Original Article

Genome Sequences of Canine Parvovirus Type 2c Prevalent in Western Mexico

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ABSTRACT

Canine parvovirus type 2 (CPV-2) is one of the main etiologies of viral gastroenteritis in dogs across the globe. This disease is mainly characterized by the presence of diarrhea, abdominal pain, vomiting, anorexia, and dehydration. This virus is responsible for high mortality and morbidity rates in unvaccinated dogs and those younger than three months. The monitoring of viral variants in our region has demonstrated that in the last seven years, variant CPV-2c has been circulating exclusively, which is unusual if we consider that in the rest of the world, at least two variants co-circulate among dog populations. To the best of our knowledge, no studies in Mexico have reported genomic sequences of CPV-2, which are relevant for population comparisons at the genetic level. Therefore, the present study aimed to sequence genomes associated with CPV-2c. To meet this objective, rectal swab samples were collected from dogs with suspected CPV-2 infection. Five positive cases diagnosed by lateral flow testing and polymerase chain reaction were selected for viral genome sequencing. Comparative analyses illustrated that the obtained genome sequences were > 99% homologous to those reported for CPV-2 in the GenBank. On the other hand, 52 nucleotide mutations were identified in the *vp1/vp2* gene, out of which three impacted amino acid transition (T226S, F267Y, and A440T). Phylogenetic analysis of the *vp1/vp2* gene demonstrated that the five sequences clustered in a clade called "III", pertaining to sequences from USA and Uruguay. To our knowledge, this was the first report of genomic sequences associated with CPV-2 in Mexico, which is of great relevance for the epidemiological-molecular understanding and evolution of the virus.

Keywords: Canine parvovirus type 2c, Genome, Mexico

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1. Introduction

Canine parvovirus type 2 (CPV-2) is one of the main etiologic agents associated with gastroenteritis in unvaccinated dogs and those younger than three months. The gastroenteric picture is characterized by the presence of clinical signs, such as vomiting, diarrhea, abdominal pain, anorexia, and dehydration (1). CPV-2 is a non-enveloped virus comprising a positive-sense single-stranded DNA genome approximately 5.2 kb in size. The genome of CPV-2 has two open reading frames (ORFs), one of which encodes two nonstructural proteins (NS1 and NS2), and the other one encodes a protein precursor from which the structural proteins VP1 and VP2 are derived. The latter represents more than 80% of the total protein of the virus since it is responsible for the formation of the viral capsid (2). The NS1 protein is a 76-kDa nuclear phosphoprotein with multiple functions. One of its primary functions is viral replication, where the N-terminal portion of the protein recognizes the ORI site of the viral sequence in a strand-specific manner (3). On the other hand, the NS2 protein has been detected in the nucleus and cytoplasm, where it has been associated with viral replication (4). The VP1 protein contains amino acids in the N-terminal region responsible for transporting the viral capsid into the nucleus (5). The VP2 protein, which is in charge of cell tropism, contains antigenic sites highly relevant for antibody-mediated viral neutralization, and its amino acid composition is analyzed for epidemiological-molecular studies that allow the identification of preponderant viral variants in canid populations (6, 7). Soon after emergence, the original CPV-2 was replaced by two antigenic variants, namely CPV-2a and CPV-2b, which differ by an amino acid substitution (Asn to Asp) at position 426 of the VP2 protein. In 2000, a new antigenic variant that emerged in Italy was classified as CPV-2c, characterized by the presence of a glutamic acid (Glu) at position 426 (8). The three viral variants co-circulate worldwide, and their distribution and genetic diversity may vary depending on the regions studied (9). For instance, in Asian countries, there is a predominance of the CPV-2a and CPV-2b variants with a lower prevalence of the CPV-2c variant (10, 11). In European countries, there is no predominance of any of the variants; nonetheless, all three co-circulate in a balanced manner in different regions (12). On the contrary, in African countries, CPV-2b infection is predominant, with CPV-2a co-circulating to a lesser extent (13). In South American countries, such as Uruguay, Brazil, and Argentina, the predominance of CPV-2c variant circulation has been identified, compared to CPV-2a and CPV-2b (9). Nevertheless, in countries such as Ecuador, Colombia, and Peru, the predominance falls on the CPV-2a variant with a lower prevalence of CPV-2b and CPV-2c (14, 15). Our group has previously reported an exclusive circulation of CPV-2c in the Western region of Mexico and possibly throughout the country, a phenomenon that could be considered unusual (16, 17). In addition, the analysis of partial sequences encoding the VP2 protein revealed that the CPV-2c circulating in our region is phylogenetically clustered; however, it is related to CPV-2c sequences reported in European and South American countries, suspecting that it was introduced to Mexico from these regions (17). Partial or complete genome sequences derived from CPV-2c have been previously reported in some countries; however, in Mexico, there are no studies on the characterization of the CPV-2 genome, which limits the knowledge of an infectious agent highly relevant to animal health. In light of the aforementioned issues, the present study aimed to sequence and analyze the genome of CPV-2c, which is prevalent in the Western region of Mexico.

2. Materials and Methods

2.1. Sample collection

Outbreaks of CPV-2 infection were identified in the municipalities of Guadalajara and Zapopan in the state of Jalisco, in western Mexico. A total of 22 of them underwent treatment at the Veterinary Hospital for Small Pets, affiliated with the University of Guadalajara, within two weeks. These dogs presented various signs, such as bloody diarrhea, vomiting, abdominal pain, anorexia, and dehydration. Rectal swabbing was performed, and the presence of CPV-2-associated antigen was confirmed by lateral flow CPV Ag Test Kit (Bionote, South Korea); rectal swab samples were frozen at -20ºC.

2.2. Confirmation by polymerase chain reaction of the presence of CPV-2

DNA extraction and purification from fecal samples were performed using the GF-1 DNA extraction kit (Vivantis, Malaysia) following the manufacturer's instructions. DNA was eluted from columns with 50 µl of DNAse and RNAse-free water and stored at -20ºC until use. Polymerase chain reaction (PCR) amplification of a partial segment within the *vp2* gene was performed with oligonucleotides described previously (18). The PCR reactions were performed in a final volume of 25 µl, with 2 µl of purified DNA, 2.5 µl of 10× PCR buffer, 10 pmol of each primer, 0.5 µl of 10 mmol/l dNTPs mix, and 0.3 µl of Taq DNA polymerase (Vivantis, Malaysia) (5 U/µl). Thermal cycler conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. DNA from a commercially available, live attenuated vaccine was used as the positive control, while pure water

and DNA extracted from rectal swabs of healthy dogs were utilized as negative controls. The PCR products were separated in 1.5% agarose gels and stained with Sybr Safe (Vivantis, Malaysia). Samples were considered PCR-positive upon visual detection of the expected 1,042-bp amplicon after gel electrophoresis (16).

2.3. Genome sequencing and *vp2* phylogenetic analysis

Four pairs of specific oligonucleotides were designed from the genomic sequence of CPV with accession number KF638400 previously reported in the GenBank database (NCBI) (Table 1). Serial Cloner software (version 2.6) was used to design and select the oligonucleotides. Of the 22 samples collected, 5 cases were selected for viral genome sequencing. The PCR reactions were performed in a final volume of 25 µl, with 3 µl of purified DNA, 2.5 µl of 10× PCR buffer, 10 pmol of each primer, 2 uL of 25 mM magnesium chloride, 0.5 µl of 10 mM dNTP mix, and 0.3 µl of high fidelity Taq DNA polymerase (Vivantis, Malaysia) (5 U/µl). The thermal cycling conditions included initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 sec, alignment at 53.5°C for 30 sec, and extension at 72°C for 1 min. An elongation step was added at 72°C for 10 min, and the samples were preserved at -20°C. The PCR amplicons were separated in 1% agarose gels in the presence of Sybr-safe dye, and the products were visualized on an LED transilluminator. Bands with corresponding molecular weights (1100 bp, 1 122 bp, 1384 bp, and 1329 bp) were obtained from the gel. The Wizard SV Gel and PCR Clean-up System kit (Promega, USA) was used to purify the PCR amplicons following the manufacturer's instructions. DNA was eluted from the columns with 50 uL of double distilled water, quantified, and its purity was determined in a Nanodrop spectrophotometer. The PCR amplicons were submitted to the DNA Synthesis and Sequencing Unit of the Institute of Biotechnology, National Autonomous University of Mexico, for DNA sequencing using the Sanger method. The sequences were quality-checked and analyzed using the FinchTV (version 1.5) and UGENE (version 44) software packages. The identity of each amplicon was checked using the NCBI-BLAST algorithm. Genome assembly from the obtained sequences was performed using UGENE software (version 44) in conjunction with the read de novo assembly (CAP3). The genomes were verified in both sense and antisense sequences, and once finalized, their identities were determined using the NCBI-BLAST algorithm. The obtained genome sequences were submitted to the GenBank Database to obtain the accession numbers.

2.4. Alignments

To compare the sequences reported herein, CPV complete sequences were searched in GenBank. In this regard, 10 countries from various parts of the world were selected due to the sequence length (four from Asia, two from the Western Hemisphere, two from Europe, one from Africa, and one from Oceania). From each country, two sequences were used for the alignment, except for the USA, where an extra sequence was used to generate the comparison root, considering the proximity of the country to Mexico. The five sequences from Jalisco were cut to fit the size of sequences found at GenBank (4269 bp). Nucleotide of the entire sequences and deduced amino acid sequences of the VP2 protein were aligned and compared to sequences belonging to the selected countries using a progressive alignment algorithm in the CLC main Work Bench software package (Version 7.7.1.) (Quiagen, Denmark, https://www.qiagenbioinformatics.com/).

Table 1. Oligonucleotide description and used in this study.

Code	Oligonucleotides	Length of amplification (bp)	Coordinates*
F1F	5'-GAATGATAGGCGGTTTGTGT-3'	1 100	0-1100
F1R	5'-TCCTGGTTGTGCCATCATTT-3'		
F2F	5'-CCTGAAGACTGGATGATGTTA-3'	1 122	1050-2172
F2R	5'-TGCCATCGTACCTTAATCCA-3'		
F3F	5'-CGTGGTCCGAAATAGAGGCA-3'	1 384	2078-3462
F3R	5'-TGTTCCTGTAGCAAATTCATCACC-3'		
F4F	5'-ATATATACCATGGTACAGATC-3'	1 329	3359-4687
F4R	5'-TAACAACATACATTAATGGT-3'		

* The coordinates are based on the CPV-2 genome sequence with GenBank accession number: KF638400.

2.5. Phylogenetic trees

The evolutionary distances were computed using the Maximum Likelihood reconstruction with the Neighbor-joining method (Saitou and Nel, 1987) and the Jukes-Cantor nucleotide substitution model with rate variation. Phylogenetic tree construction was performed with 1,000 bootstrap replicates to support the information from the internal nodes, showing confidence values to demonstrate statistical support for each clade. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are displayed next to the branches. Only bootstrap values above 70% were indicated at the node. The analysis involved 26 complete sequences. All positions containing gaps and missing data were eliminated. There was a total of 4,269 positions in the final dataset. Evolutionary analyses were conducted in CLC main Work Bench software (version 7.7.1.).

3. Results

It is well known that VP2 is the protein that has higher variability, producing a wide host range and cross-reactions between variants. Therefore, the *VP2* of our sequences was selected for genetic variation analysis. The five genome sequences presented almost identical homology compared to the US-17 sequence, presenting the highest homology rate in MX1-17 with 99.72%; the rest had homology rates of 99.46-99.67%. When comparing all sequences with US-17, 52 nucleotide mutations were identified in the *Vp2* gene, out of which 39 were synonymous and 13 involved amino acid substitutions (Table 2). When US-17 was compared with Mexico sequences (MX1-15, MX2-15, MX-16, MX1-17, and MX2-17), 12 and 3 synonymous and non-synonymous mutations were identified, respectively. One of the three identified mutations involves changing the amino acid threonine at position 226 to serine (T226S), which is present in 100% of the sequences compared to the US-17 sequence. The mutation at nucleotide 799 in *VP2* resulted in an amino acid substitution at codon position 267 (F267Y). The amino acid substitution at residue 267 was present as a mutation in all sequences used and described from countries in Asia (IN-20, IN-16, KR1-17, CN1-19, TH1-16, TH2-16, CN2-19, and KR2-17), Africa (NG1-18 and NG2-18), and one from Uruguay (UY2-11). The mutation at codon position 440 (A440T) was observed in two Jalisco sequences (MX1-15 and MX2-17). This mutation was present in some of the sequences from all the regions we used in the analysis, such as France and Finland (FR-83, FR-84, FI-80, and FI-86), Australia (AU-82 and AU-85), Thailand, China, South Korea (TH1-16, TH2-16, CN2-19, and KR2-17), Uruguay, and USA (UY1-11 and US-15). To visualize the relationship of the CPV sequences, a phylogenetic tree was constructed based on the five full-length sequences presented herein, and 21 sequences were retrieved from GenBank. The resulting phylogenetic tree demonstrated that all the Jalisco state sequences are grouped in a clade III, which is more related to sequences from the USA (US-15, US-17, and US-19) and one sequence from Uruguay (UY1-11) (Figure 1). Two more clades were generated separately, grouping the rest of the retrieved sequences. The clade I was divided into two clusters that grouped separately: Nigeria (NG1-18 and NG2-18), India (IN-16 and IN-20), China (CN1-19), and Uruguay (UY2-11) in cluster 1; and Thailand (KR1-17 and KR2-17), China (CN2-19), and Korea (KR2-17) sequences in cluster 2. Finally, clade II grouped Finland (FI-80 and FI-86) in one cluster, and France (FR-83 and FR-84) and Australia (AU-82 and AU-85) in the second cluster (Figure 1). The phylogenetic analysis considering the 21 complete CPV sequences illustrated that sequences MX2-15, MX-16, and MX1-17 were clustered together in a monophyletic clade as a branch of the root sequence from USA (US-17). Sequences MX1-15 and MXG-17 were in two different subgroups; the first was more related to US-15 (a strain from Ohio, USA) and the second pertained more closely to a strain from Uruguay (UY1-11).

4. Discussion

Previously, our group performed epidemiologic-molecular studies analyzing a fragment of the gene associated with the VP2 protein and found that the CPV-2c variant (426-Glu) has been circulating exclusively in our region and possibly in the country during the last seven years (16, 17, 19). To gain a deeper understanding of this virus, we sequenced five CPV-2c genomes. A homology of more than 99% with CPV-2 was obtained by comparing the sequences found here with others reported. The complete analysis of the *VP2* protein-associated gene allowed the identification of 12 synonymous and 3 non-synonymous mutations (T226S, F267Y, and A440Y). In the present study, the MX2-15 genome sequence presented a replacement of Phe by Tyr at position 267. This finding was interesting since it is a mutation that had not been identified in our region and is very characteristic of Asian countries (20). This mutation has been reported to be present in CPV-2a VP2 in several groups, including China (21, 22), South Korea (23), and Nigeria (24). In addition, this mutation has occurred with high frequency in the CPV-2b viral variant identified in Asian countries where, in combination with other residues (25), it has been called "new CPV-2b" since approximately 92% of the analyzed strains present this mutation (20). The residue is in the greatest variable GH loop comprising amino acids 267-498 of the VP2 protein (26), which contains antigenic antibody recognition sites (20).

Table 2. Amino acid variation of Canine Protoparvovirus samples in the VP2 protein.

GenBank Accession number	Sample	Code	Location	Codon Position												
Reference				5	87	101	106	226	267	300	305	324	370	375	426	440
MN451676	CPV603 (2017)	US-17	USA	Ala	Leu	Thr	Val	Thr	Phe	Gly	Tyr	Tyr	Gln	Asp	Glu	Ala
MN451686	CPV613 (2019)	US-19	USA	-	-	-	-	Ser	-	-	-	-	-	-	-	-
KM457142	UY370c(2011)	UY1-11	Uruguay	-	-	-	-	Ser	-	-	-	-	-	-	-	Thr
MF457594	OH20219 (2015)	US-15	USA	-	-	-	Glu	Ser	-	-	-	-	-	-	-	Thr
KM457141	UY370a(2011)	UY2-11	Uruguay	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MN451689	CPV616 (2018)	NG1-18	Nigeria	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MT441832	CPVMVC03 (2020)	IN-20	India	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MN451688	CPV615 (2018)	NG2-18	Nigeria	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MN451667	CPV58 (1983)	FR-83	France	-	-	-	-	Ser	-	-	-	-	-	-	Asn	Thr
MN661243	CPV2a (2016)	IN-16	Finland	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MN451695	RDPV124 (1986)	FI-86	Finland	-	Met	Ile	-	Ser	-	Ala	Asp	-	-	Asn	Asn	Thr
MN451666	CPV54 (1984)	FR-84	France	-	-	-	-	Ser	-	-	-	-	-	-	-	Thr
MN451669	CPV81 (1982)	AU-82	Australia	-	-	-	-	Ser	-	-	-	-	-	-	Asn	Thr
MN451694	RDPV123 (1980)	FI-80	Finland	-	Met	Ile	-	Ser	-	Ala	Asp	-	-	Asn	Asn	Thr
MN451670	CPV87 (1985)	AU-85	Australia	-	-	-	-	Ser	-	-	-	-	-	-	Asn	Thr
MK144545	K01708-2 (2017)	KR1-17	South Korea	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
KR002805	SD192014 (2019)	CN1-19	China	-	-	-	-	Ser	Tyr	-	-	Ile	-	-	Asn	-
MH711894	CU24 (2016)	TH1-16	Thailand	Gly	-	-	-	Ser	Tyr	-	-	Ile	Arg	-	-	Thr
MH711902	CU21 (2016)	TH2-16	Thailand	Gly	-	-	-	Ser	Tyr	-	-	Ile	Arg	-	-	Thr
MN840830	SH12019 (2019)	CN2-19	China	Gly	-	-	-	Ser	Tyr	-	-	Ile	Arg	-	-	Thr
MK144544	K01708-1 (2017)	KR2-17	South Korea	Gly	-	-	-	Ser	Tyr	-	-	Ile	Arg	-	-	Thr
In this study																
MT448706	MX/ZAP/H(2015)	MX1-15	Mexico	-	-	-	-	Ser	-	-	-	-	-	-	-	Thr
MT448702	MX/ZAP/149(2015)	MX2-15	Mexico	-	-	-	-	Ser	Tyr	-	-	-	-	-	-	-
MT448705	MX/ZAP/C(2017)	MX1-17	Mexico	-	-	-	-	Ser	-	-	-	-	-	-	-	-
MT448703	MX/ZAP/166(2016)	MX-16	Mexico	-	-	-	-	Ser	-	-	-	-	-	-	-	-
MT448704	MX/GDL/A(2017)	MX2-17	Mexico	-	-	-	-	Ser	-	-	-	-	-	-	-	Thr

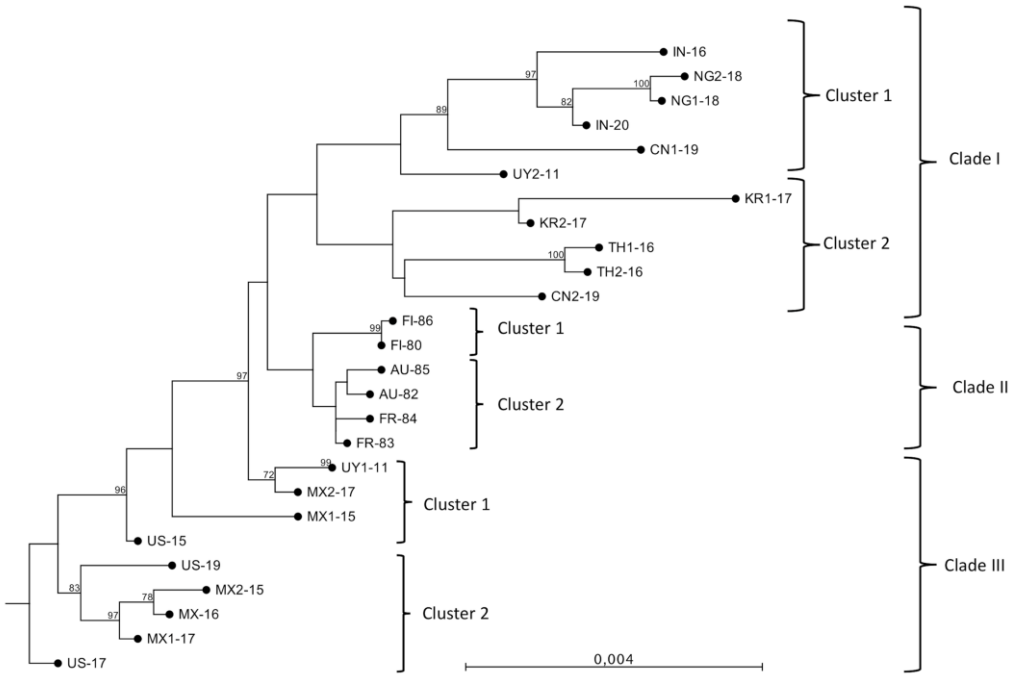

Figure 1. Phylogenetic analysis based on complete nucleotide sequences of CPV, retrieved from GenBank. The phylogenetic tree was constructed with Maximum Likelihood phylogeny using the neighbor-joining method with 1,000 bootstrap replicates in the CLC main Work Bench V. 7.7.1. software package. The scale bar indicates nucleotide substitutions per site.

Tracking this residue in future research in our region is of great importance. It is possible that viral variants with this mutation spread more frequently than others. The alanine-to-threonine substitution at position 440 was identified in all five genomes described in this study. In our previous analysis, we observed this substitution in 48% (14/29) of sequences (17), and Faz et al. reported identification in approximately 50% of sequences (19). This substitution is not limited to the CPV-2c viral variant but has also been observed in the CPV-2a viral variants in China (27) and South Africa (13), as well as in CPV-2b in Brazil (28). The relevance of this substitution has not been understood so far. Nonetheless, this residue is at the top of the threefold spike, a region characterized by high antigenicity (6). Therefore, it could be assumed that this residue could be receiving selection pressures due to vaccination, as previously reported (21,29). The phylogenetic analyses performed in the present study confirmed our previous findings, according to which the phylogenetic relationship of circulating viruses in our region was highly similar to those reported in such countries as the United States and Uruguay (17). In agreement with the analyses demonstrated in the present report and those we have previously performed (16, 17), Grecco et al. pointed out that European viral clades have spread in South America (30), allowing us to suggest that the Mexican clades have a South American origin. In conclusion, in the present study, we reported the first partial genomes of CVP-2c in Mexico, a variant that has been circulating exclusively for at least seven years. Comparative analyses with other CPV-2 genomes have demonstrated that they are > 99% homologous, and although 52 nucleotide mutations have been identified in the *VP1/VP2* gene, only three can affect the amino acid transition. Although it is still challenging to determine why only the CPV-2c variant circulates exclusively in Mexico, we can suggest that this variant is the product of viral dispersal from South America.

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Authors' Contribution

CPR and MADA conceived the study. MADA, DEQ, CGM, and AGO performed experiments. CPR and DEQ interpreted data. CPR and DEQ wrote the manuscript. All authors read and approved the final manuscript.

Ethics

The study was approved by the Biosafety, Research, and Ethics Committees of the Veterinary Hospital of the University of Guadalajara. The pets' owners were informed about the purpose and procedures of the study and conferred consent for taking a rectal swab sample from the dogs. The study followed Federal Regulations, such as NOM-087-SEMARNAT-SSA1-2002, NOM-033-ZOO-1995, and NOM-062-ZOO- 1999.

Conflict of Interest

The author(s) declares no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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