Iranian Journal of Fisheries Sciences 20(6) 1674-1683 2021
DOI: 10.22092/ijfs.2021.125449

Research Article

Molecular identification of the zoonotic parasites *Contracaecum* spp. and *Anisakis pegreffii* (Nematoda: Anisakidae) isolated from *Mesopotamichthys sharpeyi* and *Barbus grypus* in the Shadegan Marsh of Khuzestan Province

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Received: January 2019 Accepted: May 2020

Abstract

The family of Anisakidea (*Anisakis* and *Contracaecum*) includes zoonotic parasitic nematodes that have worldwide distribution. Two hundreds *Anisakis* and *Contracaecum* sp. nematode samples were collected from the intestine of *Mesopotamichthys sharpeyi* and *Barbus grypus* caught from Shadegan Wetland, where known as an important international wetland in Iran with a rich biodiversity. After extraction of the genomic DNA, PCR was used to amplify the entire ITS fragment with primers NC5-NC2. PCR products were fractionated by agarose gel electrophoresis prior to purify using gel extraction kit. The purified DNA was sequenced by an Applied Biosystems DNA sequenced in both directions. In order to compare the obtained sequence data with similar sequences from other nematodes, target sequence data were retrieved from the GenBank. The overall prevalence of *Anisakis* and *Contracaecum* in the studied fish from Shadegan Wetland was 6% and 9% in *Mesopotamichthys sharpeyi* and 4% and 6% in *Barbus grypus*, respectively. All nematodes were identified as *Anisakis pegreffii* and *Contracaecum rudolphii* based on the phylogenetic tree and genetic distance. This is the first time to report the distribution of *A. pegreffii* and *C. rudolphii* in *Mesopotamichthys sharpeyi* and *Barbus grypus* in Shadegan Wetland. Based on the results, the danger of zoonotic anisakid nematodes, including *A. pegreffii* and *C. rudolphii* in the studied area is very low.

Keywords: *Anisakis*, *Contracaecum*, ITS region, rRNA, phylogeny

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Introduction

The family of Anisakidea includes parasitic nematodes with worldwide distribution. They have a complex life-cycle among invertebrates, fish, cephalopods and mammals (Chai *et al*., 2005). The heteroxenous life cycle involves a variety of hosts that are transferred through the marine food chain (Costa *et al.*, 2003). Most important are the three genera *Anisakis* (whales), *Contracaecum* (bird and seals), and *Pseudoterranova* (seals), that can be distinguished morphologically and according to their final host spectrum (Mattiucci and Nascetti, 2008). Food-borne zoonoses via aquatic animals are most often linked to anisakid nematodes of the genera *Anisakis* (Dujardin, 1845) and *Contracaecum* (Railliet and Henry, 1912). Since the 1960s, the term anisakiasis had been used for a human disease caused by the third-stage larvae (L3) of members of the family Anisakidae (Klimpel and Palm, 2011). With increased popularity of eating undercooked or raw fish dishes, the number of anisakiasis cases may be expected to increase (Umehara *et al.*, 2008). Over 90% of cases of anisakiasis are from Japan where consumption of raw fish is popular, with most of the rest from other countries with a tradition of eating raw or marinated fish, such as the Netherlands, France, Spain, Chile and the Philippines (Chai *et al.*, 2005).

Traditional nematode taxonomy was based on a limited number of criteria, such as the shape of the esophagus, male and female reproductive organs and life cycle patterns. There were considerable obstacles for an accurate identification especially of the larval forms. For this reason, earlier classification systems were not compatible with each other, and there was no universally accepted nematode phylogeny during the last century (De Ley and Blaxter, 2002).

Anisakid nematodes can be differentiated based on their morphological characteristics and molecular data. According to, larval morphological features, including absence of a ventricular appendage and an intestinal caecum, are useful for distinction between several anisakid genera (Berland, 1961). Even accompanied with morphometric information, generic and especially species identification has been difficult, leading to a high number of erroneous identifications. This promoted molecular methods for a better and more reliable species diagnosis. Molecular anisakid nematode identification started with allozyme analyses including restriction fragment length polymorphism techniques (PCR-RFLPs of ITS-DNA) (D'Amelio *et al.*, 2000; Pontes *et al.*, 2005). A previous study has demonstrated the potential of specific PCR assays for the identification of some anisakids (Umehara *et al.*, 2008). The polymerase chain reaction (PCR) based tools have been widely used for characterization of anisakid species at multiple loci, including ribosomal internal transcribed spacer (ITS) regions (Zhu *et al.*, 1998; D'Amelio *et al.*, 2000; Pontes *et al.*,

2005). The ITS region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions. The level of variation in this region makes it suitable for detecting genetic variation within species (Umehara *et al*., 2008). Recently, in Iran, occurrences of anisakids in various fish are reported (Mokhayer, 1973; Eslami and Mokhayer, 1977; Peyghan *et al.*, 2004).

The aim of this study was to determine the species of *Anisakis* and *Contracaecum* in *Mesopotamichthys sharpeyi* and *Barbus grypus* from the Shadegan Marsh in Khuzestan Province, the largest wetland in Iran, using a molecular approach.

Material and Methods

Geographical position

The study was done on the isolated fish from Shadegan Wetland. This wetland with 537731 hectare is located at the final destination of Jarrahi River, and is known as an important international wetland with a rich biodiversity. Geographic location of Shadegan Wetland is shown in Figure 1.

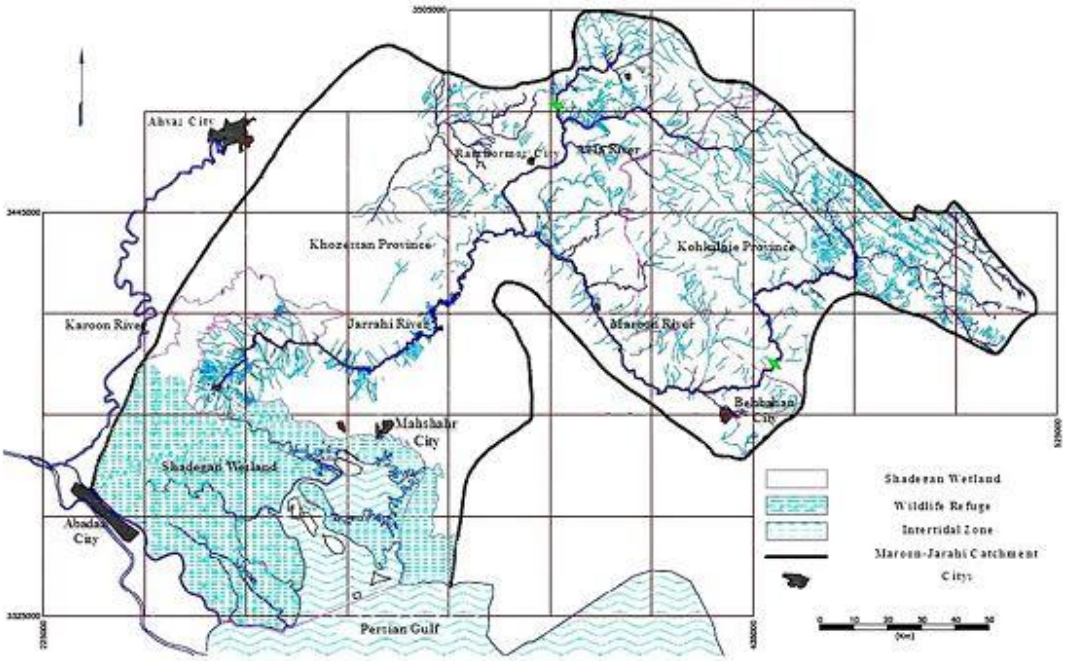

Figure 1: Location of Shadegan Wetland in Maroon-Jarrahi basin.

Parasite materials

Anisakis and *Contracaecum* were collected from intestine of *Mesopotamichthys sharpeyi* and *Barbus grypus* (n=200) caught in Shadegan Wetland. All fishes were collected from commercial catches. Specimens of *Anisakis* and *Contracaecum* nematodes were washed in physiological saline (pH 7.3). A small piece of mid-body of each nematode was removed with a scalpel and kept at -70°C freezer until use for molecular study. The rest of the nematode was cleared in lactophenol for morphological examination.

Extraction of genomic DNA

Total DNA was extracted from individual nematode tissue using Cinnapure Genomic DNA Purification Kit (Tehran, Iran) according to the manufacturer's instruction. DNA was kept at -20°C until use. Individual nematodes of Toxocara vitulorum served as outgroup for phylogenetic analyses.

DNA amplification and sequence analysis

PCR was used to amplify the entire ITS fragment with primers NC5 (forward: 5′-GTAGGTGA ACCTGCGGAAGGATCATT) and NC2 (reverse: 5′-TTAGTTTCTTTTCCTCCGCT) (Zhu *et al.*, 2007). Each PCR was performed in a reaction volume of 20 µL containing 1 µL of DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 0.4 µM of each primer and 0.25U of *Taq* DNA polymerase, in a thermocycler (Life ECO, China), under the following conditions: 95°C for 3 min, followed by 35 cycles at 94°C for 40 sec, 52°C for 45 sec, 72°C for 1 min, followed by postamplification at 72°C for 5 min. Negative controls (no DNA template) were included in all PCRs. PCR products were separated by electrophoresis in a 1.5% agarose gel (Fermentas, USA) and visualized by illumination with short wave ultraviolet light. A 100 bp DNA Ladder size marker as used to estimate the size of the PCR products. PCR products were sequenced by a dideoxy termination method and run on an Applied Biosystems 373 DNA sequenced. The sequence was determined for both strands by using overlapping fragments.

Sequences were compared with published sequences for identification in the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov). MEGA7 was used for genetic distances and phylogenetic analyses (Tamura *et al*., 2011). The nucleotide sequences were aligned using MUSCLE (Edgar, 2004), and edited manually and tested with MEGA7 model test to find the best DNA model to infer phylogenetic trees.

Results

Specimens of both studied fishes were different in size. Biometric information of studied fish is presented in Table 1. In this study 100 specimens of *Mesopotamichthys sharpeyi* and 100 specimens of *Barbus grypus* from Shadegan Wetland were examined, as a result of this review percentage of infection of these fishes to anisakid nematodes was 12.5% (25/200).

Table1: Biometric information of studied *Mesopotamichthys sharpeyi* and *Barbus grypus* specimens.

Fish	Average total length (cm)	Average standard length (cm)	Average body width (cm)	Average weight (gr)
M. sharpeyi	28.7 ±2.45	23.35 ±1.67	6.54 ±0.58	170.565 ±52.65
B. grypus	35.87 ±2.82	30.74 ±1.85	6.97 ±0.92	340.3 ±80.72

Morphological measurements

The *Anisakis* and *Contracaecum* nematodes were identified morphologically; *Anisakis* nematodes were identified based on an oblique connection between the ventriculus and the intestine, lack of a ventricular appendage and intestinal caecum (Berland, 1961; Cannon, 1977) (Fig. 2). Morphological identification of *Contracaecum* was detected based on observation of an intestinal caecum (with an average length of 0.47 mm and average width of 0.06 mm) at the interface between the esophagus and intestine (Fig. 3). The overall prevalence of *Anisakis* and *Contracaecum* sp. in the studied fish from Shadegan Wetland was 6% and 9% in *Mesopotamichthys sharpeyi* and 4% and 6% in *Barbus grypus*, respectively.

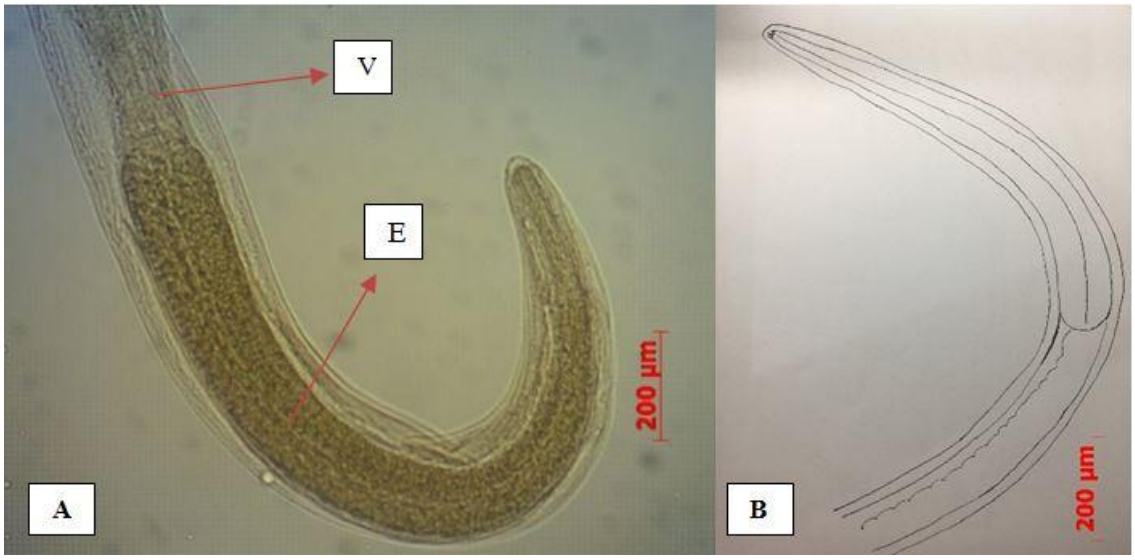

Figure 2: Morphology of *Anisakis* sp. nematodes (A). *Anisakis* nematode drowns with camera lucida (B), Ventricle (V), Esophagus (E).

PCR amplification and sequence analysis of NC5-NC2 fragment

Amplification of the NC5-NC2 fragment generated an approximately 1000 bp product. Among the amplified sequences that were isolated from the 40 collected specimens, six of them matched 100% with a sequence from *Anisakis pegreffii*, that was deposited in GenBank under the accession number MF803221. Seven of the others matched (99%) with a sequence from *Contracaecum rudolphii* that was deposited in GenBank under the accession number FJ467618.

Phylogeny and genetic distance

The nucleotide sequence of NC5-NC2 fragment with the retrieved sequence data from NCBI database was subjected to phylogenetic analysis. Phylogenetic tree was constructed based on neighbor-joining method (Fig. 4) accompanied with the morphological identification indicated that the isolated nematodes from the studied fishes were *Anisakis*

and *Contracaecum*. According to the phylogenetic tree, the samples of *Contracaecum rudolphii* and *Anisakis pegreffii* were grouped together as clusters A and B, respectively.

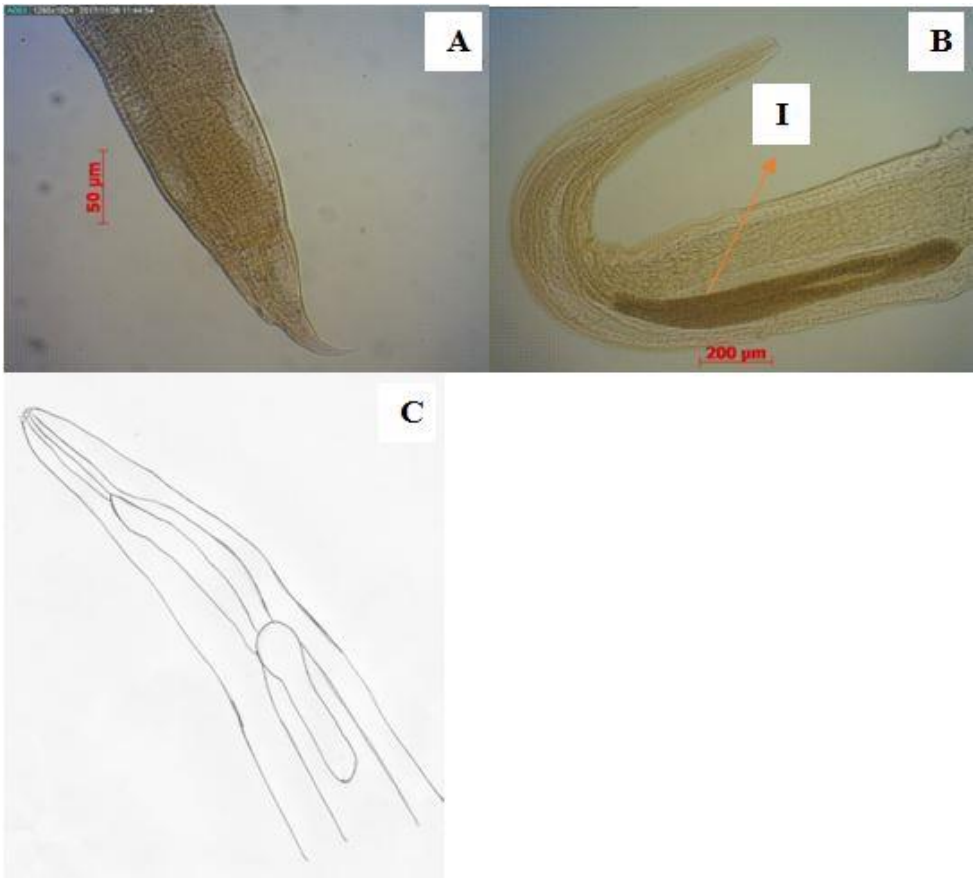

Figure 3: Anterior view of *Contracaecum* sp. (A). Posterior view of *Contracaecum* sp. (B). Anterior view of *Contracaecum* nematode drawn by camera lucida (C), intestinal caecum (I).

A minimum value of genetic distance, based on the sequence data from NC5-NC2 fragment (p distance=0.00, 0.0025), was found between the samples of *A. pegreffii* from the studied fish in this survey and A. *pegreffii* from GenBank according to Table 2. The specimen number 6 had the greatest distance (0.0025) to *A. pegreffii* from GenBank. A minimum value of genetic distance based on the sequence data from NC5-NC2 fragment (p distance =0.00, 0.001, 0.004, 0.005) was found between the samples of *C. rudolphii* from the studied fish in this survey and *C. rudolphii* from GenBank. Due to the Table 3, the specimen numbers 3 and 6 had the least distance and greatest distance to *C. rudolphii* from GenBank, respectively.

For the first time we report here the distribution of *A. pegreffii* and *C. rudolphii* in *Mesopotamichthys sharpeyi* and *Barbus grypus* from Shadegan Marsh.

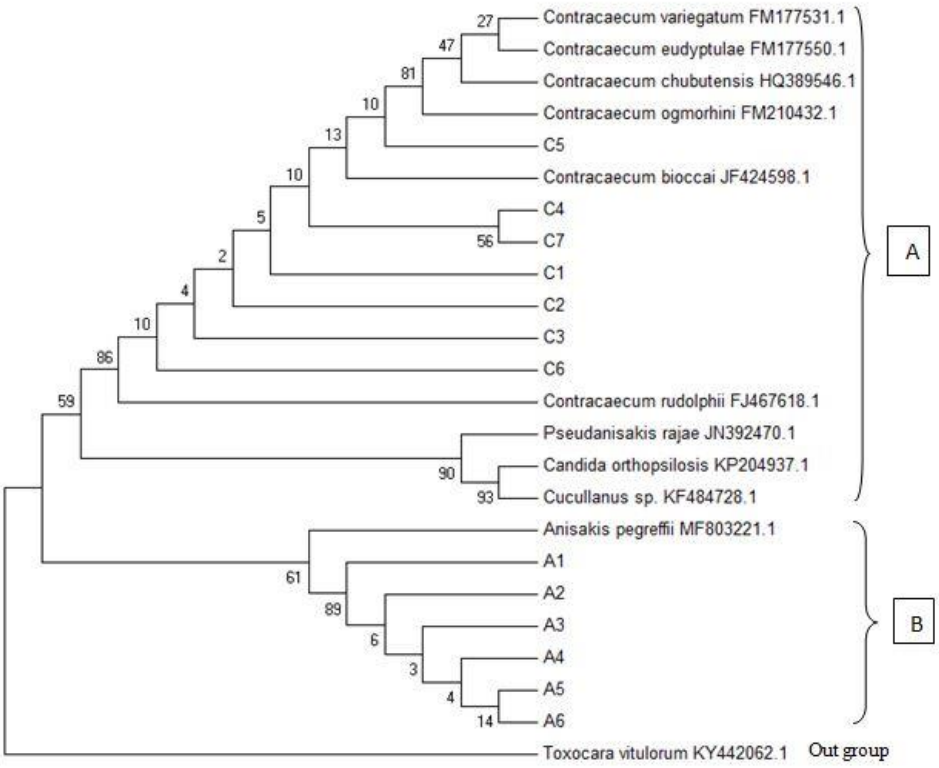

Figure 4: NC5-NC2-derived Neighbor-joining (NJ) tree for the anisakid DNA, showing genetic relationships among isolated nematodes in this study and *Anisakis pegreffii* and *Contracaecum rudolphii* and other near species from GenBank. Bootstrap values were calculated over 1,000 replicates. C: *Contracaecum sp.*, A: *Anisakis sp.*, *Toxocara vitulorum* was used as outgroup.

Table 2: The genetic distance of *Anisakis sp.* isolated from the studied fish with *Anisakis pegreffii* from GenBank database for the NC5-NC2 fragment.

	1	2	3	4	5	6	7
1. A1							
2. A2	0.00000						
3. A3	0.00000	0.00000					
4. A4	0.00000	0.00000	0.00000				
5. A5	0.00000	0.00000	0.00000	0.00000			
6. A6	0.00250	0.00250	0.00250	0.00250	0.00250		
7. Anisakis pegreffii MF803221.1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	

Table 3: The genetic distance of *Contracaecum* sp. isolated from the studied fishes with *Contracaecum rudolphii* from GenBank database for the NC5-NC2 fragment.

	1	2	3	4	5	6	7	8
1. Contracaeum rudolphii FJ467618.1								
2. C1	0.00133							
3. C2	0.00133	0.00267						
4. C3	0.00000	0.00133	0.00133					
5. C4	0.00133	0.00267	0.00267	0.00133				
6. C5	0.00133	0.00267	0.00268	0.00133	0.00267			
7. C6	0.00535	0.00670	0.00671	0.00535	0.00670	0.00671		
8. C7	0.00401	0.00535	0.00536	0.00401	0.00267	0.00536	0.00941	

Discussion

The ability to accurately identify and distinguish among species of anisakid nematodes, including *Anisakis* and *Contracaecum* spp. in different hosts and at any developmental stage has important implications for studying their population biology and ecology as well as for controlling the diseases they cause (Shamsi and Aghazadeh-Meshgi, 2010). In the present study *Anisakis* and *Contracaecum* sp. were identified morphologically, but to identify the species of *Anisakis pegreffii* and *Contracaecum rudolphii* PCR was used. The intensity of infection in *Mesopotamichthys sharpeyi* and *Barbus grypus* was low (1-3) in all fish hosts. The low infection levels could be due to the fact that most of the fish hosts sampled were relatively small in size (Table 1). In general, prevalence and parasite burden tends to increase with the size and the age of the fish host (Setyobudi *et al*., 2011). Based on previous studies, infection with anisakid nematodes has been recorded in approximately 200 fish species worldwide (Abollo *et al*., 2001), and also wide variation in prevalence and intensity of infection of anisakids in other fish hosts were reported (Setyobudi *et al*., 2011). Given the difficulty in accurate identification of anisakid nematodes, using morphological approach, the established specific PCR assays using genetic markers, should provide useful tools for unequivocal identification and differentiation of anisakids and would have implications for studying the ecology, epidemiology, and population genetics of these anisakid nematodes, as well as for the diagnosis of their infections in various hosts.

Comparison of the NC5-NC2 nucleotide sequences from this study with sequences previously deposited in GenBank resulted in 100% similarities to *A. pegreffii* found in *Conger myriaster* (Brevoort) (Anguilliformes: Congridae) (accession no. MF803221) from China, and 99% similarities to *C. rudolphii* from Northern Pacific. According to the genetic distance analysis (Tables 2 and 3) of the examined samples, *Anisakis pegreffii* and *cotracaecum rudolphii* were identified.

In conclusion, since molecular identification of *A. pegreffii* and *C. rudolphii* that is reported in this study, has not been previously recorded in Shadegan Marsh of Khuzestan Province in Iran, further studies are needed to extend the knowledge of anisakid species distributed in Khuzestan Province. Based on the results, the danger of zoonotic anisakids including *A. pegreffii* and *C. rudolphii* in the studied area is very low.

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