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Short communication

DNA detection of *Gyrodactylus* spp. in skin mucus of Nile tilapia *Oreochromis niloticus*

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ABSTRACT

Monogeneans *Gyrodactylus* von Nordmann 1832, cause outbreaks of gyrodactylosis in aquaculture settings worldwide. Detection of *Gyrodactylus* spp. is based on the morphological identification of isolated parasites after fish necropsy. Contributing to the diagnosis of gyrodactylosis, in this study, a non-destructive PCR assay was standardized; the PCR was first performed using genomic DNA of *Gyrodactylus* spp. isolated from the surface of the Nile tilapia *Oreochromis niloticus* (Linnaeus 1758), and subsequently tested with mucus samples of infected and uninfected Nile tilapia fish. The primers (Ekgyro1) were designed from the ribosomal Internal Transcriber Spacer (ITS) RNA region (ITS1, 5.8S and ITS2 rRNA gene) of *Gyrodactylus cichlidarum* Paperna 1968. The positive control group included the DNA of 30 monogeneans *Gyrodactylus* spp. The heterologous control group included 75 monogeneans *Cichlidogyrus* Paperna 1960, 75 protozoans *Ichthyophthirius multifiliis* Fouquet 1876 and 75 *Trichodina* Ehrenberg 1830. PCR products of each parasite and from the external mucus samples (described as P and M respectively), were sequenced. The average DNA concentration of the ectoparasites was of 13.5 ng/µl. The PCR test had an analytical sensitivity of 0.0039 ng µl-1 of DNA of *Gyrodactylus* spp. No cross-reactions were observed with the heterologous group. The sensitivity and specificity of the PCR test were of 100% either with genomic DNA or with DNA from mucus samples. Six DNA consensus sequences with sizes ranging from 568 bp to 571 bp were obtained and the BLAST analysis matched with DNA sequences of *G. cichlidarum*.

1. Introduction

Ectoparasites monogeneans *Gyrodactylus* von Nordmann 1832 genus, cause gyrodactylosis and serious health problems in tilapia farms worldwide (García-Vásquez et al., 2007). They are located in fins, the surface of the skin, eyes and occasionally gills of tilapia (Fajer-Ávila et al., 2017). *Gyrodactylus* species have a direct life cycle that is characterized by a polyembryonic reproduction and rapid generation time (< 24 h) resulting in exponential population growth on a single host that is favored by an increase of temperature, high concentrations of organic matter, fish crowding and seasonality (Pérez-Jvostov et al., 2015). *Gyrodactylus* feed on mucus and epithelial cells and move freely on the host by alternatively attaching to the host's epidermis with their posterior opisthaptor and anterior attachment glands. In response, the host secrete excessive mucus as a mechanism of defense.

Necropsy of fish and subsequent isolation and identification of monogeneans are used for routine diagnosis of gyrodactylids, but low or transient parasitic infections can be underestimated (Cone et al., 2013). In the Pacific side of Mexico *Gyrodactilus cichlidarum* was responsible of a massive mass mortality in Nile tilapia farms, but before this epizootic event, the parasite was imperceptible in these facilities (Grano-Maldonado et al., 2018). In this sense, detection of DNA of a given pathogen can be used in conjunction with microscopy in aquaculture, and would help to improve routine monitoring to overcome outbreaks (Priyanka et al., 2016).

In this regard, the internal transcribed spacer (ITS) that is part of the rDNA (ribosomal) array, lies between the SSU (Small Sub Unit) rDNA and the LSU (Long Sub Unit) rDNA coding regions. It is divided into ITS1 and ITS2 and are separated by the 5.8S rDNA gene. ITS sequences are specially used as complementary of morphological characterization of *Gyrodactylus* species (Zahradníčková et al., 2016).

The aims of this study were first, to standardize a PCR with genomic

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DNA of *Gyrodactylus* spp. and second, to develop a non-destructive molecular test for detection of DNA of *Gyrodactylus* spp. in external mucus swabs of the Nile tilapia *O. niloticus*.

2. Material and methods

2.1. Sample collection

One hundred fingerlings *Oreochromis niloticus* (Total length = 134 ± 13 mm) naturally infected with *Gyrodactylus* spp. were collected from the facilities of the Technologic Institute in Boca del Rio, Veracruz, Mexico in May 2016. Thirty *O. niloticus* were euthanized by a puncture in the head with a sharp scalpel and placed individually on Petri dishes. *Gyrodactylus* spp. were collected at 10X and identified up to genus level based on morphological features of the copulatory organs and haptoral sclerites at 40 X and 100 X (Vanhove et al., 2011). The positive control group included 75 *Gyrodactylus* spp. (described as P) and the heterologous group wa conformed of 75 monogeneans *Cichlidogyrus* Paperna 1960, 75 protozoans *Ichthyophthirius multifiliis* Fouquet 1876 and 75 *Trichodina* Ehrenberg 1830. Each organism was stored individually in vials with 70% ethanol for DNA extraction.

The mucus samples (described herein as Mucus = M) were collected from 45 *O. niloticus* infected with *Gyrodactylus* spp., Each fish was placed on a clean surface and was restrained firmly to rotate carefully a sterile dry cotton swab on the left and right external surface, including fins and gills, avoiding the damage of tissues and covering the total surface of the fish. Swabs were placed individually in a sterile 1.5 ml Eppendorf tube with 70% ethanol (Ek-Huchim et al., 2012). After that, fish was euthanized as mentioned above to determine the parasitic infection intensity. Also, 45 uninfected *O. niloticus* (Total length = 9 ± 0.9 mm) were euthanized and placed individually in Eppendorf tube of 1.5 ml with 70% ethanol for the mucus negative control. DNA extraction was done in the laboratory of Immunology and Molecular Biology at Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN) Mérida, Yucatán, Mexico.

2.2. DNA extraction

The Eppendorf tubes were heated at 65 °C for 1 h for ethanol evaporation. The genomic DNA was extracted at 55 °C for 2 h with 10 µl of Proteinase K (20 µg ml-1) (Sigma) and 150 µl of 10% Chelex (Sigma) (Ek-Huchim et al., 2012). Tubes with the cotton swabs with mucus were centrifuged at 10,000 *g* five mins, whereas the tubes with uninfected fishes were vortexed, fishes removed, and tubes were centrifuged at 10,000 *g* for five mins. Around 15 µl of fish mucus were used for DNA extraction using the Wizard® Genomic DNA purification kit (Promega®) according to the manufacturer's protocol. The DNA concentration of each sample was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific) to verify if samples had an absorbance relation of ≥1.8–260/280 nm for the subsequent PCR analyses (Shokere et al., 2009; Ek-Huchim et al., 2012).

2.3. PCR assays

The primers Ekgyro1 F: 5´- GTGGACTGGTTGCCTTCTG -3´ & Ekgyro1 R: 5´- ACACACCCTGTGTTGAGCTG -3´ were designed using the software primer3 (v.0.40) (Untergasser et al., 2012). They were chosen from the ITS region of *Gyrodactylus cichlidarum* sequence with accession number (AN) DQ124228.1, that include ITS1, 5.8S and ITS2 rRNA gene (García-Vásquez et al., 2007). During the PCR standardization, the set of primers Ekgyro1 were titrated in 15 serial dilutions ranging from 100 pM to 1 pM to obtain the optimal working concentration. The genomic DNA was also titrated in 15 serial dilutions (from 64 to 0.0039 ng µl-1) to determine the analytical sensitivity. Subsequently, PCR reactions were carried out in a total volume of 20 µl containing 1 µl of DNA template (˜10 ng of DNA from a single parasite), 10 pM of each primer, 1.5 mM of $MgCl_2$, 10× reaction buffer (50 mM KCl, 10 mM Tris−HCl, 0.1% Triton X-100, pH 9.0), 0.3 mM of the dNTPs mixture (Promega®) and 3.0 U of Taq DNA polymerase (BioLabs®). The PCR reactions were performed on a thermal cycler TECHNE TC-312 and the conditions were 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2.5 min, with a final extension of 72 °C for 10 min. Electrophoresis of PCR products of 610 bp and 100 bp DNA ladder was run on 2% agarose gel stained with 0.3% Ethidium Bromide. Bands were visualized and documented on a UV documentation system (MiniBis Pro®). The positive control was DNA from 10 *Gyrodactylus* spp. and negative control was and ultra-pure water. The diagnostic sensitivity and specificity of the PCR test obtained with the panels of DNA from the positive samples, negative samples and the heterologous group were evaluated by X^2 (2 × 2) (Parikh et al., 2008).

2.4. Sequencing data

The positive control (PC), 8 PCR products from each *Gyrodactylus* spp. (P) and 10 PCR products from the external mucus of tilapia infected with *Gyrodactylus* spp. (M) were sequenced in both directions by Sanger sequencing methods. The consensus sequences were aligned using ClustalX (2.0.12) (Thompson et al., 1997) and BLAST searches were performed to find homologous sequences in the GenBank™ datasets (http://www.ncbi.nlm.nih.gov/blast/Blast).

3. Results and discussion

Gyrodactylus is a hyper diverse monogenean genus with more than 400 described species from freshwater and marine teleost fish (Bakke et al., 2007; Cone et al., 2013). In hatchery facilities, fingerlings of tilapia *Oreochromis niloticus* are easily prone to gyrodactylosis characterized by high mortalities within few days of infection (Bakke et al., 2007; Grano-Maldonado et al., 2018). The rapid and accurate detection of *Gyrodactylus* spp. during early stages of a given outbreak is very important to further address parasitic management and treatment (Ye et al., 2017).

Detection of monogeneans genus-*Gyrodactylus* is based on identification of major taxonomic features such as the attachment sclerites (Ye et al., 2017). However, when the intensity of infection and prevalence are low, the detection of *Gyrodactylus* specimens can be under estimated, not to mention that microscopic examinations of skin scrapes can damage fish (Cone et al., 2013). Therefore, molecular diagnosis would help with preliminary detection of DNA of a given pathogen (Davey et al., 2011).

The DNA concentration of each *Gyrodactylus* was of

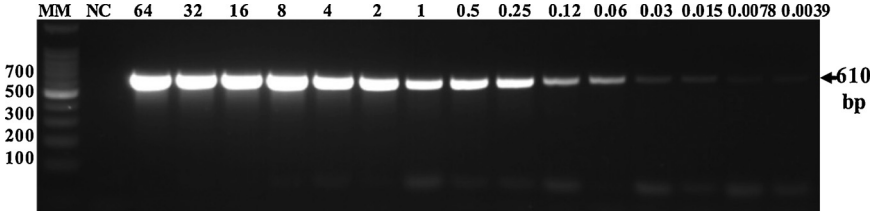

Fig. 1. Titration of DNA of *Gyrodactylus* spp.: MM = Molecular Marker, NC = Negative control and Line 64 - 0.0039 = DNA Titration ng µl-1.

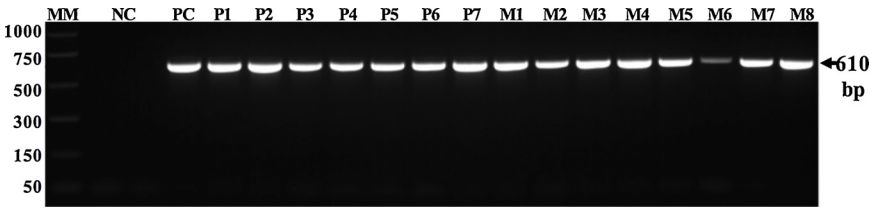

Fig. 2. PCR results of isolated parasites *Gyrodactylus* spp. (P) and fish mucus (M): MM = Molecular Marker, NC = Negative control, PC = Positive control, Lines P1 - P7 = DNA of each *Gyrodactylus* and Lines M1 - M8 = DNA from Mucus of *O. niloticus*.

27.87 ± 10.67 ng µl-1. The optimal working primer dilution was of 10 pM with an analytical sensitivity of 0.0039 ng µl-1 of DNA (Fig. 1). The DNA samples from each *Gyrodactylus* spp., amplifyed a fragment of 610 bp in the PCR test. No cross-reaction was observed with DNA from *Cichlidogyrus* spp., *Trichodina* spp. and *Ichthyophthirius multifiliis*. The diagnostic sensitivity (75 / 75) and specificity (0 / 225) of the PCR test was of 100% respectively (X^2 = 300; P = 0.0000). The sensitivity, specificity and reproducibility of the assay described herein can be attributed to the efficiency of the primers as well as to the good quality of the DNA (Santoro et al., 2013).

The mean infection intensity was of 4.03 (range 1–14) gyrodactylids per fish and the DNA concentration from the mucus of each fish was of 239.07 ± 108.72 ng µl-1. The samples from fish mucus infected with *Gyrodactylus* spp. (n = 45) also amplified at 610 bp (Fig. 2), whereas the uninfected fish (n = 45) tested negative, giving 100% of sensitivity and specificity respectively (X^2 = 90; P = 0.0000). No mortality was recorded during fish handling for mucus collection. In this sense, low parasitic burden, as one parasite, may have no little effect on the host, but initial appearances are misleading. Gyrodactylids are the only parasitic worms that reproduce *in situ* on their host (Bakke et al., 2007). Thus, a single *Gyrodactylus* can produce exponential population growth in a short period of time on suitable environmental factors (Clerc et al., 2015). The efficiency of the PCR test described here was achieved with DNA from the parasite and from the tilapia fish mucus. However, it is necessary to evaluate the PCR test with the mucus of tilapia infected with the other ectoparasites to support our preliminary findings. In special because tilapia fish are co-introduced with their pathogens to different countries with a high risk of parasite dissemination (Martins et al., 2010). In this study we did not use anaesthesia because in previous trials (data not shown), monogeneans fall out from fish skin after benzocaine treatment, and we did not want to underestimate the infection intensity of parasites. Instead, we wanted to address the detection limit of DNA from one parasite and from fish mucus as a starting point that can be used subsequently for DNA isolation and dentification without killing fish.

A total of six DNA consensus sequences with sizes ranging from 568 bp to 571 bp were obtained and submitted to the GenBank™; the DNA sequence AN MK860823 was formed by 14 similar sequences (the positive control (PC) of *Gyrodactylus*, M1, M2, M3, M8, M9, M10, P16, P17, P18, P21, P25, P27 and P29). Another DNA sequence AN MK860824 had one nucleotide variation. The DNA sequence AN MK860828 showed six nucleotide variations, AN MK860827 showed 14 nucleotide variations, AN MK860826 four nucleotide variations and AN MK860825 six nucleotide variations were observed (Supplementary Table 1). These DNA consensus sequences were homologous to 44 DNA sequences of *Gyrodactylus* from the GenBank™ database, with coverage at least 98% and a similitude range of 90% to 100%. From them, 31 were of *G. cichlidarum* whereas the other belonged to *G. ulinganisus*, *G. malalai*, *G. parisellei*, *G. occupatus*, *G. chitandiri*, *G. nyanzae*, *G. sturmbaueri* and *G. ergensi* (Supplementary Table 2). The highest similitude was observed with DNA sequences of *G. cichlidarum* that is highly pathogenic and prevalent in cultured tilapia from the world including Mexico (García-Vásquez et al., 2007; Grano-Maldonado et al., 2018). *G. cichlidarum* has also been detected in native fauna and in different order of fishes (i.e Cyprinodontiformers and Perciformes), including endangered native fish poecilids from Mexico, like *Poeciliopsis gracilis*, *Poecilia mexicana* and *Pseudoxiphophorus bimaculatus* (syn. = *Heterandria bimaculata*) (García-Vásquez et al., 2017). This PCR test can be used in several areas were gyrodactylids are a menace, because several species of *Gyrodactylus* can co-occur in aquaculture facilities and produce gyrodactylosis (Coleman and Tsongalis, 2017).

In conclusion, this paper reports the implementation of non-invasive molecular techniques either for diagnosis and/or surveillance of *Gyrodactylus* spp. in aquaculture settings and in wild organisms that can help to overcome parasitic infections without necropsy of fish.

Declaration of Competing Interest

All the authors address that there is not conflict of interest.

Ethical approval

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Center for Research and Advanced Studies (Centro de Investigacion y de Estudios Avanzados del IPN) and comply with the applicable Mexican Official Norm (NOM-062-ZOO-1999), “Technical Specifications for the Care and Use of Laboratory Animals”, as well as all applicable federal and institutional regulation.

CRediT authorship contribution statement

Juan Pablo Ek-Huchim: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Isabel Jiménez-García:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing - original draft. **Rossanna Rodríguez-Canul:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing - original draft.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.07.004.

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