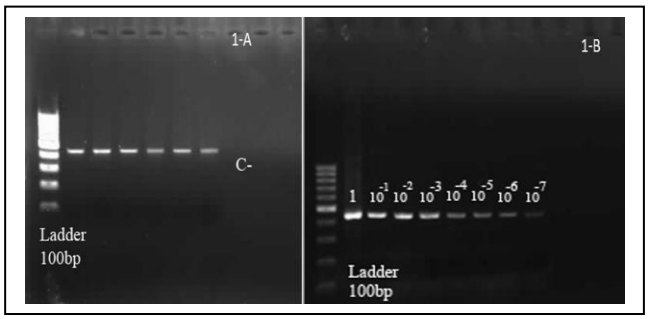

Figure 1. 1A: PCR Amplification for *vp2*, 100bp DNA Ladder, 2 PCR product 485 bp and NC: negative control. **1B:** Limit of Detection for *vp2 in* determined 10^{-7}

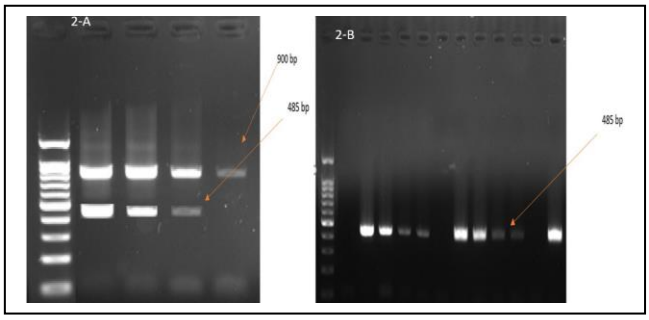

Figure 2. 2A: PCR Amplification for *Vp2* and *GAPDH*,100bp DNA Ladder, PCR product 485 bp for *Vp2* and 900 bp for *GAPDH*. **2B:** PCR Amplification for *vp2* gene and unknown Samples

Table1. Estimates of Evolutionary Divergence between Sequences of CPV2 genome

MW248921																
MW248920	001															
MW248919	000	001														
MW248918	000	001	001													
MW248917	001	000	002	001												
KX425922.1Canine_parvovirus_2a	000	001	000	001	002											
KP859577.1_Canine_parvovirus_2c	001	000	001	001	000	001										
MK675665.1_Canine_parvovirus_2b	000	001	000	000	001	001	001									
MT632754.1_Canine_parvovirus_2b	001	000	001	001	000	001	000	001								
EU659119.1_Canine_parvovirus	001	000	001	001	000	001	000	001	000							
KM457142.1_Canine_parvovirus_2c	001	000	001	001	000	001	000	001	000	000						
KM457125.1_Canine_parvovirus_2c	001	000	001	001	000	001	000	001	000	000	000					
KU508693.1_Canine_parvovirus_2c	001	000	001	001	000	001	000	001	000	001	000	000				
KC196097.1_Canine_parvovirus_2c	001	000	001	001	000	001	000	001	000	000	000	000	000			
MN810917.1_Canine_parvovirus	000	001	001	000	001	001	001	001	001	001	001	001	001	001	001	
MH545963.1_Canine_parvovirus_2a	000	001	000	000	001	000	001	000	001	001	001	001	001	001	001	001
KM457140.1_Canine_parvovirus_2a	000	001	000	000	001	000	001	000	001	001	001	001	001	001	001	001

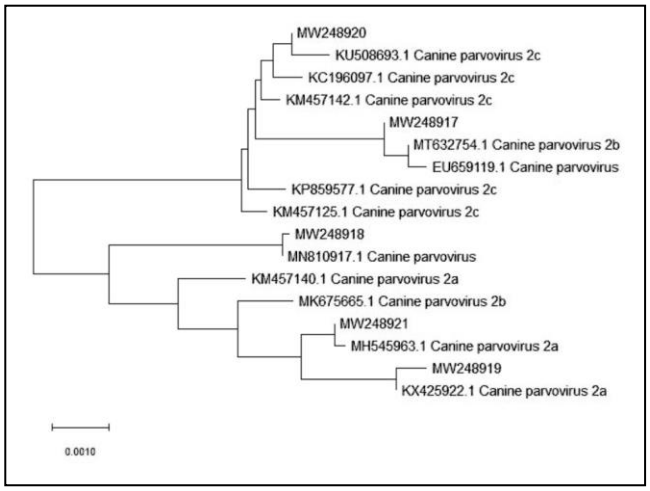

Figure 3. Phylogenetic analysis of CPV-2 based on *vp2* gene

3.4. LAMP

The un-equipped eye observed the turbidity due to isothermal amplification of the VP gene or forming the white magnesium pyrophosphate precipitation in reaction tubes. Also, owing to the combination of calcein with magnesium ions in the mixture, the emission of intense green fluorescent was observable under 254~366nm UV light. Indeed, detailed ladder-like DNA profiles corresponding to the LAMP products were shown by electrophoresis on 2% agarose gel. Moreover, among the LAMP products, only the tubes containing *CPV2* DNA showed characteristic ladder-like multi bands on gel agarose electrophoresis. This result confirmed the assay's specificity in agreement with the BLAST analysis. Preparing a serial 10-fold dilution of pTZ57R/T-VP and applying it in the LAMP reaction was a simple and reliable way to determine the assay's detection limit or analytical sensitivity. The corresponding agarose gel electrophoresis and visual detection of fluorescent emission due to forming calcein magnesium complex, the detection limit of the assay was estimated to be 25 copies per reaction tube in the case of the agarose gel electrophoresis and the real-time turbidimeter detection methods 25 copies when visual detection of the fluorescent emission (Figure 4A an 4B).

3.5. Real-Time PCR

After performing the Real-time PCR assays in serial dilutions 10- fold of the vp_2 gene, the amplification curves confirmed the increase in dilution, and the standard curves were shown (Figure 5A). Compared with the conventional PCR, the designed SYBR green REAL -TIME PCR test tenfold increased the test sensitivity. Therefore, the lower limit of detection of the assays for the *vp2* gene was 21 copies, respectively. The efficiency of R2, Y-intercept, and amplification of the regression line for the standard curve generated by the *vp2* SYBR green Real-Time PCR test was -3.527, 1, and 92%, respectively (Figure 5B).

Finally, in the 25 µl PCR reaction, the LOD for the vp2 gene was about 19 copies in the REAL TIME PCR reaction, and the LAMP technique was 21 and 25 copies, respectively.

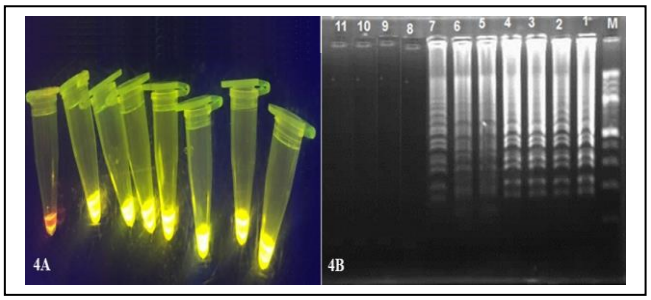

Figure 4. 4A: Visual inspection of calcein related fluorescent. Tube 1-7, tube 8, negative control. **4B:** Lane M, 100 bp DNA ladder; lanes 1–10, the serial dilution; lane 11, negative control

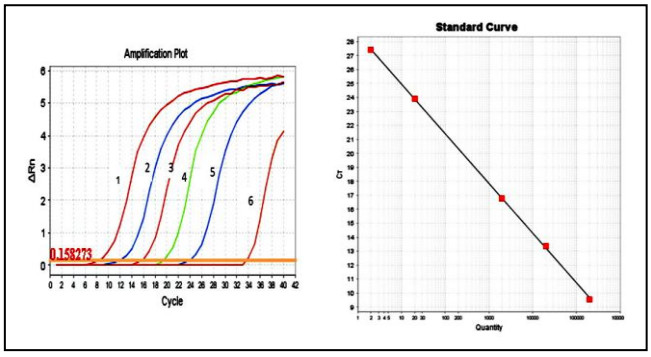

Figure 5. 5A: The Sensitivity of the SYBR Green PCR Assay for Detection of *vp2* gene. **5B:** Standard Curve Analyses and the Linearity Range of the SYBR Green Real-Time PCR for Quantitative Detection of *vp2* gene

4. Discussion

Canine parvovirus (CPV) is an infectious, highly contagious, and fatal viral disease of dogs responsible for enteritis infection (15). CPV2 is a major worldwide disease pathogen. Puppies less than three months old and all age groups are infected with the virus, but young dogs are more susceptible to adults. The original type of virus appeared about 46 years ago (15). Based on antigenic variants, three variants of CPV-2 are prevalent worldwide. There are several methods to diagnose this disease, such as IC test, electron microscopy (EM), in situ hybridization (ISH) serological tests, Virus Isolation (VI), Immunoassay process, and molecular technique (16). Some of these methods are time-consuming and expensive for use routinely in the laboratory, such as EM and VI.

The LA test has little specificity but is a rapid technique; The HA test has less repeatability, and the serological test may be less sensitive; PCR and Molecular methods based on DNA detection have been used widely in laboratory diagnosis because of their specificity and sensitivity (17). This study aimed to design and optimize the rapid detection of CPV 2 by molecular methods. The PCR technique for CPV 2 detection is very simple, convenient, fast with high sensitivity, high specificity, good repeatability, and can be performed at high speed. *vp2* encodes capsid protein in the virus; it is the major structural protein of CPV-2 (18). In this study, 25 sequences recorded for this gene in the NCBI database were used, and primers were designed based on these sequences; then, through alignment, the designed primers were used for conserved regions. The main point in developing a multiplex PCR method is the possibility of nonspecific products and primer dimers. It is essential to design primers of the same Tm values. Compared to other similar studies, all the stated points are implemented (19). Also, in this study, the specificity of primers with viruses that cause similar diseases was considered a negative control, and primers with the genome of these viruses were also negative. After amplification optimization of the reaction, the PCR products were cloned in pTZ57R/T plasmid, and in this study, the confirmed plasmids were used as positive controls.

The gene is cloned into the vector and used as a positive control in the reaction, allowing the number of plasmids to be determined. The test's sensitivity was determined by the limit of detection (LOD). The target genes had been cloned in a plasmid, and serial dilution was prepared from it to infer the LOD for each gene. Finally, in the 25 µl PCR reaction, the LOD for the vp2 gene was about 19 copies in the REAL TIME PCR reaction, and the LAMP technique was 21 and 25 copies, respectively. Fifty dog feces samples were collected from veterinary laboratories in Tehran, and their serological test results were also evaluated by this home setup method, all of which were positive. PCR assay could detect fewer CPV genomes than other conventional techniques and rapid detection of virus DNA from fecal samples. PCR method with suit primer could detect genotypes of this virus based on the conserved *vp2* region of the genome. In addition, the phylogenetic analysis compares virus strains at the genetic level, providing insights into the molecular epidemiology of the disease. A divergence of almost 1% is observed among the clusters. By knowing the genomic information and sequence of dogs, we can study epidemiology and the relationship between sequences throughout Iran and the genetic evolution of the virus.

5. Conclusion

PCR assay used in this study accurately identified the presence of correlating *vp2* virulence genes. This diagnostic assay is the first homemade diagnostic kit based on PCR for CPV-2 infection among puppies in Iran. The Molecular method based on PCR proved to be an effective detection method for CPV-2 in dogs with analytic specificity determination and analytical method sensitivity for designing primers. Design and optimization of In-house PCR assay for rapid detection of parvovirus type 2 in Iran is a significant step towards success in this field and is thought of as improved diagnostic laboratories for precise diagnosis of this

virus. Sequence 4 had a closer relationship to canine parvovirus 2c; Sequence 1 had closer to canine parvovirus type 2b, sequence 5, and sequence 3 closely related to type 2a. Additional complete genome sequences from canine parvovirus will help in the genetic evolution of the virus and elucidation of epidemiology and the same phylogenetic CPV-2 strains.

Authors' Contribution

Study was designed by H. K., S. D. M. N., T. Z. S., M. Z. and A. M.; Experiment was conducted by A. M., Data was analyzed and interpreted by A. M., H. K., and S. D. M. N.. All the authors contributed to the manuscript writing and reviewed written.

Conflict of Interest

The authors declare that they have no conflict of interest.

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