NOTE

Novel *Chlamydiales* associated with epitheliocystis in a leopard shark *Triakis semifasciata*

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ABSTRACT: The *Chlamydiales* is a diverse order of obligate intracellular gram-negative bacteria that are known to cause a wide range of diseases in terrestrial animals, including humans. Molecular analyses have revealed that these organisms are also associated with epitheliocystis in teleost fish species, highlighting the suspected deep evolutionary origin of members of this bacterial order. However, our knowledge of their fish host range and of the diversity of the bacteria themselves is still very limited. In this study, we provide molecular evidence for a novel member of the Order *Chlamydiales* in a nonteleost species, the leopard shark *Triakis semifasciata*. Based on phylogenetic analysis of the 16S rRNA gene, this novel organism appears to represent a unique lineage in the Order *Chlamydiales* despite appearing histologically similar to epitheliocystis-causing organisms in other fish species. A greater understanding of the genetic diversity of marine *Chlamydiales* will assist our attempts to manage and control epitheliocystis outbreaks and to understand the evolution of this unique obligate intracellular pathogen.

KEY WORDS: Epitheliocystis · *Chlamydia* · Leopard shark · 16S rRNA · Phylogenetic analysis · Gill pathology

INTRODUCTION

Epitheliocystis is an infectious disease of the gills and skin in many marine and freshwater fish species. Research over the last decades has provided strong ultrastructural (Paperna et al. 1981, Paperna & Alves de Matos 1984, Turnbull 1993, Crespo et al. 1999, 2001) and antigenic evidence (Groff et al. 1996) linking the causative agents of this disease with members of the *Chlamydiaceae*, which are obligate intracellular bacteria which cause disease in humans and animals. More recently, molecular studies have confirmed this description, with a number of reports detecting unique *Chlamydia*-like organisms that are phylogenetically distinct but related to the traditional terrestrial mem-

bers of this bacterial order (Draghi et al. 2004, 2007, Meijer et al. 2006, Karlsen et al. 2008). Although provisional names have been proposed for 2 of these organisms based on the salmonid host species, namely '*Candidatus* Piscichlamydia salmonis' (Draghi et al. 2004) and '*Candidatus* Clavochlamydia salmonicola' (Karlsen et al. 2008), we have virtually no data on the species specificity of these bacteria. Zoonotic potential can at least be inferred in one case, with a *Chlamydiales* with identical sequence having been discovered in conjunctival swabs from cats' eyes (von Bomhard et al. 2003) and in epitheliocystis of Arctic charr *Salvelinus alpinus* (Draghi et al. 2007). Further advances in our understanding of the host specificity and routes of transmission of *Chlamydia*-like epitheliocystis agents has

been limited by the lack of success in isolating and cultivating these obligate intracellular bacteria from infected fish.

Epitheliocystis infections have a varying effect on the health of the infected host, ranging from no apparent effect in the mildest cases to severe proliferation of gill epithelial cells, resulting in clinical signs such as sluggishness in movement caused by respiratory insufficiency. Mortality within cultured fish populations can vary greatly, especially in juvenile fish or even larvae (Katharios et al. 2008). However, as the bacteria have yet to be cultured from epitheliocystis lesions, a direct causal proof according to Koch's postulates has not been obtained for this disease (Nowak & LaPatra 2006). Apart from infecting cultured species of economic importance such as Atlantic salmon *Salmo salar*, Arctic charr *Salvelinus alpinus*, sea bass *Dicentrarchus labrax* and sea bream *Sparus aurata*, the disease has also been reported in ornamental species such as the leafy sea dragon *Phycodurus egues* (Langdon et al. 1991) (reviewed by Nowak & LaPatra 2006).

In the present report, we provide the first molecular description of a *Chlamydia*-like bacterium associated with epitheliocystis in a nonteleost fish species, the leopard shark *Triakis semifasciata*, although morphological and histological evidence for *Chlamydiales* infection in epitheliocystis of white sturgeon *Acipenser transmontanus* in aquaculture has been reported (Groff et al. 1996). Consistent with other recent reports detecting *Chlamydia*-like organisms associated with epitheliocystis, the 16S rRNA sequence detected in the gill tissue represents a novel lineage among other environmental *Chlamydia* in the Order *Chlamydiales*.

MATERIALS AND METHODS

Case history. The animal described in this report was an adult female leopard shark *Triakis semifasciata* (3.75 kg, 100 cm length). The animal was kept in a quarantine tank, together with a moray eel *Gymnothorax favagineus*, at the Zoological Garden in Basel, Switzerland. Three months prior to the death of the animal, the shark showed multiple skin ulcerations which resolved without treatment. A few days prior to euthanasia, the animal presented deteriorating symptoms including multiple skin ulcerations, reduced breathing rate and uncoordinated swimming. Antibiotic treatment with enrofloxacin was applied unsuccessfully. Since the clinical signs of disease did not resolve, the animal was euthanized and sent to the Centre for Fish and Wildlife Health (FIWI), University of Bern, Switzerland, for further investigation.

A complete necropsy was performed. Fresh mounts of skin, gill samples and the intestinal content were collected for microscopic investigation but no evidence of the presence of parasites was found. Bacteriological examination of tissue samples of skin, liver, spleen and kidney was performed by cultivation on blood agar plates (Bio Merieux) and Bromothymol blue-lactose agar plates. Besides a mixed culture from the skin sample, cultures from the inner organs were sterile. Samples of skin, gills, heart, liver, kidney, spleen, intestine and muscle were fixed in 10% buffered formalin, trimmed and embedded in paraffin blocks and routinely processed for histology and staining with hematoxylin and eosin (H&E).

Immunohistochemistry using *Chlamydiaceae*-specific antibodies. Paraffin sections were investigated for the presence of *Chlamydia* essentially as previously described (Soldati et al. 2004) using *Chlamydiaceae* family-specific mouse monoclonal antibodies (Abs) directed against the chlamydial lipopolysaccharide (cLPS; Clone ACI-P, Progen) and the EnVision kit (Dako ChemMate) according to the manufacturer's instructions. The primary antibody was prepared at a working dilution of 1:200 in antibody diluents. Intestinal tissues from gnotobiotic piglets that were experimentally infected with porcine *C. suis* strain S45 (Guscetti et al. 2009) were used as positive controls.

DNA extraction from formalin-fixed and paraffin-embedded material. Sections (30 µm) were cut from the paraffin block and stored in sterile microcentrifuge tubes. Paraffin was removed by xylene treatment, followed by repeated washing with 100% ethanol to remove residual xylene. DNA was extracted from the tissue pellet using a commercial DNA extraction kit (DNeasy tissue kit, Qiagen) according to the manufacturer's instructions.

16S rRNA Order *Chlamydiales*-specific PCR amplification and cloning of sequences. The presence of chlamydial DNA was detected by a pan-*Chlamydiales* PCR using the previously described primers 16SIGF (5′–CGG CGT GGA TGA GGC AT–3′) and 16SIGR (5′–TCA GTC CCA GTG TTG GC–3′) targeting the complete *Chlamydiales*-specific 16S rRNA signature sequence (Everett et al. 1999). Reactions were prepared by the addition of 5 µl of extracted DNA to a 45 µl PCR mastermix containing 1× PCR buffer, 1 mM dNTP, 4.5 mM MgCl$_2$, 0.3 µM of each primer and 1 U Fast Start Taq DNA polymerase (Roche Applied Science). The amplification reaction was performed with initial denaturation (94°C, 3 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 54°C (30 s) and extension at 72°C (1.5 min). The amplification reaction was completed with a final extension of 72°C for 10 min. Negative controls consisting of dH$_2$O were performed in triplicate. The presence of a resulting 298 bp PCR product was visualized by UV transillumination (254 nm) following separation of PCR prod-

ucts by agarose gel electrophoresis. Reactions were repeated in triplicate and distilled water was used in negative control reactions.

Freshly amplified 16S rRNA PCR products were ligated into the pCR2.1-TOPO cloning vector (Invitrogen), and transformed into *Escherichia coli* TOP10 chemically competent cells (Invitrogen) according to the manufacturer's instructions. Individual clones were screened using restriction digest and the inserts sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystems) or a 3100 Genetic Analyser (Applied Biosystems) using M13 forward (CGC CAG GGT TTT CCC AGT CAC GA) and M13 reverse primers (AGC GGA TAA CAA TTT CAC ACA GGA).

Molecular phylogenetic analysis. The initial identity of amplified and cloned 16S rDNA sequences was determined by BLAST-n searching (Altschul et al. 1997) of the available sequences in the GenBank database (www.ncbi.nlm.nih.gov). 16S rRNA secondary structure reconstruction and analysis was performed essentially as described previously (Meijer et al. 2006) using RNAdraw version 1.1b2 and the 16S rRNA secondary structure template provided by the European ribosomal RNA database (Wuyts et al. 2004) to authenticate the novel 16S rDNA sequences. After removal of the amplification primer sites, the novel 16S rDNA sequences were initially aligned with 16S rRNA signature sequences from members of the Order *Chlamydiales* (Everett et al. 1999), including species of the traditional *Chlamydiaecae* family and *Chlamydia*-like organisms and reported 16S rRNA sequences detected in cases of epitheliocystis in other fish species, using ClustalW (Thompson et al. 1994). The final alignment, which was used for phylogenetic analysis, was manually refined taking into account the secondary structure of each sequence included using pre-aligned 16S rRNA sequences and the 16S rRNA secondary structure template provided by the European ribosomal RNA database.

Phylogenetic analysis was carried out using the Tamura and Nei method (transversions and transitions included; heterogeneous patterns among lineages, gamma distributed rates among sites with a gamma parameter of 0.35 estimated using TREE-PUZZLE 5.2; Schmidt et al. 2002) to calculate a distance matrix. The phylogenetic tree was inferred by the neighbor-joining (NJ) method using MEGA software version 4.1 (Tamura et al. 2007). Maximum-likelihood (ML) analysis was carried out using the TREE-PUZZLE software version 5.2 (parameter estimation using NJ tree with the Tamura-Nei model for substitutions and gamma distributed rates among sites). Maximum-parsimony analysis was carried out using MEGA software version 4.1 via the close-neighbor-interchange method for finding the optimum tree. *Chlorobium tepidum* and *Piscirickettsia salmonis* 16S rRNAs were used as outgroup sequences for phylogenetic tree construction. From the 3 inferred phylogenetic trees, 1 consensus tree (topology only) was constructed.

Nucleotide sequence accession number. The partial 16S rRNA gene sequence of the leopard shark epitheliocystis agent is available at GenBank under the accession number FJ001668.

RESULTS

Histopathological description of leopard shark epitheliocystis

Histological examination of the gills revealed multifocal hyperplasia of the gill epithelium. In these areas, multiple epithelial cells were necrotic showing karyopycnosis and karyorhexis. Moderate cellular infiltration was observed, mainly with eosinophilic granular cells and few macrophages. One trematode-like structure was found in the gill section, but no amoeba was observed. At multiple locations on the lamellar epithelium, intercellular edemas with interspersed inflammatory cells were present. Multiple intracellular cysts, which were often found near these lamellar lesions, were observed in enlarged epithelial cells. This enlargement appeared to be the result of the displacement of the cytoplasm and the nucleus by a large (10 to 40 μm diameter), well-marginated vacuole containing what appeared to be fine, granular material (Fig. 1).

More detailed ultrastructural observations using transmission electron microscopy were attempted, but no epitheliocystis inclusions were found in the remaining limited tissue that was available for analysis.

Immunohistochemical analysis of the *Chlamydia*-like organism associated with leopard shark epitheliocystis

Previous reports characterizing epitheliocystis infections in different fish species have described varying cross-reactivity to antibodies that recognize immunogens expressed by the traditional members of the *Chlamydiaceae* (Nowak & LaPatra 2006). To investigate this phenomenon further, we tested tissue sections immunohistologically using a monoclonal antibody (AC-I) specific for cLPS. Previous reports on the presence of this antigen in epitheliocystis cases vary (Nowak & LaPatra 2006) and are of interest since this antigen appears to be *Chlamydiaceae* family specific and is not found in *Chlamydia*-like organisms (Corsaro & Greub 2006). Although the *Chlamydia*-infected control tissues were positive, we were unable to detect any
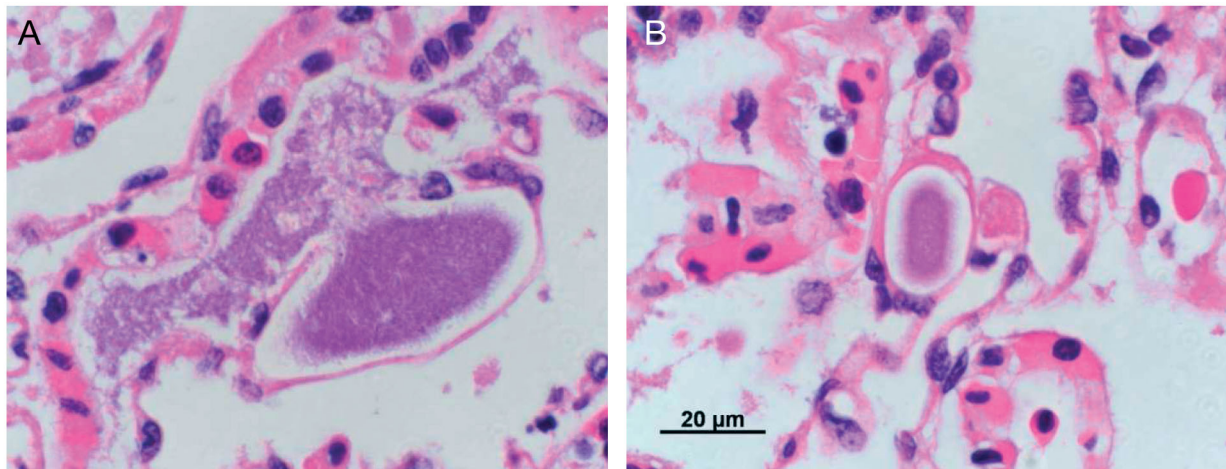
Fig. 1. *Triakis semifasciata*. Epitheliocystis inclusions in gill lamellae. (A) A ~50 µm (cross section) inclusion appears to be rupturing, releasing granular material, presumably bacteria, into the sub-epithelial space. This is an unusual feature that is rarely seen in epitheliocystis, but may imply in this case that the bacteria are thereby able to spread more widely within the host. (B) A more typical inclusion that is completely encapsulated within an epithelial cell membrane. Both (A) and (B) are reproduced at the same magnification, as indicated by the scale bar in (B)

signal with this antibody in the leopard shark sample. This suggests that the *Chlamydia*-like organisms detected in these tissue samples are sufficiently distant from the *Chlamydiaceae* so as not to carry the LPS epitope shared by members of this family (data not shown).

Molecular identification of novel *Chlamydia*-like organisms

DNA extracted from a section of the infected leopard shark gills was positive for chlamydial DNA following repeated testing with the Order *Chlamydiales* specific 16S rDNA PCR assay. Cloning and sequencing of these PCR products revealed the presence of one 294 bp sequence that could be repeatedly amplified from the same gill sample. This sequence was designated as Uncultured Fish *Chlamydiae* 1 (UFC1). BLAST-n searching of the UFC1 sequence against the NCBI database revealed closest, albeit low, sequence similarity (89%) to a 16S rDNA sequence that was obtained from a case of feline ocular disease in our previous study (WB13; von Bomhard et al. 2003). A bootstrap consensus tree was constructed from trees that were derived using 3 different analyses methods to infer the phylogenetic relationship of this novel shark epitheliocystis sequence to those of the other members of the Order *Chlamydiales* (Fig. 2). This analysis revealed that UFC1 appears to branch separately from other members of this bacterial order and away from the traditional members of the *Chlamydiaceae*, forming its own lineage among other environmental *Chlamydia*-related bacteria. This analysis confirms our morpho-logical observations suggesting that the bacterial agent of epitheliocystis, in the case of this leopard shark, belongs to a new and phylogenetically distinct branch of the Order *Chlamydiales*.

DISCUSSION

This report describes the first molecular identification of a bacterial agent associated with epitheliocystis in a nonteleost fish species, the leopard shark. Consistent with previous reports in bony fishes (Draghi et al. 2004, 2007, Meijer et al. 2006), UFC1 is genetically related to *Chlamydia*-like organisms, sharing >80% sequence similarity to other members of the Order *Chlamydiales* (Everett et al. 1999). Although not ideal for detailed phylogenetic analysis, the short 16S rRNA signature sequence that was detected in the epitheliocystis-infected tissue is unique; however, it shared only distant similarity (89%) with other previously reported and uncultured *Chlamydia*-like 16S rRNA sequences from a cat (von Bomhard et al. 2003), which in contrast were identical to sequences reported from epitheliocystis in Arctic charr (Draghi et al. 2007). The sequence that was detected in the leopard shark is clearly distinct from the latter fish epitheliocystis 16S rRNA sequences, suggesting that UFC1 is the first representative of an, as yet, unreported lineage in the Order *Chlamydiales*. Also, in contrast to the Arctic charr epitheliocystis case, we found no staining with a *Chlamydiaceae*-specific anti-LPS antibody, which further supports this conclusion.

The epitheliocystis infection was unresponsive to treatment with enrofloxacin, which would be effective

against *Chamydiaceae*, but not against *Chlamydia*-like organisms (Casson & Greub 2006, Goy & Greub 2009). Interestingly, these organisms are sensitive to tetracyclines. Oxytetracyclines have previously been found to be effective in treating epitheliocystis infec-

tions in largemouth bass (Goodwin et al. 2005), and this strategy may be the preferred treatment for future epitheliocystis outbreaks.

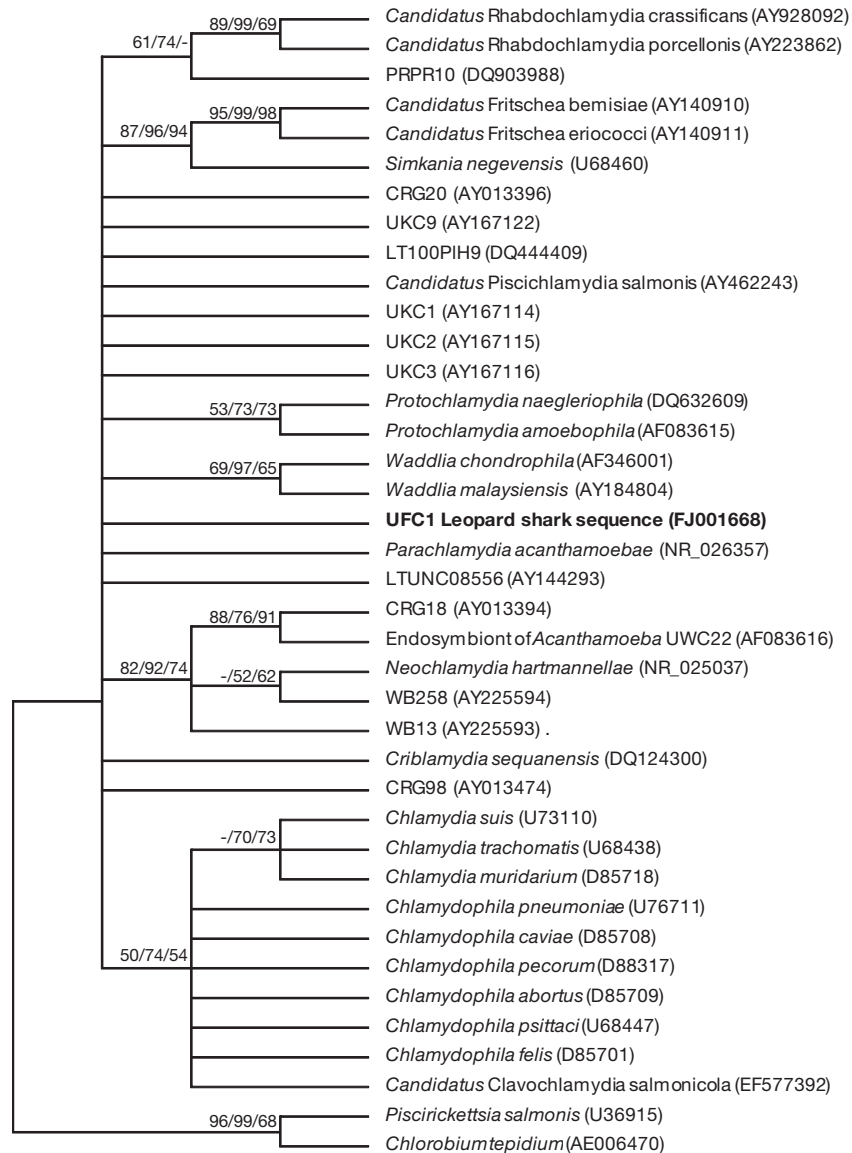We have only begun to appreciate how considerable the diversity and abundance of members of the

Fig. 2. Phylogenetic relationships between the novel leopard shark epitheliocystis 16S rRNA sequence (Uncultured Fish *Chlamydiae* 1, UFC1) and those of selected members of the Order *Chlamydiales*. The tree was inferred by using neighbor joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) analysis of a ~260 bp region of the 16S rRNA *Chlamydiales* signature sequence. The tree was rooted using the distant *Piscirickettsia salmonis* and *Chlorobium tepidium*. Numbers indicate the percentage of times each branch appeared in a tree during 10 000 bootstrap samples (NJ and MP) or puzzling steps (ML) in the order (NJ/MP/ML). Multifurcations connect branches for which the relative order could not be unambiguously determined. Branches supported by a bootstrap or puzzling reliability value of >50 % in at least 2 treeing methods are shown. GenBank accession numbers are given in parentheses. Included in the phylogenetic tree are representative sequences of type strains of classified *Chlamydiaceae*, along with the sequences of cultured *Chlamydia*-like organisms isolated from diverse hosts including amoeba, invertebrates, fish, and marsupial and placental mammals (*Criblamydia sequanensis*, *Waddlia chondrophila*, *W. malaysiensis*, *Parachlamydia acanthamoebae*, *Neochlamydia hartmanellae*, *Simkania negevensis*, '*Candidatus* Rhabdochlamydia porcellonis', '*Candidatus* Rhabdochlamydia crassificans', *Protochlamydia amoebophila*, *P. naegleriophila*, '*Candidatus* Fritschea eriococci', '*Candidatus* F. bemisiae', and an endosymbiont of *Acanthamoeba* UWC22)

Order *Chlamydiales* in the environment really is; many of these members have been isolated from rivers, lakes and water treatment plants via the amoebal co-culture method (Corsaro & Greub 2006). This takes advantage of the ability of many *Chlamydiales* to utilize amoeba as hosts, simplifying their isolation from contaminating microorganisms that are not equipped to survive amoebal predation (Thomas et al. 2006, 2008, Corsaro et al. 2009). The success of this isolation method has led to the proposal that many environmental members of the *Chlamydiales* may use amoeba as intermediate hosts (Greub & Raoult 2004), and could possibly pass from infected amoeba occupying the gills and epidermis directly to the fish host. Unfortunately, an alternative to isolation and identification of potential vertebrate *Chlamydiales* pathogens via the indirect amoebal route is to target pathogens of fish directly. For this reason, we and others (Meijer et al. 2000, 2006, Draghi et al. 2004, 2007, Nowak & LaPatra 2006, Karlsen et al. 2008) are attempting to molecularly identify and categorize the agents of epitheliocystis. Initial investigations have identified the novel bacteria relative to their hosts, as evident in their names '*Candidatus* Piscichlamydia salmonis' (Draghi et al. 2004) and '*Candidatus* Clavochlamydia salmonica' (Karlsen et al. 2008), raising the question of host specificity. However, it was observed that *Chlamydiales* discovered in epitheliocystis of Arctic charr from Virginia (USA) (Draghi et al. 2007) were partly identical to *Chlamydiales* identified in conjunctivitis infections of cats in Zurich (von Bomhard et al. 2003); this raised the very real possibility that these *Chlamydiales* are zoonotic pathogens that are widely spread geographically and are able to cause disease over a broad spectrum of vertebrate hosts.

We now extend these observations to provide the first molecular evidence for *Chlamydiales* infection of a nonteleost fish. This result should help stimulate others to investigate the molecular basis of epitheliocystis infections and encourage efforts to isolate these organisms directly from their fish hosts, the success of which is still proving to be elusive. This would be an important step as only when we have successfully isolated and cultivated *Chlamydiales* bacteria from fish with epitheliocystis will we be able to establish experimental fish models to test whether the bacteria can directly infect their hosts or whether an intermediate host, e.g. an amoeba, is necessary.

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