Genetics of Aquatic Organisms 7(3), GA669
https://doi.org/10.4194/GA669

RESEARCH PAPER

Molecular Investigation of Aquatic Reoviruses in Rainbow Trout in Northern Türkiye: The First Molecular Detection of Aquareovirus A in Türkiye

Semra Gunduz[1], Hanne Nur Kurucay[1], Emine Dogan[1], Harun Albayrak[1], Cuneyt Tamer[1,*]

[1]Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Virology, Samsun/TÜRKİYE

How to Cite

Gunduz, S., Kurucay, H.N., Dogan, E., Albayrak, H., Tamer, C. (2023). Molecular Investigation of Aquatic Reoviruses in Rainbow Trout in Northern Türkiye: The First Molecular Detection of Aquareovirus A in Türkiye. *Genetics of Aquatic Organisms, 7(3), GA669*. https://doi.org/10.4194/GA669

Article History
Received 25 July 2023
Accepted 20 November 2023
First Online 21 November 2023

Corresponding Author
E-mail: cuneyt_tamer@hotmail.com

Keywords
AqRV A
PRV
RT-PCR
Phylogenetic analyses

Abstract

Aquareovirus A (AqRV A) has been known to cause low or intermediate diseases among various salmon species and mollusks. Additionally, *Piscine orthoreovirus* (PRV), divided into subtypes PRV-1, PRV-2 and PRV-3, has been reported in rainbow trout, coho salmon and Atlantic salmon. In this study, we investigated the presence of these aquatic reoviruses in rainbow trout in Turkiye. For this purpose, fish were collected from 5 cities (Sinop, Samsun, Ordu, Trabzon and Rize) in Turkiye's Black Sea Region. In total, 150 fresh rainbow trout (30 fish per city) of 25–35 cm were collected from fishmongers. All the genotypes of Piscine orthoreoviruses (PRV) and Aquareovirus A were investigated using RT-PCR with degenerated pan-specific primers. No PRV genotypes were detected, but 4 pools of 30 pools were positive for AqRV A. According to phylogenetic analyses of segment 10 (VP7), all viruses were closely related to the Chum salmon reovirus (CSRV) belonging to AqRV A (GeneBank Accession number: NC_007590.1). This is the first report of AqRV A in naturally infected rainbow trout cultivated in sea cages in the world, necessitating further investigation of the pathobiology of AqRV A in rainbow trout.

Introduction

Aquareovirus (AqRV) and *Orthoreovirus* are the only genera containing aquatic reoviruses belonging to the family *Spinareoviridae* and order *Reovirales* (ICTV, 2023). The AqRV virion consists of 11 segmented double-stranded RNA and icosahedral capsids and is divided into 7 distinct species (*AqRV A*, *AqRV B*, *AqRV C*, *AqRV D*, *AqRV E*, *AqRV F* and *AqRV G*) according to RNA–RNA hybridization and nucleotide sequence analyses. The 11-segmented genome encodes 12 proteins (7 structural proteins, 5 non-structural proteins). Of the structural proteins, VP1 is the turret protein; VP2 is an RNA-dependent RNA polymerase located on the inner surface of the capsid shell; VP3 serves as both an inner capsid protein and a helicase protein; VP4 is the outer capsid protein that helps to penetrate the virion into the host cell membrane; and VP5 is the RNA-binding protein in the core, and is present along with VP7 in the outer coat of the virion (Chen et al., 2015). These proteins are removed via trypsinization, resulting in the release of core particles (Makhsous et al., 2017). Lastly, VP6 is the clamper protein that stabilizes the core of the virion. The non-structural proteins NS1, NS4, NS2, and NS3 are encoded by segments 4, 7, 9 and 11, respectively. NS5 is also encoded by segment 7 (ICTV, 2023).

Aquareoviruses have been known to cause mild diseases among various fish species, crustaceans and mollusks (Kibenge and Godoy, 2016). Most of the AqRV A variants isolated from Salmonidae spp. are low-

virulent, but some strains can cause high mortality in rainbow trout (*Oncorhynchus mykiss*), as reported in an experimental study (Meyers, 1983). A few reported viruses belonging to AqRV A have also been known to be highly virulent in salmonids (Lupiani et al., 1995). For instance, experimental studies have shown that these viruses cause restricted pathologic lesions like focal hepatic necrosis and hemorrhages in internal organs (Lupiani et al., 1995; Zainathan, 2013).

Piscineorthoreoviruses (PRV) are classified in the genus *Orthoreovirus* in the family *Spinareoviridae* of the order *Reovirales*. These viruses have a double-stranded RNA genome consisting of 10 segments. According to phylogenetic analysis of the 1081-bp-long S1 segment encoding PRV's outer capsid protein, these viruses fall into three subtypes (PRV-1, PRV-2, and PRV-3; ICTV, 2023; Kibenge et al., 2013). PRV-1 was first reported in 1999 in Atlantic salmon (*Salmo salar*) from a trout farm in Norway. This virus is the causative agent of heart and skeletal muscle inflammation, a disease that occurs in farmed Atlantic salmon and rainbow trout (Cartagena et al., 2018; Dhamotharan et al., 2020; Polinski et al., 2022). Morbidity rates close to 100% and mortality rates up to 20% have been reported in affected cages (Kongtorp et al., 2004). PRV-2 was also shown to cause erythrocytic inclusion body syndrome in coho salmon (*Oncorhynchus kisutch*) in Japan in 2016, though the causative agent has not been reported outside Japan (Takano et al., 2016). PRV-3 was first identified in 2013 following a comprehensive investigation of unexplained mortality in juvenile rainbow trout (*Oncorhynchus mykiss*) cultured in freshwater in Norway (Olsen et al., 2015). PRV-3 was also shown to cause anemia and associated skin darkening and idiopathic syndrome in coho salmon in South America (Cartagena et al., 2018; Cartagena et al., 2020). Since 2015, PRV-3 has caused significant losses in rainbow trout populations in Denmark, Scotland and Germany (Adamek et al., 2019; Cartagena et al., 2020).

Infectious pancreatic necrosis virus (IPNV) and Piscine novirhabovirus, formerly known as Viral hemorrhagic septicemia virus (VHSV), are the major viral threat for the aquaculture industry in Turkiye (Işıdan & Bolat, 2011; Tamer et al., 2022). Furthermore, aquatic reoviruses, potentially affecting the fish supply, threaten fish populations and therefore food supply in Turkiye. Considering that Turkiye imports fish eggs and fry from Europe, it is necessary to reveal whether novel viral diseases circulating in Europe cause trout mortality in Turkiye. In this study, we investigated the presence of these aquatic reoviruses in rainbow trout in Turkiye, which pose a potential novel threat to the aquaculture industry.

Material and Methods

Sampling

The fish were collected at 2% estimated prevalence, 100% specificity, and 100 percent sensitivity according to the Aquatic Animal Disease Surveillance manual of the World Organization for Animal Health (WOAH, 2023). For this purpose, fish were collected from the cities of Sinop, Samsun, Ordu, Trabzon and Rize in Turkey's Black Sea Region (Figure 1). In total, 150 fresh rainbow trout (30 fish per city) of 25–35 cm that had been cultured in Black Sea cages were bought from fishmongers. The kidney, heart, spleen and liver were collected from each fish and dissected using a sterile mortar. The organ samples were pooled in each consisting of 5 fish. So, 6 pools were created from each city (Si1-Si6, Sa1-Sa6, O1-O6, T1-T6, R1-R6).

Figure 1. Map of the study area in which the fish samples were collected.

RT-PCR and Sequencing

RNA extraction was performed using a High Pure Viral Nucleic Acid Kit (Roche, cat. no: 11858874001) according to the manufacturer's instructions.

For PRV, all genotypes were investigated using the Biorad CFX Connect Real-Time PCR Detection System with degenerated pan-specific primers, which is specific for all PRV genotypes (Zhao et al., 2021). For this purpose, an iTaq Universal Probes One-Step Kit (Biorad, cat. no: 1725141) was used in the Biorad CFX Connect Real-Time PCR System with the following reaction steps: 10 min RT at 50°C, 3 min predenaturation at 95°C, 5 s at 95°C and 10 s at 60°C after 40 repeat cycles. Each RT-PCR reaction contained 10 µl 2× iTaq buffer, 320 nM forward primer (5'-TGGGTAACTATCAGACAAGTAACAAC-3'), 320 nM reverse primer (5'-GTAGARTCGAGTCCGCCTTCAG-3'), 160 nM probe (5'-FAM- CAATTTTGGGTAACTGGCGACGGCAATGA -TAMRA-3') and 5 µl template RNA.

For AqRV A, an iScript™ cDNA Synthesis Kit (Biorad, cat. no: 1708891) was used for the cDNA synthesis. DreamTaq DNA Polymerase (Thermo, cat. no: EP0702) was used for the amplification of VP7 (Segment10) under the following reaction conditions: 3 min predenaturation at 95°C, 30 s at 95°C, 30 s at 55°C and 60 s at 72°C after 35 repeat cycles. Each RT-PCR reaction contained 5 µl 10× Taq buffer, 320 nM forward primer (5'-1811F: CRCCATGGAGACCAAACC-3'), 320 nM reverse primer (5'-1812R: CTSTGRTTCATCATAGCGTG-3'), 200 nM dNTP and 5 µl template RNA (Makhsous et al., 2017).

The raw sequence data were initially assembled using BioEdit Sequence Alignment Editor software (BioEdit version 7.2.5, 12/11/2013). The consensus sequences of AqRV A T6 and AqRV A T3 were aligned with sequences of AqRV and PRV in the Genbank database using Mega X. The Bayesian method was used for the phylogenetic analysis of 361 bp of VP7 sequences by using maximum likelihood estimation with 1000 bootstraps from Mega X (Mega Version 10.2.6).

Results

Clinical and Necroscopic Findings

There were no clinical lesions found upon microscopic inspection. Petechial hemorrhages in the liver were observed in the two fish pooled in T6 pools at necropsy (Figure 2). Spleens, hearts and kidneys had normal anatomical appearances in all fish.

RT-PCR and Phylogenetic Analyses

Unlike the ponds with no detected PRV nucleic acid, 4 of 30 pools (T3, T6, R3 and R5) were positive for AqRV in Trabzon and Rize. Then, RT-PCR was performed

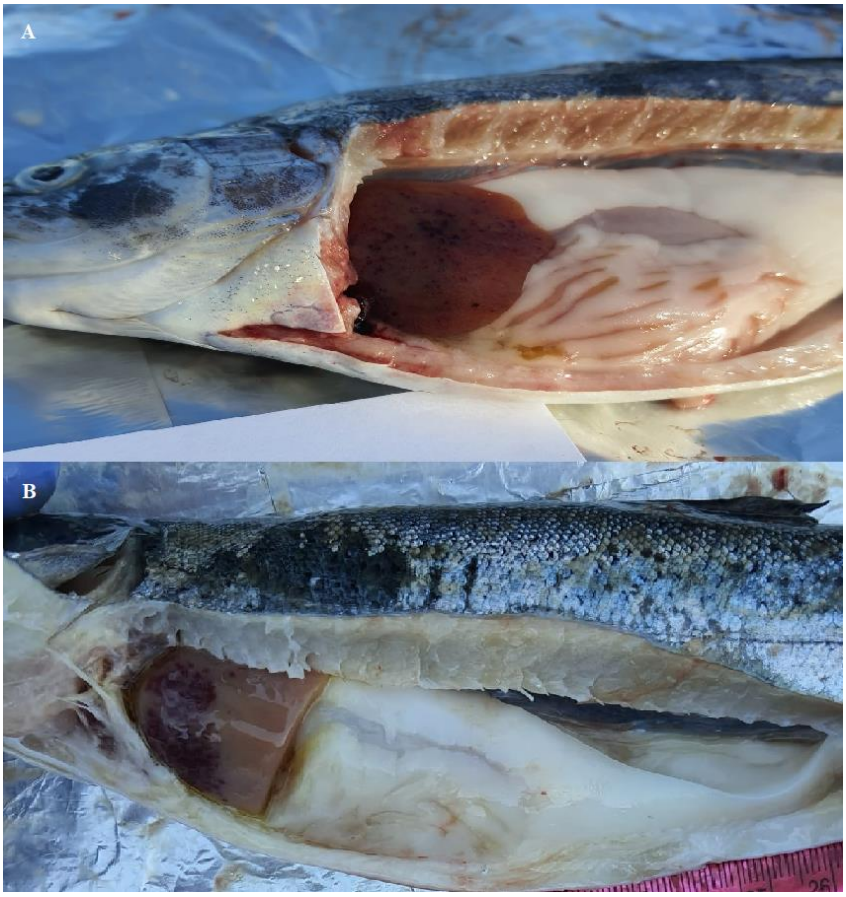

Figure 2. Necropsy findings of the fish collected from the Trabzon region (T6 pool). **A-** Fish numbered 2 in pool T6. **B-** Fish numbered 3 in pool T6.

to each fish separately in those pools and these fish samples were positive except for numbers 1, 4 and 5 in the T3 pool (Figure 3). However, only the T6 and R3 pools' sequence data were reliable.

These data were edited in BioEdit Sequence Alignment Editor Software, and the consensus sequences of the AqRV A were uploaded to the NCBI database with accession numbers Trabzon 6: OQ749649 and Rize 3: OQ749650 (realization date: 24.05.2024). Trabzon 6 and Rize 3 isolates were subsequently found to have 99.17% nucleic acid similarities with Chum salmon reovirus (CSRV; GenBank Accession no: AF418303.1; Figure 4).

Discussion

Turkiye exports 1.3 billion US dollars and imports 200,000 dollars in fish products annually. Within its fish production, rainbow trout breeding is the widest-spread branch of aquaculture, producing 144,347 tonnes in inland waters and 45,454 tonnes in marine waters in 2022 (TUIK, 2023). Thanks to increasing demand in Europe, these rainbow trout are exported to countries such as Russia and Greece (TUIK, 2022). However, infectious diseases like IPNV and VHSV threaten the Turkish aquaculture industry. As the fishing industry grows in Turkiye, it is important to know the presence and rapid diagnosis of these infectious diseases.

In this study, we investigated the presence of aquatic reoviruses in rainbow trout in Turkiye's central and eastern Black Sea Region, which have not been studied before. As a result of RT-PCR, no PRV genotypes were detected, but 4 pools were positive for AqRV A. According to phylogenetic analyses of segment 10 (VP7) of T6 AqRV and R3 AqRV, both viruses were closely related to the CSRV belonging to AqRV A (GenBank accession number: NC_007590.1). CSRV has previously been isolated from chum salmon (*Oncorhynchus keta*) in Japan (Winton et al., 1981). While these results offer a novel understanding of these viruses, there are not enough similar epidemiological and phylogenetic studies from European and Asian countries that have close and commercial relations with Turkiye. Moreover, there are only 7 VP7 sequence data in GenBank (until 14/07/2023). This makes this study the first report of AqRV A in naturally infected rainbow trout in Europe.

The only known lesion of AqRV A is focal necrosis hepatitis (Kibenge and Godoy, 2016; Winton et al., 1989). In this study, the fish in pool T6 were observed to have macroscopic lesions of the liver. Fish infected with AqRV A are generally of low or no virulence, but high mortality has been reported in rainbow trout infected with some strains of AqRV A (Kibenge and Godoy, 2016; Meyer, 1983), meaning the virus has the potential to generate yield loss in rainbow trout production. On the contrary, the infection of AqRV A has been reported to increase resistance to other viral diseases, such as IHNV (LaPatra et al., 1995). The pathogenicity of AqRV A in rainbow trout should thus undergo further study in relation to interference with other viral infections.

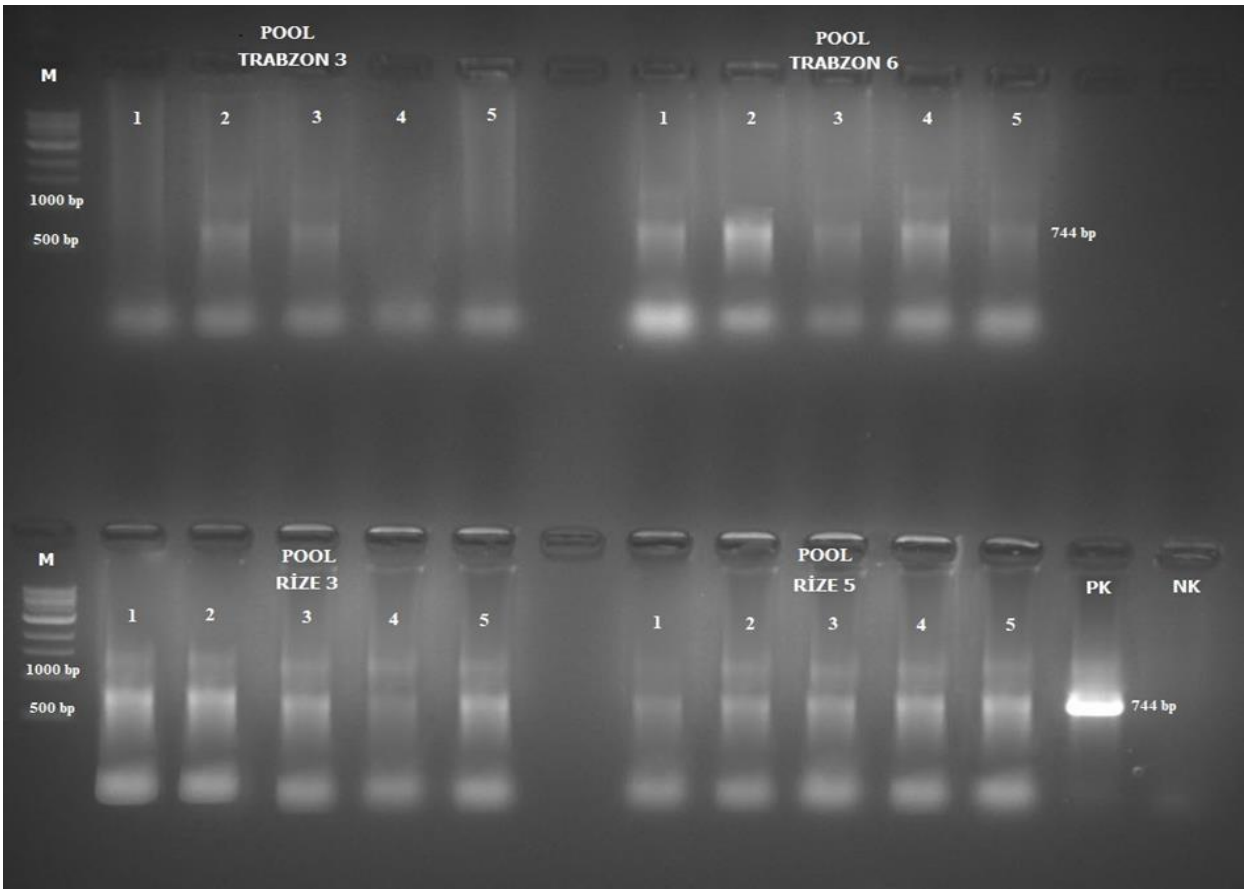

Figure 3. RT-PCR results of segment 10 (VP7) of AqRV. 744 bp length of amplicon was detected in 4 pools (T3, T6, R3 and R5).

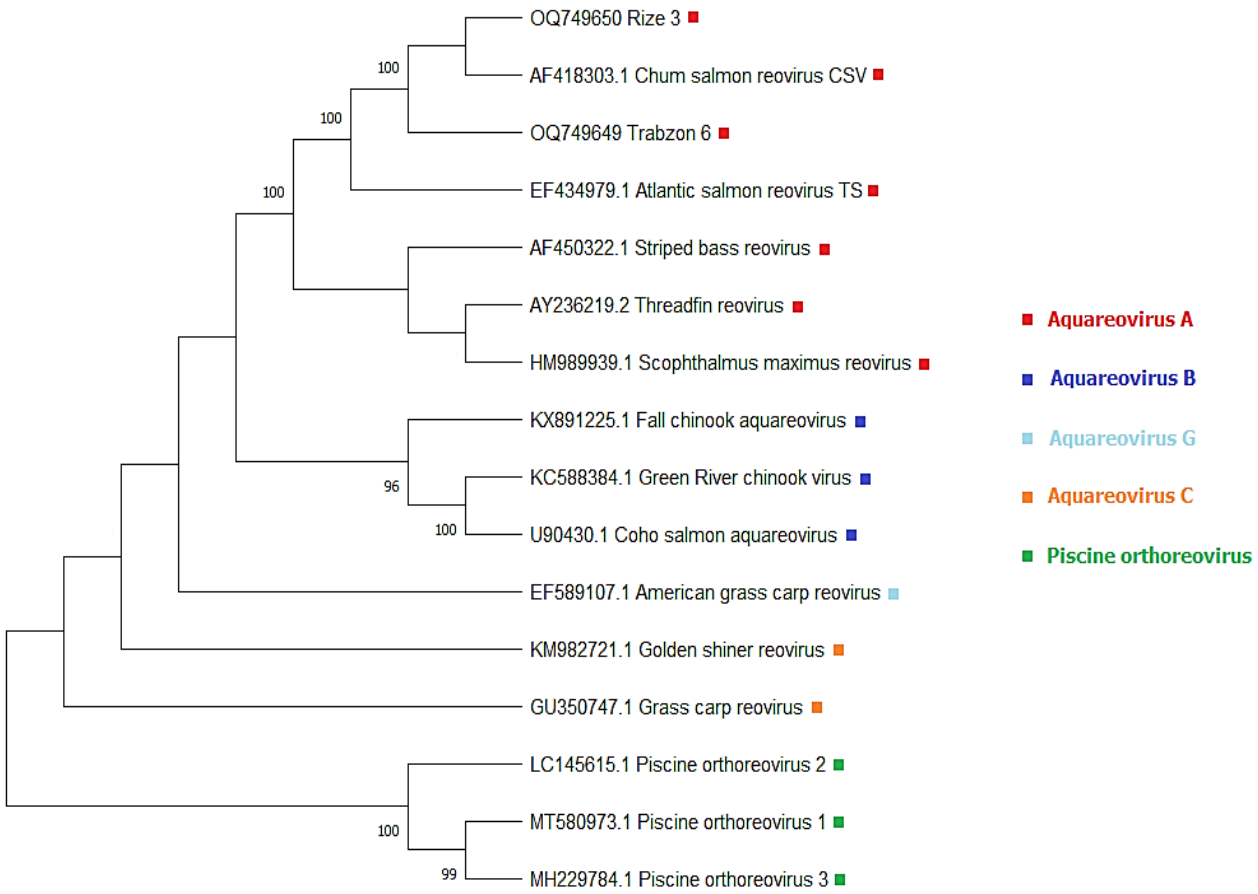

Figure 4. A Bayesian phylogenetic tree of the Aquatic reoviruses based on 361 bp of segment 10 (VP7) sequences, as generated with 1000 bootstraps using the maximum likelihood estimation method..

PRV has been detected in Salmonidae spp., especially in rainbow trout in South America, Europe and Japan (Cartagena et al., 2020; Kibenge et al., 2013; Takano et al., 2016). PRV-3 has caused significant economic losses in rainbow trout in Denmark, Scotland and Germany since 2015 (Adamek et al., 2019; Olsen et al., 2015; Sorensen et al., 2020). PRV-1 has also caused significant losses in rainbow trout in Chile (Cartagena et al., 2020). Considering that Turkiye imports fish eggs and fry from Europe and America, there is demand for more studies of whether PRV-3 is the cause of unexplained trout mortality in Turkiye. Although we did not find PRV genotypes in this study, larger-scale and more frequent epidemiologic research should be conducted to verify these conclusions.

Conclusions

This study was conducted to inform about aquatic reoviruses in Turkish fish samples, which have not previously been investigated epidemiologically. According to these results, AqRV A's pathobiology in rainbow trout should be further investigated. For PRV infections likely to contaminate Turkeiye, further epidemiology studies and the necessary preparations for rapid diagnosis should be realized by relevant diagnostic laboratories as well.

Ethical Statement

No transactions were made on live fish. All fish are sold at fishermen's stalls which collect from sea cages

Funding Information

This study was supported by the Scientific Research Fund of the Ondokuz Mayis University (Project no: PYO.VET.1904.22.020).

Author Contribution

Conceptualization: CT, Funding Acquisition: CT, Investigation: SG, HNK, ED, Methodology: HA, CT, Writing – Original Draft Preparation: HA, CT

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was carried out as a part of the Master thesis of Semra GUNDUZ (First author) with the Supervisor of the Cuneyt TAMER in the Department of Veterinary Virology, Ondokuz Mayıs University, Samsun/Türkiye.

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