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ARTICLE

Prevalence of Kudoa in Fish Fillets Caught in Para State

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ABSTRACT

Kudoa is a myxozoan that causes myoliquefaction in marine fishes. Most of species only affect fish, but a *K. septempunctata* outbreak was reported in 358 people. Although many species of *Kudoa* are known, none was described in *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus* or *Oxydoras niger* until now. Due to the economic cost of eliminating seafood presenting myxozoan lesions, this study aimed to describe lesions found at necropsy and histopathology, as well as to detect this myxozoan by molecular techniques. For this purpose, were sampled 85 fish of the following species: *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* from Colares and Vigia, Pará, Brazil. Necropsies were carried out to describe lesions and molecular techniques (PCR and sequencing) were applied for identification. Although muscle lesions were not observed at necropsy, histopathology revealed bacterial colonies, coagulative necrosis, dystrophic calcification, eosinophils, hemorrhage, parasitic pseudocysts, protozoan, and vacuolization. After sequencing, *K. shiomitsui* (GENBANK: LC128646) was identified as the most similar causative agent of fishes infection, but due to phylogenetic results and identity we suggest that the myxozoan found could be a new specie. Also, high parasitism of this myxozoan was observed in fishes sampled, i.e., 90 % in Colares and 100% in Vigia.

1. Introduction

The genus *Kudoa* is a myxozoan of Multivalvulida order and Kudoidae family. Until now 63 species of this protozoan were described in fish species. This parasite is well described in the musculature of seafood, causing myoliquefaction or cyst formation [1], which can depreciate the product, leading to economic losses.

Kudoa was found in many species of fish and countries, such as *Thrysites atun* and *Beryx splendens* in Australia and South Africa [2], *Oncorhynchus kisutch* in Northwest Pacific of North America [3], *Merluccius productus* and

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Oncorhynchus kisutch in Canadian Pacific [3-5], *Salmo salar* in Washington Pacific [4], *Crypsilurus* and *Engraulis japonicas* [4], *Zeus faber* in Mauritania [4], and *Scomber sombrus L*. in North Sea [1].

In Brazil, it is described in the following hosts: *Aequidens plagiozonatus* [5], *Steliffer minor* [6], *Chaetobranchopsis orbicularis* [7], *Mugil liza* [8], *Mugil platanus* [9], *Scomberomorus brasiliensis* [10], *Odontesthes bonariensis*, and *Micropogonias furnieri* [11].

Due to no report and impact evaluation in the fish fillets with *Kudoa* from Para state, this study aimed to describe lesions by necropsy and histopathology, as well as to detect and identify this myxozoan by molecular techniques.

2. Materials AND Methods

2.1 Sampling

Eight five fishes of the following species were randomly sampled through net fishing: *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* from Colares and Vigia, Pará, Brazil (Table 1).

Table 1. Number and species of fishes sampled in Colares and Vigia, Pará, Brazil

Specie	Colares	Vigia	Total
Brachyplatystoma filamentosum	10	12	**22**
Brachyplatystoma rousseauxii	10	10	**20**
Mugil curema	0	13	**13**
Oxydoras niger	10	10	**20**
Plagioscion squamosissimus	10	0	**10**
Total	**40**	**45**	**85**

2.2 Necropsy and Histopathology

After caught, fish necropsies were carried out according to Noga [12] to observe macroscopic lesions and sampling of the muscle fragments for histopathology due to the high occurrence of *Kudoa* in this tissue. For this purpose, a 1-cm³ of muscle portion was fixed in 10% neutral buffered formalin followed by processing using standard histological techniques and embedded in paraffin [12]. Hematoxylin and eosin was used for staining.

2.3 PCR and Sequencing

For PCR (Polymerase Chain Reaction), it was weighted 20 mg of muscle sampled during necropsy for each fish analyzed. Besides, it was added 500 µL of buffer (50 mM EDTA, 50 mM Tris, 150 mM NaCl, pH 8.0) into a tube with the muscle weighted. After this step, samples were frozen (-80ºC) and thawed (20ºC) for 4 hours. All this treatment of temperatures was performed 3 times. Then, the lysis was made with 50µL of proteinase K (10 mg mL) under 50°C for 4 hours. Finally, lysate was extracted with Wizard® SV Genomic DNA Purification System (PROMEGA®) kit, according to manufacturer´s recommendations.

The PCR was performed with the primers 18f (5' CAC-CAG-GTT-GAT-TCT-GCC 3') and 1492r (5' GGT-TAC-CTT-GTT-ACG-ACT-T 3') as described by Baker et al. [13]. Thereby, it was used Platinum®Taq DNA Polymerase (Invitrogen®) mix with the following concentrations: Buffer 1X, dNTP 0.2mM, $MgCl_2$ 1.5mM, primers (18f and 1492r) 0.2 µM each, and Platinum®Taq DNA Polymerase 1 unit. The reaction protocol was the following: denaturation under 95ºC for 5 minutes; followed by 40 cycles of 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute, and final extension of 72ºC for 5 minutes. The amplicon was visualized under ultraviolet light by eletrophoresis with agarose 1.5%.

The 1200 bp amplicons were purified with an Ilustra Microspin™ S-400 HR Columns Kit (GE Healthcare®) according to the manufacturer's instructions for identification by Sanger sequencing. For this, the purified amplicon was sequenced in both directions using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems capillary 3500 Genetic Analyzer. The quality of the electropherograms was assessed in Sequencing Analysis version 5.4 (Applied Biosystems). After this step, sequences were identified by similarity using BLAST (Basic Local Alignment Search Tool) algorithm.

A nucleotide sequence of approximately 1200 bp was used to query the GenBank library to arrive at the closest strain type and thus attain a species affiliation and possible identification to that level. To compare the sequences from different strains found in the GenBank library, the nucleotide sequences were aligned with ClustalW from MEGA software, version 7. For MEGA, the item "Find Best DNA/Protein Model" recommended the Maximum Likelihood method based on the General Time Reversible model with gamma distribution as model evolutionary rate differences among sites (2 categories, parameter = 1.3963) for our sequence as the most parameter-rich evolutionary model. We used codon positions included were 1st+2nd+3rd+Noncoding, 1935 positions in the final dataset, and bootstraps with 500 replicates.

The sequences used for phylogenetic tree were our sequence, *Kudoa amamiensis* (genbank: AY152748), *K. anatolia* (genbank: MH310914), *K. barracudai* (genbank: KU212177), *K. hexapunctata* (genbak: LC316999), *K. iwatai* (genbank: AB693041), *K. lateolabracis* (gen-

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bank: AY382606), *K. neothunni* (genbank: LC317001), *K. niluferi* (genbank: MH310915), *K. ogawai* (genbank: KX163082), *K. ovivora* (genbank: AY152750), *K. puraishii* (genbank: KF413764), *K. rayformis* (genbank: KR140014), *K. shiomitsui* (genbank: LC128646), *K. septempunctata* (genbank: AB693040), *Kudoa* sp. (genbank: AY302723), *K. thalassomi* (genbank: LC382036), *K. thyrsites* (genbank: AY382607), and *Pseudopolystoma dendriticum* (genbank: FM992707).

2.4 Statistical Analysis

The prevalence of *K. shiomitsui* detected by PCR and evaluation of risk factors were calculated for each place of sampling, also for sex and host specie. The comparison between factors cited was performed with Fisher´s exact test with confidence interval of 95%. This statistical analysis was performed and visualized in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results

During necropsy, it was observed lesions in the liver, kidney, spleen, and stomach, such as hemorrhage, increase in the size of the organ, and necrosis. Despite no muscle injury was observed, bacterium colonies, coagulative necrosis, dystrophic calcification, eosinophils, hemorrhage, parasites pseudocysts, protozoan and vacuolization were confirmed by histopathology (Figure 1). The most common alterations were coagulative necrosis, hemorrhage, and protozoan presence (Figure 2).

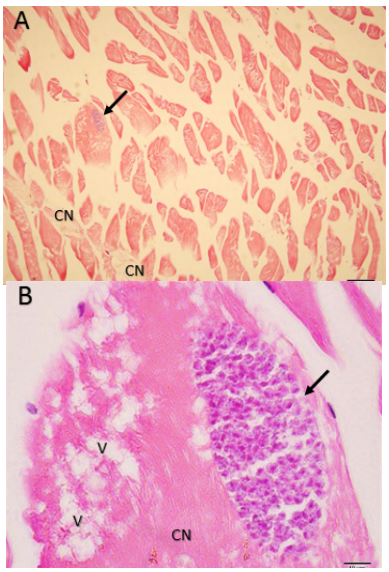

Figure 1. Muscle histopathology. A: Observation of coagulative necrosis (CN) and parasite cyst (arrow). B: Visualization of coagulative necrosis (CN), parasite cyst (arrow), and vacuolization (V). HE staining

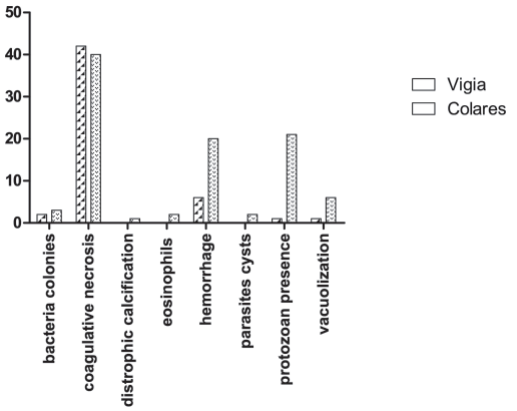

Figure 2. Prevalence of microscopic lesions in the muscle of fish analyzed in Vigia and Colares municipality

The PCR amplified a product of 1200 bp, which presented 86% of identity with *Kudoa shiomitsui* (GENBANK: LC128646), confirming that the myxozoan observed in histopathology. Analyzing phylogeny results (Figure 3), we observed that our sequence grouped with some species of *Kudoa*, such as *K. shiomitsui*, *K. ogawai*, *K. rayformis*, *K. thyrsites*. As showed Figure 3, our sequence formed another clade, which suggests that the myxozoan detected in our study could be a new species.

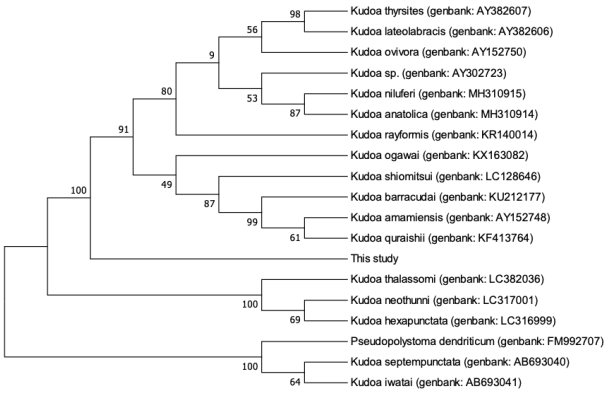

Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method based on the General Time Reversible model with gamma distribution as model evolutionary rate differences among sites (2 categories, parameter = 1.3963). The tree with the highest log likelihood (-14660.88) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap with 500 replicates

The species analyzed in this study presented highly parasitism, since 70% to 100% (Table 2). Thereby, *Branchyplastoma* filamentosum, *B. rousseauxi*, and *Oxydoras niger* presented 100% of parasitism, *Mugil curema* with 92,30% and *Plagioscion squamosissimus* with 70%.

Afterwards, it was also observed that 97.77% of fishes sampled in Vigia and 92.50% in Colares (Table 2) were positive for this myxozoan, which was statistically significant ($p < 0.05$) by Fisher´s exact test. On the other hand, when gender was evaluated as a factor for this disease, no statistically significant differences were observed ($p = 0.4368$), the opposite for host species ($p < 0.05$), i.e., only place and host species were identified as risk factor for this parasitosis.

Table 2. Prevalence of *Kudoa shiomitsui* in fishes sampled in Colares and Vigia, Pará, Brazil

Specie	Colares	Vigia	Total
Brachyplatystoma filamentosum	10 (100%)	12 (100%)	**22**
Brachyplatystoma rousseauxii	10 (100%)	10 (100%)	**20**
Mugil curema	*	12 (92,30%)	**13**
Oxydoras niger	10 (100%)	10 (100%)	**20**
Plagioscion squamosissimus	7 (70%)	*	**10**
Total	**37 (92,50%)**	**44 (97,77%)**	**85 (100%)**

Note: * Fish species that were not sampled.

4. Discussion

Although *Kudoa* species cause myoliquefaction in fish muscle, Tsuyuki et al. [14] reported that proteolytic activity seems to be linked to the stage of parasitic infection, pH value, as well as to the level of infection [15]. This information explains why the present study found lesions only at microscopy.

During infection, the parasite destroys the sarcolemma, which lead to the development of a fibroblast layer around it and development of pseudocyst with black appearance. Although, in some species of fish there is no formation of black pseudocyst and in the macroscopy the muscle is intact [16], such as *Scomberomorus braziliensis* [10], which also could be the case of the fishes sampled in this study.

In Brazil, there were reported *Kudoa* in *Lutjanu analis, L. jocu, Bagre marinus, Aspitor luniscutis, Scomberomorus braziliensis* [10], and others species, but the fish species studied in this research has no report of myxozoan. According to Eiras et al. [11] investigation of *Kudoa* in fish along the Brazilian coast is very scarce, which supports the importance of this study due to economic loss that this protozoan can cause in seafood.

Although we observed that only locality is a risk factor for the parasitism, St-Hilaire et al. [6] and Levsen et al. [4] also described that age and size of fishes could lead to higher parasitism, being the larger (>600 g) and sexually mature more susceptible. This is an important point since the fishes caught normally are adults, increasing the risk of parasitism.

The diagnosis of *Kudoa* in fish fillets that not present myoliquefaction is difficult in sanitary inspection by observation of the muscle. For this reason, this study showed that histopathology and PCR help and increase detection of this myxozoan. Shaw et al. [17] also reported that even when visualization and histopathology are negative for this parasite, PCR detected *Kudoa*. Due to the sensitivity, specificity, cost, and time spent, molecular techniques can be used for surveillance and diagnosis of this protozoan in fish fillets.

Although the main problem of *Kudoa* parasitism is associated to myoliquefaction in fish fillets, the species *K. septempunctata* were responsible of a 358 people outbreak, after consumption of *Paralichthys olivaceus* [16], showing the zoonotic potential of this group of parasites.

Other studies also show samples with detection of *Kudoa shiomitsui*, such as Kasai et al. [18], which reported this specie of myxozoan in fish samples collected in different markets in Japan from the Inland sea and the west, the east China sea and the Pacific Ocean in the period of July 2013 to December 2015. In the study, gills and viscera were collected and examined, then the presence of myxozoan of the genus *Kudoa* was observed through a dissection microscope confirmed by PCR. From the 75 analyzed samples of monocantid fish, 8 presented four different species of *Kudoa*, being in two fishes *K. septempuctata*, in other three *K. thyrsites* and *K. shiomitsui*, located in the pericardium with the presence of more than 80 cysts.

In another study, two sampling boxes containing 25 fish with three different species of *Merluccius*. They found parasites in nodules and cysts that were observed through a dissection microscope and DNA sequenced. As a result 89% presented the presence of different species of *Kudoa* [19].

5. Conclusions

With this study we conclude that *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii*, *Mugil curema*, *Plagioscion squamosissimus*, and *Oxydoras niger* were highly parasite by *Kudoa*. It was also observed that municipality and host species could increase the risk of this infection. With this, is crucial surveillance of this parasite in those fishes to decrease the economic loss and guarantee food safety.

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Conflict of Interest

For this research, there are no conflict of interest.

Availability of Data and Material

All samples and DNA are deposited in Biotechnology Institute of São Paulo State University.

Code Availability

Not applicable.

Author's Contribution

Marianna Vaz Rodrigues: the author contributed in sampling, as also performing necropsy, histopathology, molecular techniques and write of manuscript.

Patrícia Tidori Miura: the author contributed in molecular techniques and write of manuscript.

Jéssica Fernandes de Oliveira: the author contributed in molecular techniques and write of manuscript.

Maria das Dores Correia Palha: the author contributed in sampling, necropsy and write of manuscript.

João Pessoa Araújo Júnior: the author contributed in molecular techniques and write of manuscript.

Ethics Approval

Due to the study used fish caught and marketed in Colares and Vigia, there were no need for ethics approval.

Consent to Participate

All authors agree to participate of this study.

Consent for Publication

All authors agree in publication this study.

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