Aquaculture 454 (2016) 237–242

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Nonlethal sampling methods for diagnosis of *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus* (L.)

Guilherme Campos Tavares, Frederico Augusto de Alcântara Costa, Raquel Ribeiro Dias Santos, Gustavo Morais Barony, Carlos Augusto Gomes Leal, Henrique César Pereira Figueiredo *

AQUACEN — National Reference Laboratory of Aquatic Animal Diseases, Ministry of Fisheries and Aquaculture, Veterinary School, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

ARTICLE INFO

Article history:
Received 9 September 2015
Received in revised form 18 December 2015
Accepted 22 December 2015
Available online 23 December 2015

Keywords:
Kidney aspiration
Venipuncture
Nonlethal sampling
Oreochromis niloticus
Streptococcus agalactiae

ABSTRACT

Streptococcus agalactiae is a major etiologic agent of septicemia and meningoencephalitis outbreaks in Nile tilapia (*Oreochromis niloticus* L.) farms and the presence of carrier fish can be a significant source of infection. Nonlethal methods to obtain samples for microbiological and molecular diagnostic tests are an option for detecting carrier fish. The aim of this study was to standardize nonlethal sampling methods for the diagnosis of *S. agalactiae* infection in Nile tilapia using microbiological and molecular assays. For this, 140 Nile tilapia juveniles were used in three different experimental trials. In the first trial, nonlethal sampling methods – kidney aspiration, venipuncture, nasal wash, gill mucus swabs, and fecal sample collection – were evaluated. The second trial was performed to determine the routes of *S. agalactiae* shedding, and the third trial was conducted to evaluate the efficiency of using nonlethal samples for the diagnosis of *S. agalactiae* infection in experimentally infected fish. The nonlethal sampling methods used to collect samples from Nile tilapia were successfully standardized. There was no mortality and no signs of infection observed in fish subjected to the sampling methods tested (kidney aspiration, venipuncture, nasal wash, gill mucus swabs, and fecal sample collection). *S. agalactiae* was successfully reisolated from all samples with the exception of nasal washes. The diagnostic methods used were able to detect the presence of *S. agalactiae* from carrier and diseased Nile tilapia. Our results demonstrate that nonlethal sampling methods, including kidney aspiration and venipuncture, can be used for the detection of *S. agalactiae* by bacteriology, PCR, and qPCR. Kidney aspiration and venipuncture in combination with qPCR were highly sensitive for the detection of *S. agalactiae* (93.04%).

Statement of relevance: The use of nonlethal sampling is an alternative method for the detection of *S. agalactiae* infection in diseased fish and carriers, allowing the diagnosis of this disease in broodstocks, or useful for surveillance programs and health monitoring in hatcheries without having to sacrifice fish. This diagnosis is crucial to avoid the maintenance of the pathogen in the culture system and the transmission of the disease to other farms.

1. Introduction

Streptococcus agalactiae has emerged as a major etiologic agent of septicemia and meningoencephalitis outbreaks in fish farms around the world (Jiménez et al., 2011; Mian et al., 2009). Currently, the diagnosis of *S. agalactiae* infection in fish is based on lethal sampling of brain and kidney tissues of diseased fish for bacteriology or PCR assays (Evans et al., 2002; Itsaro et al., 2012; Mata et al., 2004). Lethal sampling can be impractical for health monitoring and surveillance, particularly in hatcheries where a large number of broodstock fish with high economic value may need to be sacrificed (Cutrín et al., 2005). In addition, fish can be carriers of *S. agalactiae* without showing signs of disease (Evans et al., 2002) and the clinical sensitivity of existing lethal methods for detecting the presence of the pathogen in carrier fish is unknown at present. The presence of carrier fish can be a significant source of infection in the epidemiological dynamics of streptococcosis. Thus, detection of the carrier state, mainly among convalescing fish after outbreaks, is crucial to avoid the maintenance of the pathogen in the culture system and the transmission of the disease to other farms (Austin and Austin, 2007).

Nonlethal methods to obtain samples for microbiological and molecular diagnostic tests are an option for detecting carrier fish and have been used previously to diagnose viral infections (Cornwell et al., 2013; Drennan et al., 2007; Gahlawat et al., 2004; Monaghan et al., 2015), parasitic infections (Ek-Huchim et al., 2012), and some bacterial diseases (Altinok et al., 2001; Cipriano et al., 1996; Noga et al., 1988; White et al., 1996). Venipuncture, biopsy, and mucus swab are the most common nonlethal sampling methods used to diagnose infectious diseases in fish (Altinok et al., 2001; Cornwell et al., 2013; Monaghan et al., 2015; Noga et al., 1988). Currently, however, there are no

* Corresponding author.
E-mail address: figueiredoh@yahoo.com (H.C.P. Figueiredo).

http://dx.doi.org/10.1016/j.aquaculture.2015.12.028
0044-8486/

standardized nonlethal sampling methods for the diagnosis of *S. agalactiae* infection in Nile tilapia (*Oreochromis niloticus* L.).

The aim of this study was to standardize nonlethal sampling methods for the diagnosis of *S. agalactiae* infection in Nile tilapia using microbiological, PCR, and qPCR assays.

2. Material and methods

The study was performed using three experimental trials. In the first trial, nonlethal sampling methods—kidney aspiration, venipuncture, nasal wash, gill mucus swabs, and fecal sample collection—were evaluated. After assessing the feasibility and safety of the sampling techniques, trial 2 was performed to determine the routes of *S. agalactiae* shedding, and trial 3 was conducted to evaluate the efficiency of using nonlethal samples for the diagnosis of *S. agalactiae* infection in experimentally infected fish.

2.1. Fish

A total of 140 juvenile Nile tilapia with an average weight of 120 g were obtained from commercial fish farms. They were acclimated in several 57-L aquaria supplied with flow-through dechlorinated tap water (0.5 L h^{-1}) at 28 °C, under a 12:12 h light/dark photoperiod, and were fed to apparent satiation with VITAFISH 32% PB (Matsuda, São Paulo, Brazil) twice per day. Prior to the experimental trials, five fish were randomly collected and submitted to microbiological and *S. agalactiae*-specific PCR assays (Mata et al., 2004) to demonstrate freedom from bacterial infections. All of the in vivo protocols were approved by the Ethics Committee for Animal Use of the Federal University of Minas Gerais (CEUA-UFMG – 399/2013).

2.2. Nonlethal sampling methods (trial 1)

In trial 1, two experimental groups (test group, n = 30; control group, n = 10) were used to evaluate the following sampling methods: kidney aspiration, venipuncture of the caudal vein, nasal wash, gill mucus swab, and fecal collection. Fish were anesthetized by immersion in 100 mg L^{-1} benzocaine (Sigma-Aldrich, Saint Louis, USA). Fish in the control group were anesthetized, but samples were not taken using nonlethal methods. All nonlethal sampling methods were applied to all fish in the test group. Fish in the test group were first submitted to kidney aspiration using a needle biopsy. The operculum was opened cranially and a 1-mL syringe with a 22-gauge needle was inserted ventrodorsally and then introduced craniocaudally at an approximate angle of 45° into the cranial region of the kidney. Approximately 0.2 mL of kidney tissue was collected. The presence of kidney tissue in each aspirate was confirmed microscopically by smearing a subsample on a glass slide and staining the tissue using a May Grünwald Giemsa stain fast kit (Laborclin, Pinhais, Brazil). Blood samples (0.5 mL) were taken aseptically from the caudal vein with a syringe and 22-gauge needle and dispersed into a tube without anticoagulant. Nasal wash samples were obtained by administering and recovering 0.1 mL of sterile saline solution in each nostril with an automatic 20–200 μL pipette (HTL, Warsaw, Poland). The wash solution was administered and recovered three times in each nostril then collected and stored in a sterile 0.5 mL microtube. Fecal samples were obtained by squeezing the ventral abdomen in the caudal direction. These samples were stored in sterile 0.5 mL microtubes. Additionally, gill mucus was collected by swabbing following the method described by Ek-Huchim et al. (2012). Fish in both the test and control groups were monitored three times a day for 7 days to evaluate the occurrence of clinical and behavioral alterations caused by sampling.

2.3. Pathogen shedding routes (trial 2)

The fish were experimentally challenged with *S. agalactiae* SA 20-06 to identify possible shedding routes of the pathogen. This strain was previously isolated from diseased Nile tilapia and identified by phenotypic and molecular methods (Mian et al., 2009). The LD_{50} of this strain has been previously determined ($6.14 \times 10^{1.17}$ CFU), and its complete genome sequence is available (Godoy et al., 2013; Mian et al., 2009; Pereira et al., 2013). A variant of SA 20-06 was selected that had reduced virulence following serial passages in culture (LD_{50} of 10^6 CFU) and was able to induce a carrier state in Nile tilapia (unpublished data). Two experimental groups (challenge group, n = 10; control group, n = 10) were used in the infection assays. The experimental infection was performed following Mian et al. (2009). The *S. agalactiae*-challenged group were anesthetized and injected intraperitoneally with 0.1 mL of the bacterial suspension (10^6 CFU $fish^{-1}$). The control group were inoculated with 0.1 mL of sterile BHI broth (Brain Heart Infusion, Himedia, Mumbai, India). At 2, 4, 7, 10, and 15 day post-infection (dpi), two fish were randomly collected from each experimental group, and kidney aspirate, blood, nasal wash, gill mucus, and fecal samples were taken from each fish. After sampling, fish were euthanized with an overdose of benzocaine (250 mg L^{-1}) in order to take brain and kidney samples. The aseptic samples (brain, kidney, kidney aspirate, and blood) were streaked onto 5% sheep blood agar and incubated at 28 °C for 72 h. A volume of 0.01 mL of each sample considered to be contaminated (nasal wash, gill mucus, and feces) was inoculated into Granada biphasic broth (BioMerieux, Madrid, Spain) immediately after collection and incubated at 28 C for 48 h as an enrichment step to select *S. agalactiae* from other microorganisms present in these samples. Following culture, 0.1 mL of the broth was streaked onto 5% sheep blood agar and incubated at 28 °C for 72 h. The isolates were submitted to Gram stain, catalase, and oxidase tests. *S. agalactiae* isolates were identified serologically using Slidex Latex Agglutination (BioMerieux, Marcy l'Etoile, France) and by *S. agalactiae*-specific PCR (Mata et al., 2004). The most efficient nonlethal samples for bacteriological detection of *S. agalactiae* were chosen for use in trial 3.

2.4. Efficiency of nonlethal sampling methods for diagnosis of S. agalactiae (trial 3)

The fish were experimentally challenged with *S. agalactiae* SA 20-06 to evaluate the effectiveness of each sampling method for the detection of the pathogen. Two experimental groups (challenge group, n = 45; control group, n = 30) were used in the infection assays. The experimental infection was performed as described for trial 2. The fish were monitored three times a day for 15 days to evaluate clinical signs of disease and mortality. Upon observation of severe clinical signs of disease (moribund fish with complete loss of equilibrium and no response to stimuli), kidney aspiration and blood sampling were conducted. Moribund fish were then euthanized with an overdose of benzocaine and subjected to bacteriological examination and tissue sampling for PCR and qPCR. At the end of the experiment, all surviving fish were subjected to nonlethal sampling methods. Immediately after taking nonlethal samples, these fish were euthanized and subjected to the lethal sampling methods.

2.5. Diagnostic methods

For bacterial isolation, samples of brain and kidney tissue (lethal sampling method) were aseptically collected. These samples, in addition to kidney aspirate and blood samples, were streaked onto 5% sheep blood agar and incubated at 28 °C for 48 h. The isolates were characterized biochemically and serologically following the methods described in trial 2.

For PCR and qPCR assays, the bacterial DNA from the tissues sampled using lethal and nonlethal methods was extracted using a Maxwell 16 Tissue DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) and stored at −20 °C until use.