Molecular characterization and pathogenicity of Vibrio atlanticus and Vibrio rotiferianus associated with mortality of Cownose Ray Rhinoptera bonasus

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Abstract

The vulnerability of cownose rays (Rhinoptera bonasus) to anthropogenic pressures, including overfishing and habitat degradation, underscores the urgent need for comprehensive conservation efforts to safeguard this species and maintain the ecological equilibrium of marine ecosystems. Still, the susceptibility of cownose rays to Vibrio bacteria underscores the importance of understanding this host-pathogen relationship. Six cownose rays, obtained post-mortem from a public aquarium, were transferred to the laboratory under controlled conditions for necropsy. Internal organ samples, including kidney, liver, spleen, and uterine abscess, were collected and cultured on tryptic soy agar with 1.5% marine salts, marine agar, and blood agar. For identification and characterization, multilocus sequence analysis (MLSA) was performed. PCR was conducted to target housekeeping genes atpA, gyrB, pyrH, and recA, and the products were sequenced. The gene fragments, ranging from 462 to 570 base pairs, were concatenated into a 2,022-bp sequence. The MLSA data and sequences from 60 closely related Vibrio species were used to construct Maximum Likelihood and Neighbor-Joining phylogenetic trees. Despite good overall body condition and no visible lesions on the dorsal aspect, internal examination unveiled significant abnormalities. Notable observations included extensive muscle and uterine hemorrhage, petechial hemorrhage on the heart, valvular intestine dilation, uterine pus accumulation, pyometra, and severe endometrial hemorrhage. In-depth bacteriological analysis resulted in dominant gram-negative, fermentative, motile, oxidase- and catalase-positive comma-like bacteria. Identification and characterization through PCR and sequence analysis revealed two distinct Vibrio isolates. MLSA analysis exhibited a close relationship between the concatenated tree and V. atlanticus and V. rotiferianus. The study identified a novel sequence type, ST 247, for the isolates, characterized by allelic differences in key gene regions compared to closely related strains. This study sheds light on the intricate association between cownose rays and Vibrio bacteria, uncovering the potential role of these bacteria in the observed pathological conditions.

Introduction

Rays are cartilaginous fish that fall under the group of elasmobranch. The species of elasmobranchs are one of the most popular fish that have been exhibited in public aquariums for centuries (American Elasmobranch Society, Census Data, 2008 http://elasmo.org/). Cownose Ray, Rhinoptera bonasus, is a large, coastal pelagic fish that moves in large schools along the United States East Coast and the Gulf of Mexico for the aim of migration. Cownose Rays, also known as Cowfish or Skeete, are considered “Vulnerable” by the International Union for Conservation of Nature’s assessment in June 2019, and that is due to their low productivity as it is the least productive elasmobranch (https://www.iucnredlist.org/species/60128/3088381#assessment-information). Cownose rays are commonly displayed in touch pool exhibits at accredited zoos and aquariums across the country, but there has been little research to explore how these practices might affect the health of the animals.

Current standards of care utilize physical examination, imaging, hematology, ultrasonography, and cytology. Physical examinations can reveal grossly evident abnormalities such as trauma and body condition scores,
imaging modalities, such as ultrasound, are used to evaluate individuals for structural abnormalities of the internal organs and nutritional and reproductive status. While studies in several elasmobranch species have determined internal abnormalities and an understanding of specific responses to disease, the microbiological approach is one of the best practices for understanding exact pathogenesis, and it enables successful treatment. Zoos and aquariums are places where deep interactions between animals and humans cause serious stress for fish kept in captivity.

*Vibrio* is a ubiquitous bacteria, mainly found in marine environments because of its halophilic characteristics, and they are capable of causing disease in various marine fish in stress conditions. Although crayfish had been suspected as a potential vehicle for many pathogenic agents, they were found as a reservoir of *Vibrio* to transmit the pathogen to animals and humans via foodborne. *Vibrio* sis was first isolated from brown sharks (*Carcharhinus plumbeus*) held in captivity and *V. harveyi* was identified as the causative agent. The pathogen was also experimentally infecting lemon sharks (*Negaprion brevirostris*) and spiny dogfish (*Squalus acanthias*). Infections have also been recorded in wild sand tiger sharks (*Carcharias taurus*), linked to meningitis or skin ulcers. Additionally, *V. alginolyticus* infection was reported in aquarium-maintained dark-spotted (*Himantura uarnak*) and Tahitian (*H. fai*) stingrays. While several *Vibrio* agents were reported as a causative agent of marine fish kept in captivity in aquaria, there is an important gap in the effect of uncommon *Vibrio* species, such as *V. atlanticus* and *V. rotiferianus*, which can be easily transferred via fish transport throughout countries.

We aimed that the determination of causative agents of Cownose rays transferred from the Atlantic region by detailed macroscopic, histologic, and bacteriological examination. The causative agents of infections were deeply characterized by Multi Locus Sequence Analyzing (MLSA) and Multi Locus Sequence Typing (MLST).

**Material and Methods**

**Fish and Bacteriology**

Sampling was carried out following the guidelines for diagnosing fish diseases and in consideration of international and national guidelines for animal welfare. Cownose rays, *Rhinoptera bonasus* (n=6) were obtained from a public aquarium after natural death and were transferred to our laboratory under cold-chain conditions for necropsy. Dissections were made following techniques. Samples from the internal organs, kidney, liver, spleen and abscess from the uterus of cownose rays were inoculated in tryptic soy agar with 1.5 marine salts, marine agar and blood agar, and were incubated in aerobic conditions at 22°C and 28°C for 48-72 h. Isolated colonies were biochemically characterized by oxidase, catalase, and glucose fermentation in aerobic and anaerobic conditions (O/F basal medium), and also with gram staining and motility test.

**Identification and Characterization by multilocus sequence analysis (MLSA) and sequence typing (MLST)**

The genomic DNA of the bacterial isolates was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The amount and purity of DNA in each sample were measured at wavelengths of 260 nm and 260/280 nm with a spectrophotometer (Multiskan Go, Thermo).

Following the previous MLSL database, the housekeeping genes used were *atp A* (ATP synthase α subunit), *gyr B* (DNA gyrase β subunit), *pyr H* (uridylate kinase) and *rec A* (recombinase A). The PCR was performed as previously described. All PCR products were sequenced by Macrogen (Republic of Korea). The DNA fragments used for the analysis were 570 bp for *gyr B*, 501 bp for *pyr H*, 462 for *rec A*, and 489 for *atp A*, and the concatenated sequences produced a 2,022-bp fragment. Sequences obtained for *atp A*, *gyr B*, *pyr H* and *rec A* gene regions of two *Vibrio* isolates were deposited in the PubMLST database (https://pubmlst.org/organisms/Vibrio-spp), allele numbers and sequence types (STs) were generated by pubmlst database.

Housekeeping genes used in MLSA analysis were submitted to the GenBank RefSeq Representative genomes database and 60 *Vibrio* species (n=58 from NCBI database) which have a high similarity to submitted
sequences were used to construct two different concatenated phylogenetic tree by Maximum Likelihood method and Neighbor-Joining methods. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-14412.70) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3135)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 30.86% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Neighbor-Joining method was also used to infer the evolutionary history. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

Both two analyses involved 60 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1011 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Results

Necropsy

External examination revealed a good body condition and no macroscopical lesions in the dorsal side of the head, trunk and tail. The color of ventral body parts of the cow nose rays were white to grey. Internal examination, mass hemorrhage in muscle and uterus and petechial hemorrhage on heart were observed. Dilated and hyperemic valvular intestine, pus in the uterus, pyometra, and severe hemorrhage in the endometrium were the major lesions in the necropsy findings.

Bacteriology

Strains of gram-negative, fermentative, motile, oxidase- and catalase-positive comma-like bacteria were purely and dominantly isolated from the kidney, liver, uterus, pus and valvular intestine of affected animals. The isolated bacteria were identified and characterized by PCR and sequence analysis resulted from 570 bp for gyrB, 501 bp for pyrH, 462 for recA, and 489 for atpA, and the concatenated sequences produced a 2,022-bp fragment.

Identification and Characterization of the isolates

Two different Vibrio isolates named gv9 and gv10, having different colony types, were characterized by ST539 and ST540, respectively. The isolates were assigned allele numbers 262 for gyrB, 82 for pyrH, 248 for recA and 65 for atpA and 264 for gyrB, 186 for pyrH, 253 for recA and 179 for atpA to the strains of gv9 and gv10. Sequences obtained for atpA, gyrB, pyrH and recA of two representative Vibrio isolates were deposited in the PubMLST Database (https://pubmlst.org/organisms/Vibrio-spp), allele numbers and sequence types (STs) were generated by PubMLST database. Moreover, the sequences of all strains were deposited in PubMLST (https://pubmlst.org/organisms/Vibrio-spp).

Isolated bacteria were found to be closely related (above 99%) with Vibrio hepatarius (DSM 19134) by each gene region in the GenBank RefSeq Representative genomes database. By MLSA analysis, the concatenated tree has resulted in a close relationship with V. atlanticus and V. rotiferianus but far from V. tasmaniensis, V. harveyi, V. owensii, V. campbellii and other low similar species shown in Figure 1 and 2.
Sequences of gyr B, ppy H, rec A, and atp A gene regions were submitted to the PubMLST database (https://pubmlst.org/organisms/Vibrio-spp) and allelic numbers of each gene were determined as 147, 111, 140 and 105, respectively (Table 1). Strains were uploaded to the PubMLST database with the names V2 and V3, and a novel sequence type, ST 247, was described for the study isolates. While our isolate had a close relationship with V. hepatarius DSM 19134, our isolates had allelic differences in nine positions at gyr B, four positions at rec A, and one at atp A genes.

Discussion

The Cownose Ray (Rhinoptera bonasus) is a species of eagle ray found in the Western Atlantic Ocean, particularly along the coasts of North and South America. They are known for their distinctive appearance, with a broad, kite-shaped body and a pointed snout resembling a cow’s nose, which gives them their name. As of the last knowledge update in September 2021, the Cownose Ray is listed as ”Near Threatened” on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. In recent years, a growing interest has been in conserving these rays and managing their populations to prevent declines. Regulations and conservation efforts are being implemented to ensure their protection and minimize their vulnerability to overfishing and habitat loss. In our study, we especially focused on the disease of cownose rays that emerged in a public aquarium, one of the biggest thematic aquariums worldwide, and imported a considerable number of cownose rays for monitoring. We first researched why the aquarium imports this species in huge numbers and then why the fish live shorter than the natural lifespan. Our findings showed that one of the more critical reasons for the short lifespan of Cownose Ray was that some imported fish were infected showing disease symptoms such as decreased movement and feed intake and red spots in the ventral body side. While other ones were asymptomatic, only severe bacteria accumulation was detected in the direct gram-staining from tissues. When observing asymptomatic fish in the aquarium, we noticed that fish died within 1-3 months, either red spotted in the ventral body side or no clinical lesion/symptom.

Vibrio infections in aquarium fish are a significant concern within the realm of aquatic health and disease management. Vibrio is a genus of bacteria that encompasses various species, some of which can cause infections in fish kept in both captive and natural aquatic environments. These infections can lead to considerable morbidity and mortality among fish populations. A significant number of Vibrio species are part of the normal microbiota in marine environments, but certain strains can become opportunistic pathogens under specific circumstances, such as when fish are stressed or their immune systems are compromised. Vibrio sis infection in ornamental fish species commonly reported due to V. anguillarum, V. harveyi, V. alginolyticus, V. parahaemolyticus, V. vulnificus and V. mimicus, but we identified V. atlanticus and V. rotiferianus from Cownose rays for the first time. The bacteria were identified by multilocus sequence analysis (MLSA) and characterized by multilocus sequence typing (MLST) methods in the present study. The phylogenetic tree was constructed by obtaining all sequences deposited in the PubMIST database, and strains were characterized as having a close relationship with validated strains in this study. The identification of uncommon species, namely V. atlanticus and V. rotiferianus, among ornamental fish populations has raised due to several implications: (1) due to the high diversity present within Vibrio populations and the fact that genetic interactions within the genus modify population expression at different levels; (2) the utilization of outdated bacterial identification methods within the aquarium fish industry; and (3) the lack of disease survey investigations concerning ornamental fisheries—a relatively marginalized sector in animal breeding.

The isolation of V. atlanticus and V. rotiferianus predominantly from diseased and asymptomatic fish without any other detected bacterial agent strongly supported that our suggestions, such as valid bacterial identification methods and disease surveillance studies, need to be conducted. V. atlanticus was initially described from Portus Magnus Artabrorum in Galicia, north-west Spain and V. rotiferianus from Artemia Reference Centre, University of Ghent, Belgium, in 2005 and 1999, respectively, where neighboring countries of North Atlantic Ocean. Considering that Cownose rays commonly live in the Atlantic Ocean. The isolation of V. atlanticus and V. rotiferianus supported our findings that fish transport could possibly transfer agents from Atlantic regions to our country.

Public aquariums often play a crucial role in protecting and conserving marine species like the Cownose
Ray. They serve as educational and research centers, raising awareness about the importance of marine ecosystems and the need for conservation efforts. Public aquariums provide opportunities for visitors to learn about Cownose Rays and their role in the ecosystem. Many public aquariums research Cownose Rays to better understand their biology, behavior, and migration patterns. Protecting and restoring key habitats for Cownose Rays, such as coastal areas and seagrass beds, can positively impact their populations. They can use their influence to support regulations against overfishing, habitat destruction, and other threats to Cownose Rays. In cases where injured or stranded Cownose Rays are rescued, some aquariums have the facilities and expertise to rehabilitate and release them back into the wild after recovering. For the mentioned positive actions of conservation of Cownose rays, our study clearly showed that public aquariums need a disease survey study for imported fish and the natural species captured by health professionals noted as diseased.

In conclusion, public aquariums contribute to the protection of Cownose Rays by combining education, research, advocacy, and practical conservation efforts. Through these combined efforts, they strive to ensure the long-term survival of these species and promote a healthier marine ecosystem. However, these positive conservation actions need effective observation in nature and aquariums, and practical, non-invasive disease survey programs should be implemented. Another crucial conclusion to be noted from this study is that live fish imported from captured in nature between aquariums causes transmission of important bacterial agents cross-continental. We suggest a quarantine program, effective disease determination and thus treatment measurement have to be applied.

References

Table 1. Allelic profiles and ST information of the study isolates

<table>
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<tr>
<th>ID</th>
<th>ST</th>
<th>gyrB</th>
<th>pyrH</th>
<th>recA</th>
<th>atpA</th>
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<tr>
<td>Gv9</td>
<td>539</td>
<td>262*</td>
<td>82</td>
<td>248*</td>
<td>65</td>
<td><em>Vibrio atlanticus</em></td>
</tr>
<tr>
<td>Gv10</td>
<td>540</td>
<td>264*</td>
<td>186*</td>
<td>253*</td>
<td>179*</td>
<td><em>Vibrio rotiferianus</em></td>
</tr>
</tbody>
</table>

*: The allele numbers were identified in this study