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Original Article

Detection of canine parvovirus type 2c (CPV-2c) in Palestine

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Abstract

Introduction: The objective of the present study was to report, for the first time, the presence of canine parvovirus type 2c (CPV-2c) in domesticated dogs with acute gastroenteritis and to characterize the antigenic variants circulating in Palestine.

Methodology: A veterinary clinical-based epidemiological study was carried out between December 2022 and April 2023. Fifty fecal samples were collected from dogs with gastroenteritis and screened for CPV-2 infection by polymerase chain reaction. The distribution of positive cases according to various epidemiological factors was studied. Partial sequencing of the *viral protein 2* (*VP2)* gene was performed for the analysis of CPV-2 variants.

Results: Most of the investigated samples (60%; n = 50) during the study period were found positive for CPV-2 infection. There was no difference in the distribution of positive cases of CPV-2 infection based on age group, gender, location, and vaccination status. The analysis of nucleotide and amino acid sequences from amplified products, as well as phylogenetic analysis, revealed the presence of CPV-2c clustered with Asian CPV-2c variants.

Conclusions: In summary, this study represents the initial genetic analysis of CPV-2 present in Palestinian dogs with gastroenteritis and provides evidence that confirms the existence of the CPV-2c variants. To determine the prevailing CPV-2 variant associated with the infection, it is crucial to conduct further sequence analysis using large populations of both domestic and wild canines.

Key words: CPV-2c; dogs, Palestine; phylogenetic analysis; *VP2* gene; canine parvovirus.

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Introduction

Canine parvovirus (CPV) is the etiological agent of one of the most infectious diseases in dogs worldwide. According to the current classification of the International Committee on Taxonomy of Viruses (ICTV), CPV belongs to the species *Carnivore protoparvovirus 1* (CPPV), genus Protoparvoviruses, subfamily Parvovirinae, of the family Parvoviridae. The species CPPV includes a wide range of viruses that cause diseases in canine and feline species, mainly the canine parvovirus (CPV), also referred to as canine parvovirus type 2 (CPV2); and the feline parvovirus (FPV), also known as feline panleukopenia virus (FPLV) [1]. CPV-2 emerged as a dog pathogen in 1978 [2]. Some hypotheses suggest that CPV originated from FPV or FPV-like viruses of wild carnivores [3].

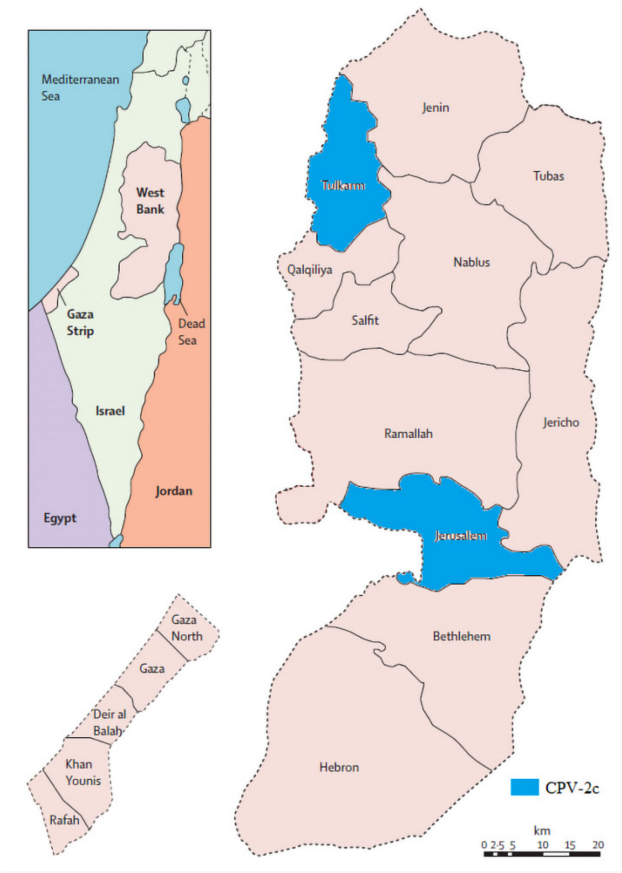
CPV-2 penetrates through the oronasal route, and the virus replicates in lymphoid cells infecting leukocytes causing acute lymphopenia and disseminating the virus throughout the body [4]. The virus attacks rapidly dividing cells in intestinal crypts in young and adult dogs causing the most characteristic clinical form represented by hemorrhagic enteritis. In addition, the virus replicates in cardiac cells causing myocarditis in young pups [5].

The infected dogs show clinical signs including depression, high fever, anorexia, vomiting, and severe hemorrhagic diarrhea [6]. The disease is more severe in growing pups between six weeks and six months of age. Adult and vaccinated dogs show mild or subclinical disease [7]. Similarly, FPV infects mainly kittens of all members of the Felidae family and causes fever, enteritis, bloody diarrhea, and profound leukopenia (white blood cell below 1000 cells per mL of blood). Perinatal or in-utero infection of kittens can cause cerebellar hypoplasia [8].

The virion of the species CPV-2 is small in size (23-28 nm), non-enveloped, and the genome is a single-stranded DNA (around 5 kb) containing two open reading frames (ORFs). The first ORF encodes two

overlapping structural proteins named viral particle 1 (VP1) and VP2, and a third protein VP3 is generated by cleavage of 15-20 amino acid residues of the amino terminus of VP2 by the cellular protease. The second ORF encodes two nonstructural proteins (NS1 and NS2) [1]. NS1 plays an essential role in viral replication and is responsible for inducing cell apoptosis [9]. NS2 controls chromatin remodeling and DNA damage response during parvoviral replication [10]. VP1 plays a role during entry into the host cell and contributes to virus transport to the nucleus [11]. VP2 represents 90% of the capsid protein that encapsulates viral DNA. Moreover, VP2 is the major antigenic protein that determines viral host range and tissue tropism. Certain amino acid residue substitutions at position 426 of VP2 account for new CPV-2 variants into sub-species; asparagine (N) for CVP-2a, aspartic acid (D) for CVP-2b, and glutamic acid (E) for CVP-2c [5,2]. VP3 in full capsids may play a role in facilitating viral DNA release during infection [13].

Figure 1. Map of Palestine showing the distribution of CPV-2. The samples were collected from dogs with gastroenteritis in Tulkarm in the north and Jerusalem in the Middle of West Bank. Uniform blue shading indicates the presence of CPV-2c variant identified in this study.



CPV-2 emerged as a result of a mutation in the VP2 protein from FPLV, enabling binding to the canine transferrin receptor [5]. The high mutation rate (10^{-4} per nucleotide site per year) suggests that CPV-2 is still evolving as additional amino acid changes occurred within the main antigenic regions of CPV-2 capsid, altering the antigenic profile of the virus and stressing the need for implementing diagnostic assays [14,15]. New emerging CPV-2 infections rapidly transmit among the dog and cat populations globally [5].

CPV-2 represents a major concern because of its high morbidity and mortality. The disease is thought to exist for a long time in Palestine, however, to the best of our knowledge, no studies on the genetic diversity of CPV-2 in Palestine have been conducted so far. Therefore, we aimed to identify the presence of CPV-2 in suspected clinical samples using conventional polymerase chain reaction (PCR) and to perform phylogenetic analysis to understand the evolutionary relationship.

Methodology

Study region and samples

This study included samples obtained from diseased dogs in Tulkarm in the north and Jerusalem in the Middle of West Bank, Palestine (Figure 1). A total of 50 fecal swab samples from dogs with diarrhea suspected of CPV-2 infection were collected in Noah's Ark Pets Care Hospital (Anata, Jerusalem) and Dr. Belal Veterinary Center (Tulkarm, Palestine) as reference private veterinary service providers, between December 2022 and April 2023. The samples were stored at -20 ºC until used. Data regarding breed, location, gender, age, and vaccination status were recorded for each sample.

Clinical findings and dog characteristics

The samples were obtained from puppies of different breeds (Pekingese, Maltese, Malinois, Husky, Pomeranian, German Shepherd, and Canaan) and ages (between one and six months) with suspected CPV-2 infection. The presented puppies showed symptoms such as diarrhea, fever, vomiting, anorexia, and dehydration. The Primodog® (Boehringer Ingelheim, Lyon, France) vaccine was used in the vaccinated dogs. Data on the breed, age, gender, and vaccination status are summarized in Table 1.

Sample processing, DNA extraction and PCR

DNA was extracted from fecal swabs as described previously [16]. Briefly, samples were collected by rubbing sterile swabs in the inner surface of the rectum,

Table 1: Univariate analysis of factors associated with CPV-2 positivity in the PCR screening of dog fecal samples in Palestine between December 2022 and April 2023.

Variable	Category	Tested samples	Number of positive (%)	*p* value
Breed	Pekingese	1	0 (0.0%)	0.017*
	Maltese	2	0 (0.0%)	
	Malinois	32	25 (78.13%)	
	Husky	3	2 (66.7%)	
	Pomeranian	1	0 (0, 0.0%)	
	German Shepherd	8	2 (25.0%)	
	Canaan	3	1 (3.33%)	
Location	Anata	34	20 (58.82%)	0.528
	Tulkarm	16	10 (62.50%)	
Gender	Male	26	13 (50.0%)	0.133
	Female	24	17 (70.83%)	
Vaccination	Vaccinated	4	1 (25.0%)	0.329
	Partially Vaccinated	8	5 (62.50%)	
	Unvaccinated	38	24 (63.16%)	
Age (months)	1	2	2 (100.0%)	0.454
	1.5	11	6 (54.54%)	
	2	14	9 (64.29%)	
	2.5	3	3 (100.0%)	
	3	7	4 (57.15%)	
	4	9	5 (55.56%)	
	5	2	1 (50.0%)	
	6	2	0 (0.0%)	

*Factors statistically significant at $p < 0.05$

and then suspended in 1 mL phosphate buffer saline. The suspension was centrifuged at 12,000 ×g. The cleared supernatant was frozen at -20 °C until processing. DNA extraction was performed with Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

All samples were tested for parvovirus using conventional PCR for specific detection of the *VP2* gene as previously described [17]. A commercially available inactivated vaccine (Primodog®, Boehringer Ingelheim, Lyon, France) was used as a positive control of CPV, and a fecal swab from a healthy adult dog processed similarly was used as a negative control. A specific primer pair - 555-for (5'-CAGGAAGATATCCAGAAGGA-3') and 555-rev (5'-GGTGCTAGTTGATATGTAATAA-ACA-3') - was used to amplify 583 bp of the *VP2* gene as described previously, and the data was used for detection and phylogenetic analysis of CPV-2 variants [16,17]. Each PCR reaction tube contained 25 µL of 2× GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 µM of each primer, 5 µL of total DNA (approximately 100 ng) and nuclease-free water up to 50 µL. PCR conditions were as follows: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 46 °C for 30 s, and 72 °C for 45 s; followed by a final extension step at 72 °C for 5 min. The amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel with 0.005% ethidium bromide.

Sequence analysis

PCR products of the expected size from two positive reactions were submitted to Sanger sequencing (Syntezza Bioscience, IDT, Jerusalem) with the forward and reverse primers. The nucleotide sequence results were retrieved by Finch TV 1.4 (https://finchtv.software.informer.com/1.4/) software and submitted to GenBank. The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology (NCBI) was used to search for the most similar CPV-2 (http://blast.ncbi.nlm.nih.gov/) [18]. The sequences we generated and reference CPV variants sequences were aligned and analyzed using the MEGA

Table 2. Amino acid residue variations in VP2 capsid protein sequences for characterization and identification of CPV-2b variants and strain.

Variant/reference	Acc. Nr.	VP2	
		426	440
CPV-2a	AB054215.1	N	T
CPV-2b	MF177226	D	T
CPV-2c	MF177228	E	T
CPV_2c/IA-T1/Palestine/2022	OP756333	E	T
CPV_2c/IA-T1/Palestine/2022	OP756334	E	T

D= Aspartic acid, E= Glutamic acid, N= Asparagine, T= Threonine.

X® software [19]. The phylogenetic tree was obtained by using the maximum-likelihood method with 1,000 bootstrap replications.

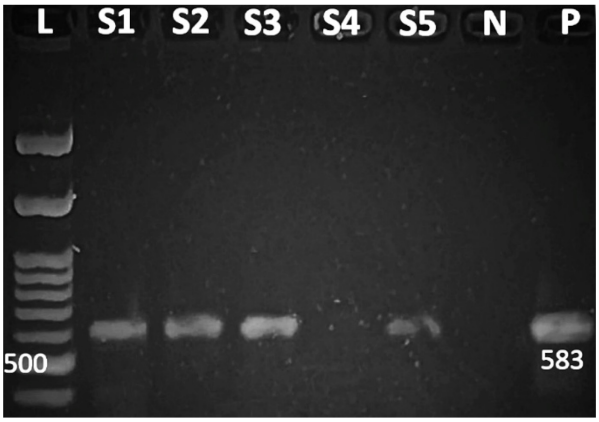
Statistical analysis

The associations of PCR results with breed, location, gender, age, and vaccination status were tested using the Fisher's exact test. $p < 0.05$ was used to denote statistical significance. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 21 (IBM Corp, Armonk, NY, USA).

Results

In this study, an investigation on clinical canine gastroenteritis cases was conducted in the West Bank regions of Palestine. Fecal samples were collected from a total of 50 cases from two geographically distinct locations, with an average distance of 110 kilometers separating them. The samples were collected from puppies with a median age of two months (one to six months), comprising of 26 males and 24 females, representing seven recognized breeds within this age group. Thirty-seven out 50 dogs were between one and three months of age; and 13 out of 50 were between four and six months of age. Four cases were vaccinated, eight cases were partially vaccinated, and the remaining 38 puppies were unvaccinated. Data regarding the breed, age, gender, and vaccination status are described in Table 1. All presented puppies showed clinical signs of gastroenteritis; the most common clinical sign observed was diarrhea (92%), anorexia (86%), dehydration (72%), fever (72%), and vomiting without blood (66%).

Figure 2. Agarose (1.5%) gel electrophoresis of the PCR products for the identification of CPV-2 in fecal samples collected from dogs with gastroenteritis. Lane L = 100 bp ladder, P = positive control, N = negative control, S1-S5 = fecal samples



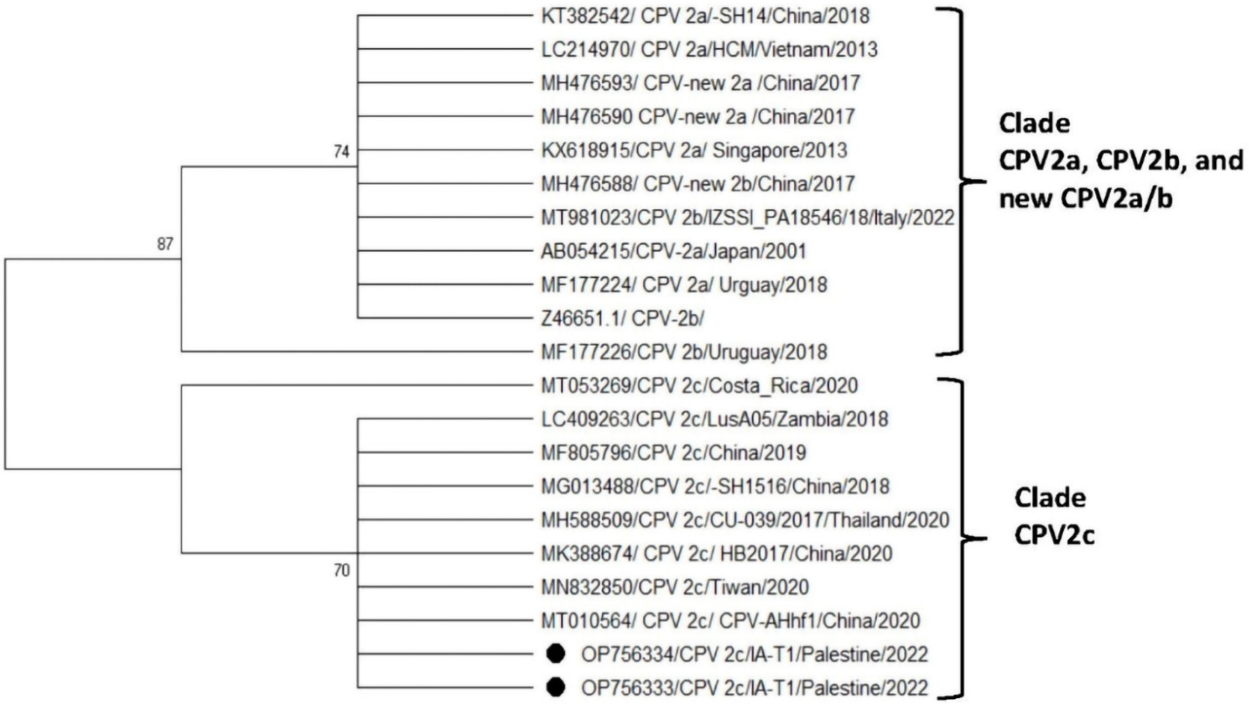
In our study, 60% of the samples tested positive via conventional PCR (30 out of 50 samples). These positive samples yielded a 583-base pair fragment of the *VP2* gene including primers (Figure 2). Positive samples were observed in unvaccinated, partially vaccinated and vaccinated animals (Table 1). The vaccination status of the dam was unknown. No statistical differences were obtained between the PCR results based on age, gender, and vaccination status. (Table 1).

Sanger sequencing was performed on two samples and deposited in GenBank (accession numbers: OP756333.1 and OP756334.1). The obtained sequences showed high nucleotide identity (98.84%). BLAST analysis identified the glutamic acid (E) amino acid residue at position 426 of VP2 which belongs to CPV-2c variants (Table 2). The NCBI BLAST analysis of the Palestinian CPV-2c sequences had 97.89% to 100% pairwise nucleotide identity when compared with Asian CPV-2c variants sequences reported from Zambia, China, Thailand, and Taiwan. Finally, the phylogeny was in concordance with BLAST analysis and the Palestinian CPV-2c was clustered with Asian CPV-2c variants from China, Thailand, and Taiwan (Figure 3).

Discussion

This is the first report of the emergence of the CPV-2c strain in domestic dogs in Palestine. CPV-2 was identified by characterizing the *VP2* gene through diagnostic activity in domestic dogs. The results of the

Figure 3. Phylogenetic analysis of CPV-2 nucleotide sequences encoding for the VP2 capsid protein. The evolutionary history was inferred using the maximum evolution method [1]. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Evolutionary analyses were conducted in MEGA X [19].



current study could provide a baseline and enable updated information on the circulation and distribution patterns of this virus. Canine parvovirosis, caused by CPV-2, is an extremely contagious infectious disease that is considered the primary cause of viral gastroenteritis in dogs worldwide [20,21]. The spread of CPV-2 has been increasingly reported in neighboring countries in recent years [22,23]. The domesticated dog population in Palestine has experienced a significant increase in number, with dogs being kept both as pets and for guarding purposes. Despite this rise, there is a notable lack of reports and studies investigating the evolution of CPV-2 variants in Palestine. Currently, the diagnosis of CPV-2 primarily relies on clinical signs and rapid tests, which are not completely reliable and can yield false positive and false negative results [5].

The parvoviral infection targets rapidly dividing cells, such as intestinal crypts and lymphoid tissue, leading to symptoms like diarrhea, fever, vomiting, anorexia, and dehydration, as observed in this study [24,25]. Similar symptoms have been reported in other studies, including anorexia, lethargy, vomiting, and hemorrhagic diarrhea [22,26].

The present study aimed to characterize CPV-2 field variants circulating in the West Bank of Palestine from December 2022 to April 2023. Most of the samples subjected to PCR testing were found to be positive for CPV-2, highlighting its significant role in causing gastroenteritis, particularly in puppies. However, these results also indicate the necessity for further investigation of other potential causative agents associated with severe diarrhea or mixed infections. The highest occurrence of CPV-2 infection was found in dogs aged one to three months representing 74% of all cases. This finding correlated with those who reported increased prevalence in dogs between the age of one and three months [27]. This could be attributed to immature development of immune organs and lymphoid tissues, resulting in weakened resistance of the body in young puppies [28]. During this study, more female dogs were found with CPV-2 than males (17/24 cases vs. 13/23 cases). However, due to the small number of samples obtained, it would be difficult to conclude this result, although other studies found that males were more affected than females. This is in agreement with other reports that showed no

association of CPV-2 enteritis with the gender of the dogs that were tested [29,30].

The canine parvovirus vaccine is considered a core vaccine globally; however, in Palestine (unlike rabies virus vaccination), parvovirus vaccination is not required by law [31]. Despite the diligent vaccination practices carried out by veterinarians in clinics, the prevalence of clinical signs associated with CPV-2 continues to persist [32]. In our study, 92% (46 out of 50) of all samples analyzed and tested positive by PCR were from unvaccinated dogs or dogs with an incomplete vaccination schedule, reinforcing the need to increase vaccination effort to reduce CPV2 prevalence. Moreover, epidemiological surveys have shed light on the emergence of the CPV-2c variant, which is becoming increasingly prevalent across different geographic regions [21]. Alarmingly, this variant is frequently associated with severe disease even in adult dogs and those that have completed the recommended vaccination protocols [33]. It is important to note that the potential factors contributing to vaccination failure in infected dogs include poor vaccine quality, improper storage conditions, incorrect vaccine administration, inadequate hygiene practices, and interference from maternally derived antibodies [5,34,35]. Addressing these factors is crucial to improve vaccine effectiveness and enhance protection against CPV-2 infections.

CPV-2 remains a significant pathogenic agent, causing enteritis and mortality in dogs worldwide [20,21]. Since their emergence in 2000, new variants of CPV-2 have continued to emerge due to a high mutation rate [15]. Various genetic variants of CPV-2 have been reported across different countries [36]. This study represents the first effort to describe the genetic diversity of CPV-2 in Palestine based on partial *VP2* sequence. In our study, due to limited resources and constraints, we were able to obtain only two sequences from the 30 positive samples. To prioritize the most informative samples or to manage limited sequencing capacity, we might have selected specific positive samples for sequencing based on geographic distribution. The nucleotide and amino acid BLAST analysis as well as phylogenetic analysis of *VP2* gene confirmed the identification of CPV2-c variants in concordance with the Asian-origin CPV-2 strains in puppies affected by diarrhea during the study period. Our findings expand the existing evidence of CPV-2c being the predominant variant in the Middle East region [37]. Therefore, it is crucial to conduct routine surveillance of CPV-2 in domestic dogs in different countries and regions to gather epidemiological information that can support effective prevention and control practices.

The small sample size of target cities is the main limitation of this study. Hence, the results may not be representative of the genetic and antigenic diversity of CPV-2 in the entire country. However, our results confirm the presence of CPV-2c in West Bank-Palestine and emphasize the necessity of intensifying the effectiveness of the available commercial vaccines used against parvovirus in the country. Future studies, with more resources and improved sequencing capabilities are likely to provide a more comprehensive understanding of the viral variants and their distribution in the studied population.

Conclusions

This study confirmed the circulation of CPV-2c as a main cause of gastroenteritis in dogs in Palestine. This is the first report of CPV-2 infection and the first time CPV-2c has been reported in Palestine. Further studies are needed throughout the country to establish the current epidemiological profile and prevalence of antigenic variants in domestic dogs. A particular emphasis on genomic surveillance, prevention, and education is needed to effectively control and fight the spread of this virus.

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Authors' contributions

IA, manuscript design and draft; BAH, clinical symptom evaluations and sample collection; AF, HA, NJ, molecular detection and sequence analyses. All the authors contributed and accepted the final manuscript.

Data availability statement

All data from this study are available from the corresponding author upon request. The partial *VP2* gene sequences are available in the NCBI database with the accession numbers mentioned in the manuscript.

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