Unexpected occurrence of *Aeromonas* species in the cutaneous mucus of Barbour's seahorses (*Hippocampus barbouri*)

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Short Report

Keywords: *Aeromonas*, Illumina sequencing, *H. barbouri*, seahorse

Posted Date: February 2nd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1294475/v1>

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Abstract

Because the skin microbiota is valuable for elucidating health status and common disorders in the host, this study aimed to describe the bacterial community composition of the skin of Barbour's seahorse (*Hippocampus barbouri*) using high-throughput sequencing of 16S ribosomal RNA genes. Water and sediment samples from the surrounding environment were also analyzed for comparative purposes. The results revealed the presence of operational taxonomic units (OTUs) affiliated to the *Aeromonas* genus in the skin, whose abundance was slightly similar to the sediment samples. Given that some *Aeromonas* species are known to be important pathogens in humans and fish, these results may provide information to show the dependency of the skin microbial composition on the environment, as well as determine whether the presence of *Aeromonas* species has implications on seahorse health.

Introduction

Although seahorses (*Hippocampus*) play important roles in the ecosystem, they are constantly at risk because of trade for medicinal and ornamental purposes (IUCN 2019). Moreover, seahorses are not exempt from diseases, which could make the situation even more critical. In fact, some studies have reported skin diseases of diverse etiology in wild and farmed seahorses (Balcázar et al. 2010a, 2011; LePage et al. 2014). In a recent study carried out by our research team, we observed the presence of *Aeromonas* in the cutaneous mucus of healthy *Hippocampus barbouri* (Ortega et al. 2021). It should be noted that *Aeromonas* species are ubiquitous bacteria primarily recovered from aquatic ecosystems (Janda and Abbot 2010), as they are commonly found in freshwater bodies, estuaries (Chaix et al. 2017), and even seawater (Chenia and Duma 2017). Despite that some *Aeromonas* species are potential pathogens of fish (Vivekanandhan et al. 2005), there is limited information on the prevalence of these microorganisms in seahorses. Thanks to the implementation of cutting-edge sequencing technologies, a full picture of bacterial community composition can be obtained, thereby increasing our knowledge on the role of skin microbiota in health and disease. Here we used the Illumina sequencing technology to explore the bacterial community structure and composition within the skin ecosystem of Barbour's seahorses, with particular emphasis on members belonging to the *Aeromonadaceae* family.

Materials And Methods

Eleven healthy Barbour's seahorses and samples from the surrounding environment (water and sediment) were collected from coral reefs off the coast of Cantiasay Island, San Pedro, Surigao del Norte. Samples were immediately transported to the Oceanography Laboratory of the Premier Research Institute of Science and Mathematics, Mindanao State University-Iligan Institute of Technology for further analysis. Seahorses were gently washed with sterile seawater twice to remove debris without compromising the microbial community on the skin (Nurul et al. 2018). Skin mucus samples were then collected by scraping the seahorse's dorsal surface using a sterile swab (Balcazar et al. 2010b), which were placed on a 2 ml microcentrifuge tube and stored at -65°C until DNA extraction.

Genomic DNA was extracted from the skin microbial community of six female Barbour's seahorses (HBFS) and five male Barbour's seahorses (HBMS), as well as from water (WS) and sediment (SS) samples. Samples were pooled according to their origin and HiPurA™ DNA Purification Kit (HIMEDIA; Mumbai, India) was used for DNA extraction, according to the manufacturer's instructions. Universal primers targeting the V1-V3 regions of the 16S rRNA gene were used for PCR amplification, whose purified products were sent to Macrogen Inc. (Seoul, South Korea) for high-throughput sequencing on the Illumina MiSeq platform. Raw data were processed using the Quantitative Insights Into Microbial Ecology (QIIME 2) pipeline (Bolyen et al. 2019). Operational taxonomic units (OTUs) were defined at 99% sequence similarity of 16S rRNA genes. This value was used to define a core set of representative sequences, which were used for phylogenetic analyses. The weighted UniFrac test was applied to determine whether two or more communities have the same structure (Lozupone et al. 2007). A heatmap was also generated showing the relative abundance of OTUs assigned to the *Aeromonadaceae* family across the samples, which were classified using the EzBioCloud database (Yoon et al. 2017). Phylogenetic analyses were performed by using MEGA version 6.0 (Tamura et al. 2013). Distances (distance options according to the Kimura 2-parameter model) and clustering with the neighbor-joining method were determined by using bootstrap values for 1,000 replications.

Results And Discussion

After normalizing to avoid any bias due to the difference in the total number of sequences, bacterial community structure was analyzed using the weighted UniFrac test (sensitive to abundances of taxa), whose results demonstrated that the relative abundance of OTUs (defined at 99% similarity) was significantly different ($p < 0.001$) among groups.

Overall taxonomic characterization of the bacterial community was conducted at the family level (Figure 1A). Sequences affiliated with the *Shewanellaceae* family were dominant in the skin of female Barbour's seahorses and sediment samples (41.6 and 37.8%, respectively), whereas sequences affiliated with the *Bacillaceae* family were dominant in the skin of male Barbour's seahorses (59.1%). Moreover, water samples (WS) were dominated by sequences affiliated with *Flavobacteriaceae* and *Vibrionaceae* families (7.2 and 6.7%, respectively). Although sequences affiliated with the *Aeromonadaceae* family were found in all samples, its abundance was very low in water samples (0.4%). Specifically, sequences affiliated with the *Aeromonadaceae* family had relative abundances of 7.6 and 5.5% in the skin of female and male Barbour's seahorses, respectively. Interestingly, a relatively high abundance of sequences affiliated with the *Aeromonadaceae* family was found in sediment samples (21.4%). Because the sequences belonging to this family were abundant, a phylogenetic analysis was carried out to establish its taxonomic affiliation. A phylogenetic dendrogram of selected OTUs was then constructed using the neighbor-joining method (Fig. 1B). The phylogenetic analysis revealed that these OTUs grouped with known *Aeromonas* species. The closest described relative of OTU 6 and OTU 588 was *A. taiwanensis* LMG 24683, whereas OTU 91 was closely related to *A. tecta* CECT 7082, OTU 104 to *A. sanarellii* LMG 24682, OTU 462 to *A. bivalvium* CECT 7113, OTU 582 to *A. jandaiei* CECT 4228, and OTU 708 to *A. rivipollensis* LMG 26313. Although *Aeromonas* species share high levels of similarity based on 16S rRNA gene analysis (Carnahan and Joseph 2005), our analyses provide valuable information on the diversity of *Aeromonas* species, thereby validating the reliability of our findings. The above mentioned *Aeromonas* species have been also observed in fishes intended for human

consumption such as tilapia and salmonids (Figueras and Beaz-Hidalgo 2014), bivalve mollusks (Galbis et al. 2007), whereas other *Aeromonas* species have been reported in clinical and environmental samples (Carnahan et al. 1991; Demarta et al. 2008; Alperi et al. 2010; Marti et al. 2015).

The relative abundance of each OTUs classified as *Aeromonas* species in the skin of *H. barbouri* was compared to sediment and water samples through a heatmap (Fig. 2). The map showed that the abundance of *Aeromonas* species from the skin of *H. barbouri* was more related to the sediments compared to water. This is consistent with the overall OTU representations. *Aeromonas* species detected in the skin of *H. barbouri* samples were greatly influenced by the environment where they thrive. Based on Larsen *et al.* (2013), the structure and composition of the skin microbiota are likely to be impacted by several variables including abiotic factors linked to geographic locality and season, as well as biotic factors related to the nutrient potential or antimicrobial components of fish mucus. Given the interface influencing this skin microbiota, water and sediments were considered (Chiarello et al. 2015), and in this case, the sediments had a higher influence on skin microbiota. Our results were also supported by recent findings, where the skin-microbiome assemblage of marine organisms is strongly associated with the surrounding sediments (Kawamoto et al. 2021).

The genus *Aeromonas* is a member of the *Aeromonadaceae* family, which consists of Gram-negative bacteria commonly found in aquatic environments, some of which are capable of causing disease in humans, fish, and other aquatic animals (Abu-Elala et al. 2015; Bravo and Figueras 2020). As inhabitants of marine environments, fish and other seafood are the most common sources for isolating these microorganisms (Pessoa et al. 2019), supporting their occurrence in seahorses. Moreover, *Aeromonas* spp. are known to be opportunistic pathogens for fish, and under stressful conditions such as an increase in water temperature, poor water quality, excessive handling, etc., they can cause epidemic outbreaks (Beaz-Hidalgo et al. 2010). However, the severity of disease cases depends on the concentration of these microorganisms (Hu et al. 2012). Some changes of the skin microbiota based on its phylogenetic composition may affect its functions, thereby upsetting its homeostatic interactions with the host and eventually favoring disease development (Chiarello et al. 2015). Considering that *H. barbouri* specimens in this study were directly collected from the wild with no trace of skin disease, they can be considered apparently healthy. As a result, further research is needed to understand the differences in the composition of a healthy and diseased *H. barbouri*.

Conclusions

High-throughput sequencing technology revealed the presence of sequences affiliated to *Aeromonas* species in the skin of *Hippocampus barbouri*. Interestingly, *Aeromonas* species were also observed in the sediments, which seem to be the most probable source of these species. Although some *Aeromonas* species are known to be important pathogens, the presence of these species in this study may not have been affecting the health status of *Hippocampus barbouri* yet. However, further studies are required to explore the implications of these species on seahorse health.

Declarations

Acknowledgement

The authors would like to thank DOST-ASTHRDP, BFAR, MSU-IIT PRISM, LGU Surigao and locals for supporting this study.

Author contributions

RCMHO: Conceptualization, methodology, investigation, formal analysis, visualization, writing - (both original draft and review and editing), funding acquisition. SRMT: Conceptualization, methodology, supervision, funding acquisition. JGTM: Conceptualization, methodology, supervision. JLB: Methodology, investigation, formal analysis, visualization, supervision, writing - (review and editing).

Funding

This study is funded by DOST under DOST-ASTHRDP scholarship grant to RCMHO.

Data availability

All sequences obtained in this study are available and will be submitted to GenBank at the NCBI.

Consent to Participate

Not applicable.

Consent for Publication

All authors consent to the publication of this article.

Conflict of Interest

The authors declare no competing interests.

Ethical Statement

Legal requirements from the BFAR national office under a project “Seahorses and Pipefishes with pharmaceutical potentials in Mindanao” was acquired and granted and their guidelines for the care and use of animals have been followed.

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Figures

Figure 1

(A) Relative abundance of dominant bacterial families found in the skin samples from Barbour's seahorses, as well as in water and sediment samples from the surrounding environment. (B) Phylogenetic dendrogram of selected OTUs with the most closely related *Aeromonas* species, based on 16S rRNA gene sequences and constructed by the neighbor-joining method. Bootstrap percentages (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.002 estimated substitutions per site.

Figure 2

Heatmap of OTUs assigned to the *Aeromonadaceae* family, which were compared with their abundances in each sample. The color intensity (log2 scale) in each panel shows the percentage of each OTU.