



Full length article

Validation of IgY for the diagnosis of *Streptococcus agalactiae*-caused endocarditis and bacterial meningitis in Nile tilapia (*Oreochromis niloticus*)



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ABSTRACT

Streptococcus agalactiae (*Sta*), which belongs to Lancefield group B, causes sepsis, endocarditis and bacterial meningitis in human neonates and Nile tilapia. Because the pathophysiology of *Sta* infection is partially similar in both species, the identification of biomarkers for the diagnosis and study of this disease is of importance for human and animal health. Therefore, in the present study, we produced an immunoglobulin Y (IgY) by immunizing laying hens with *Sta* proteins and evaluated its ability to detect *Sta* in paraffinized tilapia brain and cardiac tissue by direct immunofluorescence (IMF) and indirect immunohistochemistry (IHC). The IgY produced was effective in the diagnosis of *Sta* infection in Nile tilapia, justifying the use of this species as a biomodel for the study of this disease.

1. Introduction

Streptococcus agalactiae (*Sta*) is a Gram-positive bacterium that is cosmopolitan and commensal and has the ability to infect a wide variety of species, including fish, reptiles, amphibians, birds, mammals and humans [1]. To date, it has been classified into nine serotypes: Ia, Ib and II–IX [2]. Serotypes Ia, Ib and III and serotypes Ia, III and IV are associated with infection in Nile tilapia and humans, respectively [3]. In humans, it has etiological prevalence in neonatal bacterial meningitis and adult endocarditis and pneumonia [4].

In the Nile tilapia, *Sta* colonizes cardiac tissue, injuring cardiac fibers and central nervous tissue, and causes suppurative meningitis, resulting in severe sequelae in the host [5]. The migration of *Sta* from systemic circulation to the central nervous system (CNS) is related to the virulence factors expressed by the pathogen, such as the polysaccharide capsule, pili, serine-rich (SR) proteins, β -hemolysin, cytolysin, fibronectin and teichoic acid [6]. All these factors contribute to the intracellular survival of *Sta* and its use of phagocytes to cross the blood-brain barrier (BBB) and migrate to the CNS, which is referred to as a “Trojan horse” from a Greek literary epic [7].

In addition to the similarity between tilapia and human *Sta* infection

mechanisms, an important serotype III-4 variant isolated from aquatic organisms was associated with emergent infections in humans in Asia, indicating the zoonotic potential of this bacterium and its impact in the health sector [8]. These findings highlight the importance of this pathogen in veterinary and human medicine, as well as its direct impact on the global economy, through losses in the aquaculture sector.

The efficacy of immunoglobulin Y (IgY), extracted from the egg yolk of laying hens, in the diagnosis and treatment of microbial infections in aquatic organisms has been demonstrated for *Vibrio anguillarum* in *Cynoglossus semilaevis* [9], *Vibrio alginolyticus* in *Haliotis* sp. [10], and for the treatment of shrimp White Spot Syndrome Virus [11]. Therefore, the present study produced an anti-*Sta* IgY and validated its application for the diagnosis of endocarditis and bacterial meningitis in tilapia, indicating that this species could be a model for this disease.

2. Materials and methods

2.1. Bacteria and separation of bacterial proteins

Sta was isolated from naturally infected fish and identified by PCR following the method described by Berridge et al. [12] and El-Razika

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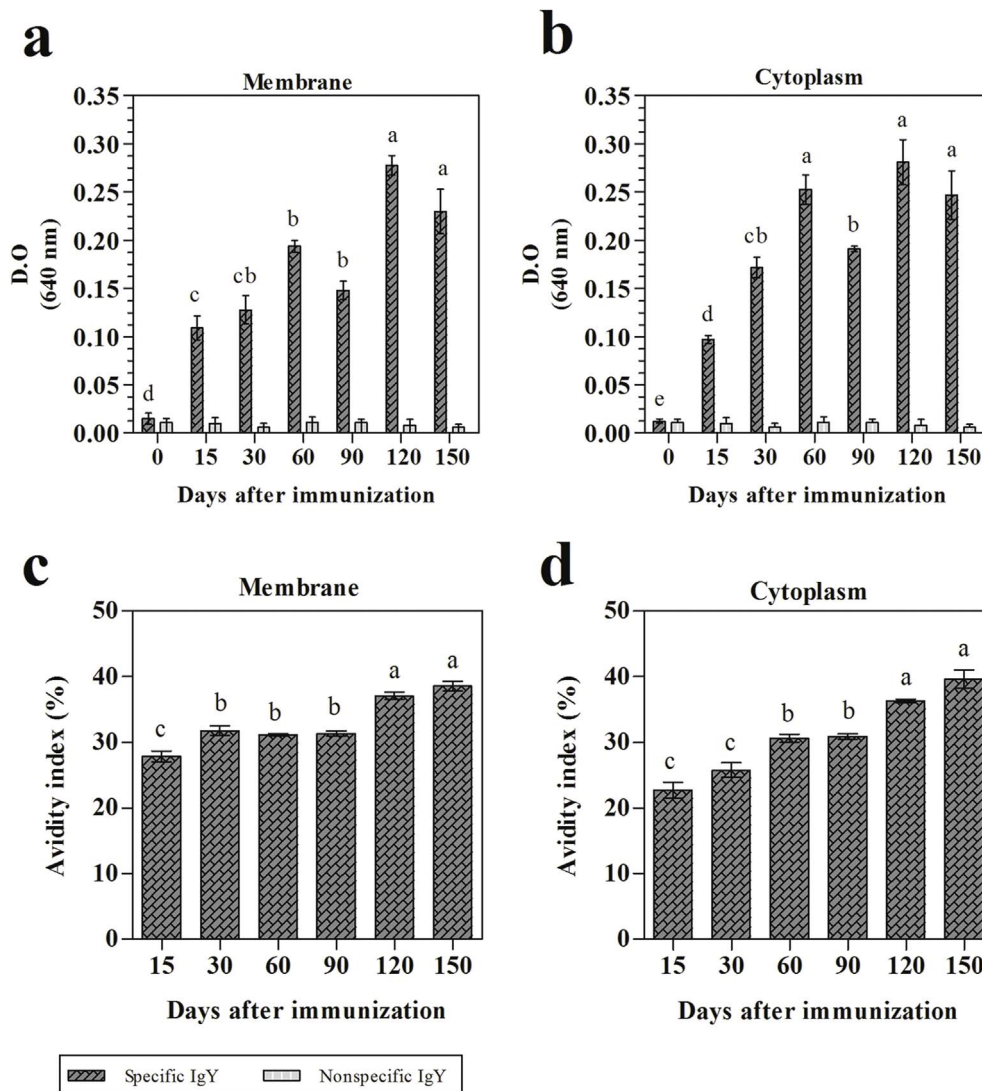


Fig. 1. Kinetics of IgY production in the serum of immunized laying hens with membrane proteins (MPs) and cytoplasmic proteins (CPs) from *Streptococcus agalactiae* (*Sta*). (a–b): Systemic production of IgY for CPs and MPs. (c–d): IgY avidity index for MPs and CPs in serum (1:100). Averages and their respective standard deviations for experiments performed in triplicate are presented, and different letters indicate significant differences (Tukey's test, $p < 0.05$) between the observed times.

et al. [13]. The protein separation method followed the protocol described by Sayuri et al. [14]. *Sta* was cultured in BHI broth (Difco, Detroit, MI, USA) at 28 °C with shaking for 48 h. The culture medium was removed by centrifugation at 10,000 g for 10 min at 4 °C, and the cell pellet was washed four times with 0.15 M PBS, pH 7.2. Subsequently, the pellet was resuspended in 25 mM Tris-HCl, pH 7.4, and the cells were lysed in a Sonifier 250 ultrasonic sonicator (Branson Ultrasonics, Danbury, CT, USA). The sample was centrifuged at 2000 g at 4 °C for 10 min to precipitate intact cells. The supernatant was then filtered through a 0.22- μ m filter and centrifuged at 100,000 g for 2 h at 4 °C, and the supernatant containing cytoplasmic proteins (CPs) was collected. The precipitated membrane proteins (MPs) were resuspended in 2 mL of 25 mM Tris-HCl, pH 7.4, and the samples were stored at –70 °C.

2.2. Hens immunization

Fourteen 16-week-old hens from the *Isa Brown* lineage were randomly assigned to two groups ($n = 7$): control (non-specific IgY) and experimental (specific IgY). Hens were maintained in individual cages with 12 h of light and 12 h of darkness, an ambient temperature between 26 °C and 29 °C, a relative humidity of 63%–75%, drinking water

ad libitum and commercial stall feed (100 g/bird/day). The experimental group received three doses of the total extract containing the two protein fractions from *Sta* MPs and CPs at a final concentration of 200 μ g/mL emulsified with incomplete Freund's adjuvant (Sigma-Aldrich Corp., St. Louis, MO, USA) at 30-day intervals, injected at four distinct points on the pectoral muscle. The control group was injected with 0.15 M PBS, pH 7.2. Serum was collected prior to immunization and at 15, 30, 60, 90, 120 and 150 days after the initiation of the protocol.

2.3. Enzyme linked immunosorbent assay (ELISA)

2.3.1. Monitoring systemic production of specific IgY

The ELISA method followed the protocol described by Tong et al. [15]. A flat-bottom 96-well plate (Costar; Sigma-Aldrich Corp., St. Louis, MO, USA) was sensitized with a 100 μ l/well volume of 10 μ g/mL MPs and CPs and incubated overnight in a wet chamber at 4 °C. It was then washed with PBS-Tween and blocked with 250 μ l of PBS containing 5% Molico skim milk (Nestlé Brazil, São Paulo, Brazil) for 1 h at room temperature. Subsequently, the serum was diluted (1:1000 v/v) in 1% PBS-milk, distributed, incubated for 1 h, and washed with 0.05% PBS-Tween. Additionally, 100 μ l/well of anti-IgY peroxidase (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted 1:1000 in PBS was

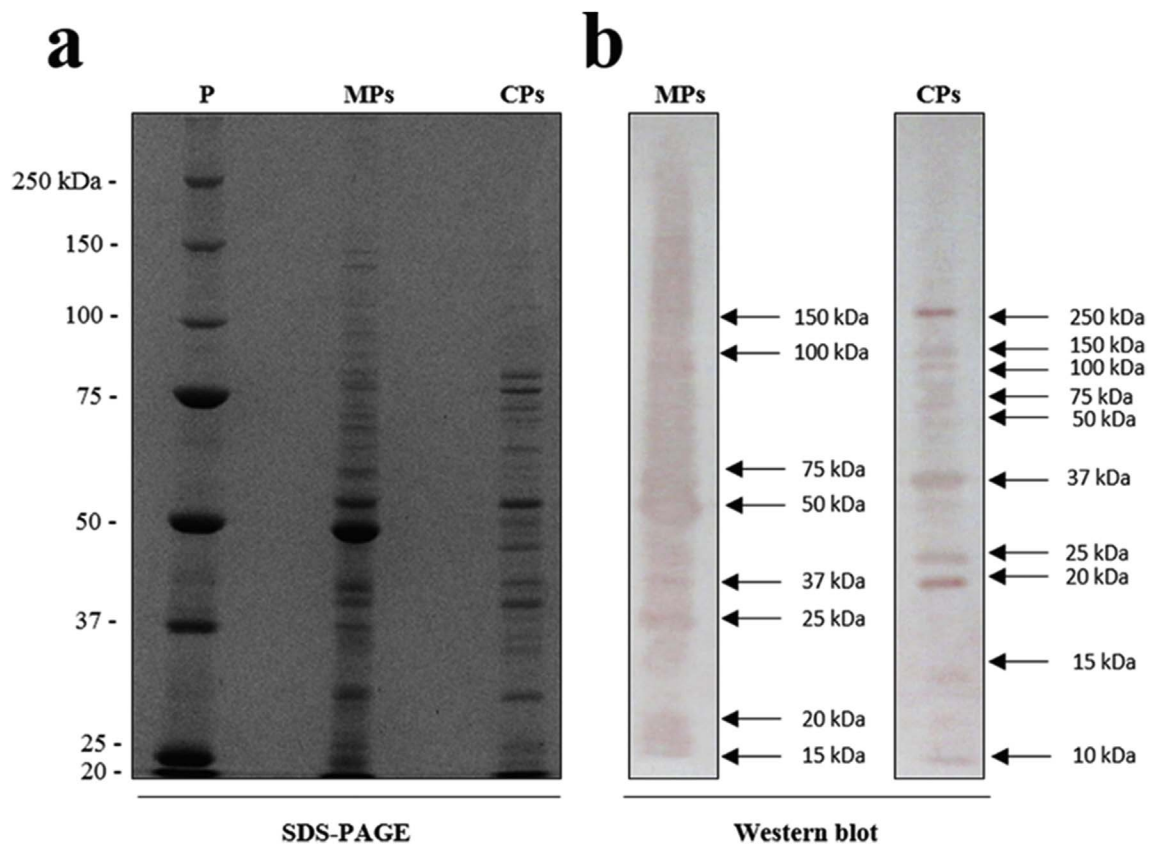


Fig. 2. Multi-specific potential of anti- *Streptococcus agalactiae* (Sta) IgY. (a) Electrophoretic profile on standard (P) SDS-PAGE gel, membrane proteins (MPs) and cytoplasmic proteins (CPs) of Sta. (b) Western blot using the serum of laying hens immunized with total proteins of Sta (1:100).

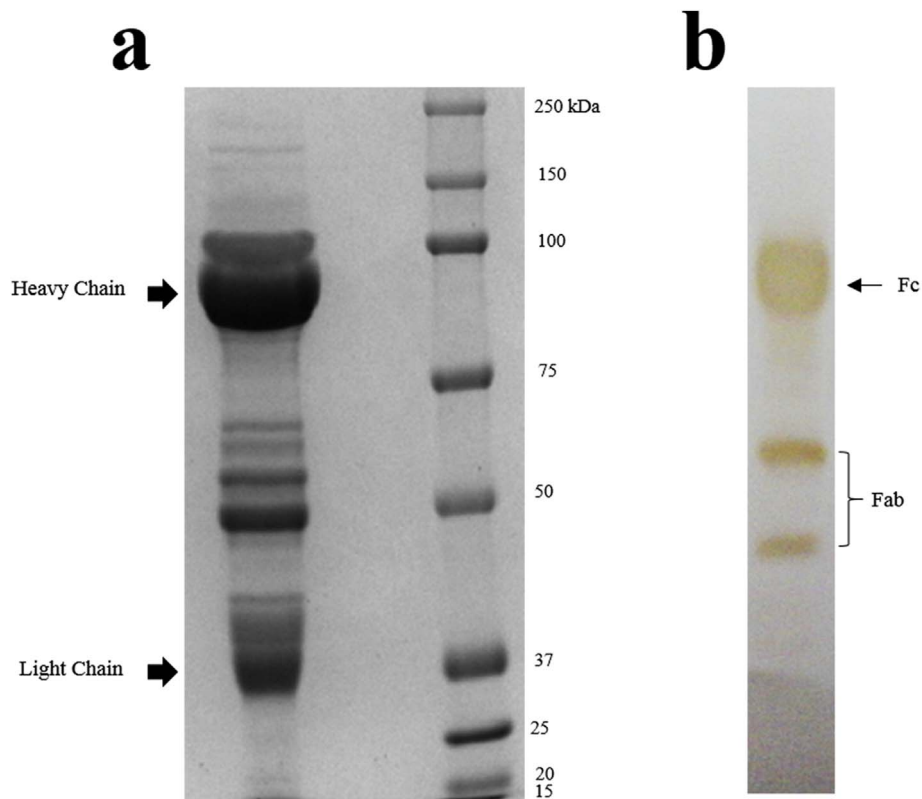


Fig. 3. Characterization of IgY in the purified extract. (a) SDS-PAGE, IgY precipitated with pectin at the concentration of 40 µg/mL; arrows indicate the molecular weight of 70–75 kDa (IgY heavy chain) and 20–25 kDa (IgY light chain). (b) Western blot-positive immunoblotting for IgY fractions. PM: molecular standard.

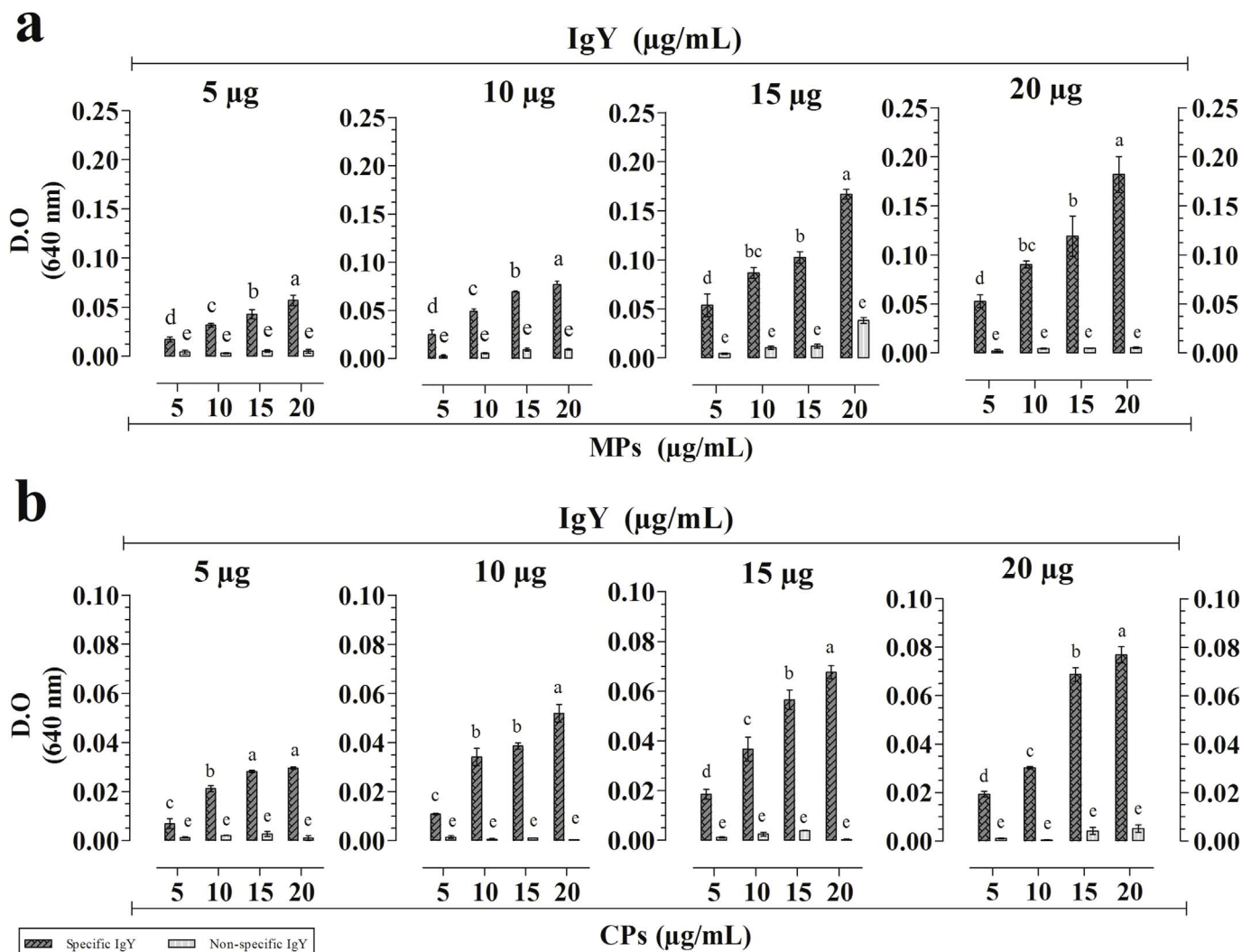


Fig. 4. Sensitivity of IgY detection for *Streptococcus agalactiae* (*Sta*) proteins *in vitro*. One hundred and fifty day purified extract, specific IgY and non-specific IgY. (a) Membrane and (b) cytoplasmic proteins. Different concentrations of IgY (upper axis) and homologous concentrations of *Sta* proteins (lower axis). Averages and their standard deviations of experiments performed in triplicate are presented. Different letters indicate significant differences by Tukey's test ($p < 0.05$).

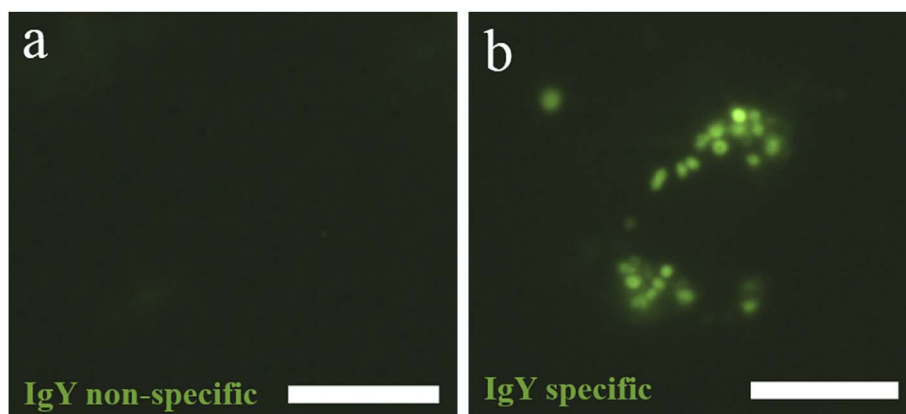


Fig. 5. Detection of *Streptococcus agalactiae* (*Sta*) cells with anti-*Sta* IgY conjugated to fluorescein isothiocyanate (FITC); (a) non-specific IgY and (b) specific IgY. Bar: 10 µm.

added, and the mixture was incubated for 1 h. It was then washed, and the substrate (H_2O_2 /ABST) was added; the reaction was quenched with a 5% SDS solution. The absorbance was determined at 640 nm on a microplate reader (Molecular Devices SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

2.3.2. IgY affinity

After incubation of the serum as described in the previous section, the wells were washed with PBS-Tween. A 1 M urea solution was added to a final volume of 100 µL/well (in triplicate). Saline solution (0.9% NaCl) was used for the control samples, and the reaction was incubated for 30 min at room temperature. The wells were washed and incubated

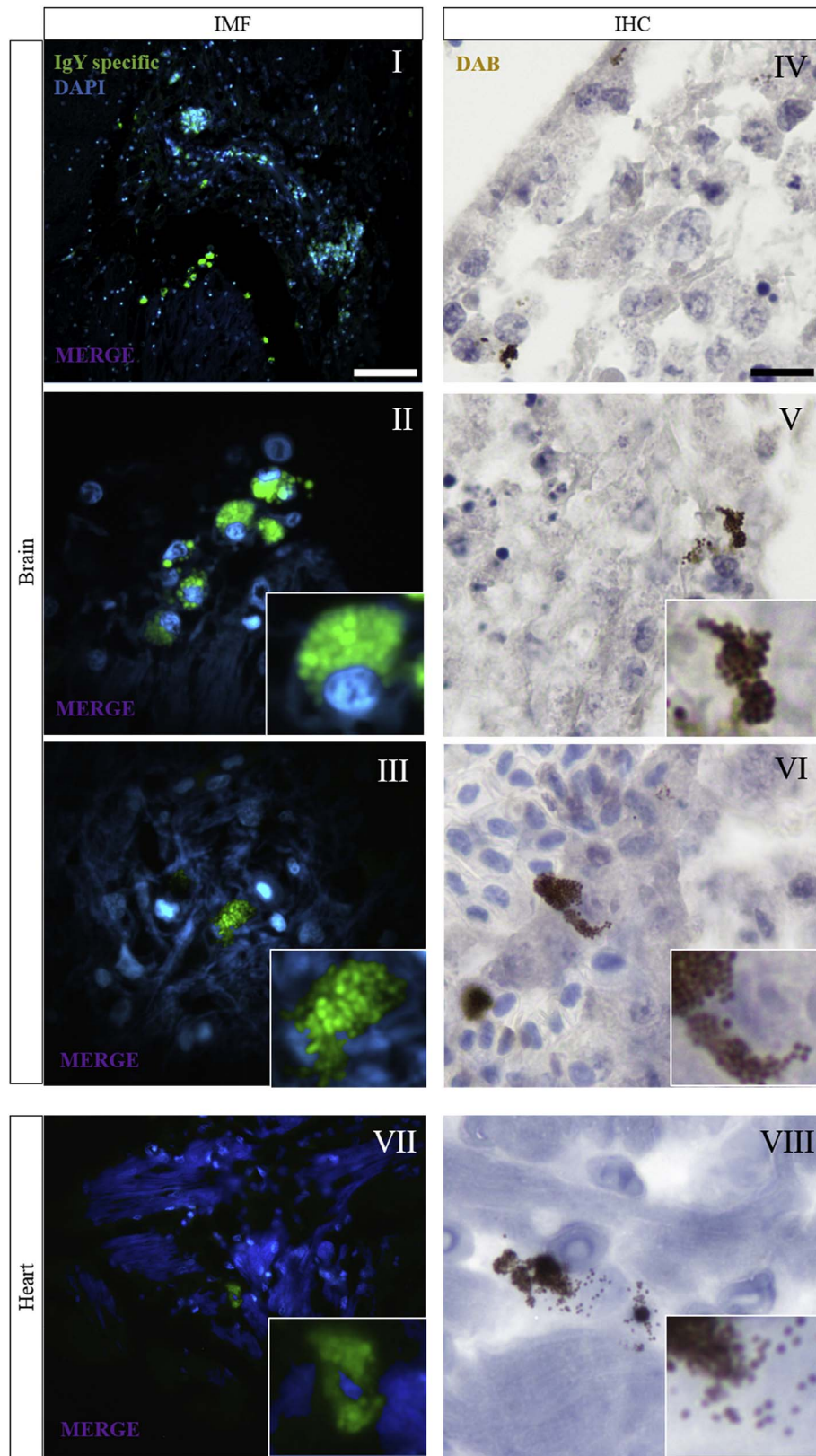


Fig. 6. Potential of anti-*Sta* IgY for immunolabeling of *Streptococcus agalactiae* (*Sta*) bacterial cells using immunofluorescence (IMF) and immunohistochemistry (IHC). Positive in cardiac and cerebral tissue of *Oreochromis niloticus* (I, IV–V) (large increase); (II–V), intracellular visualization of bacterial cells with coccus morphology internalized in phagocytes in the meninges and endocardium and (III–VI, VII–VIII) cocci in the free phase. Bar: 10 μ m; IHC stain, Harris hematoxylin.

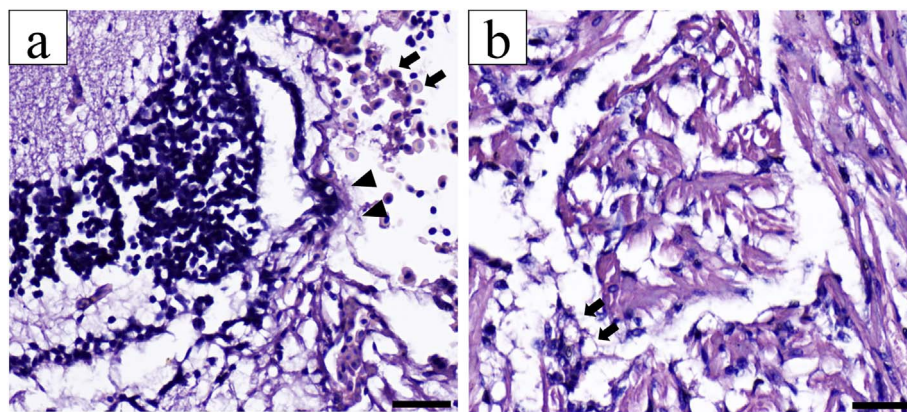


Fig. 7. Histopathological changes in cardiac and cerebral tissue of *Oreochromis niloticus* infected with *Streptococcus agalactiae* (*Sta*). (a) Brain tissue, arrows pointing to suppurative lesions in the meningeal and (b) cardiac tissue with hemorrhagic lesions and leukocyte infiltrates. Bar: 10 µm; IHC stain, Harris hematoxylin.

for 1 h with anti-IgY peroxidase (HRP); the absorbance was measured at 640 nm. The values were transformed into an affinity index.

2.4. IgY purification

The IgY purification method followed the protocol described by Tong et al. [15]. The yolk was separated, precipitated with pectin (Sigma-Aldrich Corp., St. Louis, MO, USA), dialyzed and stored at -20°C until use.

2.5. SDS-PAGE

Dissociative electrophoresis was performed according to Laemmli [16], and 7.9 µg of the bacterial fractions and 80 µg of purified IgY extract were loaded onto a 9.0% SDS-polyacrylamide gel in TA buffer at 100 V. The gels were fixed and stained with Coomassie brilliant blue R 0.25% (Sigma-Aldrich, St. Louis, MO, USA) and scanned and analyzed with Image Lab software (Bio-Rad, Hercules, CA, USA).

2.6. Western blotting

The proteins present in the above gel were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) under constant voltage (90 V) at 4°C for 1 h. The membrane was then incubated with PBS-5% skim milk at room temperature for 1 h with continuous shaking, washed, and incubated for 1 h with anti-*Sta* IgY solution (1 mg/mL) diluted at 1:2000. It was subsequently washed with PBS-Tween and the anti-IgY peroxidase conjugate (diluted 1:2000, added and incubated for 1 h at room temperature). The reaction was revealed with diaminobenzidine (DAB) substrate (Dako, Carpinteria, CA, USA) and stopped with deionized distilled water, and the membranes were scanned and analyzed with Image Lab software (Bio-Rad, Hercules, CA, USA).

2.7. Sensitivity test

Membrane proteins and CPs at concentrations of 5, 10, 15 and 20 µg/mL were adsorbed onto the 96-well plate in a volume of 100 µL/well ($n = 3$) and incubated overnight at 4°C . After washing and blocking as described in section 2.3.1., the specific IgY was diluted to the same concentrations as the antigen. Detection, staining, blocking and reading of the reaction were as described in section 2.3.1.

2.8. IgY conjugation with fluorescein isothiocyanate (FITC)

Specific and non-specific IgY were conjugated with FITC (Sigma-Aldrich Corp., St. Louis, MO USA) following the manufacturer's

recommendations. Thus, a 1 mg/mL solution of FITC was prepared, and 50 µL was added to a 1 mg/mL solution of IgY diluted in PBS, pH 7.4, and allowed to rotate for 4 h at 4°C . The solution was then saturated with 1% bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA), and the reaction was blocked with 25 mM ammonium nitrate solution (NH_4). Xylene cyanol was added, and the solution was filtered through a Sephadex G-100 resin column (Sigma-Aldrich Corp., St. Louis, MO, USA).

2.9. Experimental infection and biological sample collection

Nile tilapia (90.5 ± 7 g) were anesthetized by immersion in benzocaine solution (Sigma-Aldrich Laboratory, Steinheim, Germany) (1:20,000 v/v) diluted in 98% ethanol (0.1 g/mL) and challenged with a pre-determined lethal dose 50 (2.0×10^5 CFU/mL) of live *Sta* strains following the recommendations of Wedemeyer [17]. The fish that showed signs indicative of the disease were euthanized by immersion in benzocaine (1:500 v/v), and the collected brain and cardiac tissue were fixed in 10% buffered formalin, processed in paraffin, sectioned into 3–5-µm-thick cuts, and mounted on slides for use.

2.10. Immunofluorescence and immunohistochemistry

The direct IMF method was carried out as described by Robertson et al. [18]. The tissue was deparaffinized and hydrated, and the antigenic recovery was made in moist heat in citrate buffer for 15 min. Blocking of endogenous peroxidase was achieved using a commercial solution (Dako Dual Endogenous; Dako, Carpinteria, CA, USA), and nonspecific sites were blocked with Dako Protein Block Serum-Free (Dako, Carpinteria, CA, USA). The heart and brain slides were then incubated with specific IgY and the non-specific IgY at 1:1000 dilutions. Both were conjugated to FITC and incubated overnight in a dark, moist chamber at 4°C . The slide was washed, the core was labeled with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Corp., St. Louis, MO, USA), and the cut was mounted with Fluoromount for fluorescence microscope analysis (Olympus BX56 series; Olympus Life Science, Center Valley, PA, USA). Indirect IHC was performed following the method of Marin de Mateo [19]. Deparaffinization, antigen retrieval and blocking were carried out by the methodology described for IMF. After blocking and washing, the slide was incubated with the specific IgY, and a duplicate was incubated with non-specific IgY at the 1:1000 dilution overnight at 4°C . Then, the peroxidase-bound anti-IgY rabbit IgG antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) at a 1:2000 dilution was incubated for 2 h, and the reaction was revealed with DAB (Dako, Carpinteria, CA, USA). The slides were mounted and analyzed by light microscopy, and images were captured by Olympus Stream Capture software (Olympus Life Science, Center Valley, PA, USA).

2.11. Statistical analysis

The data were analyzed by ANOVA and Tukey's test at a significance level of 5% [20].

3. Results

3.1. Characterization of anti-*sta* IgY

The production of anti-*Sta* IgY in bird serum started on day 15 after the first dose of the antigen, and the response pattern was similar for PMs e PC de *Sta*. There were increases in antibody titers on the 60th day after the 2nd dose of the antigen and on the 120th day after the 3rd dose. The increase in the IgY affinity index was progressive for MPs and CPs and was significant on the 150th day relative to the 15th, 30th, 60th, and 90th days (Fig. 1).

3.2. Immunogenic profile of bacterial proteins

The SDS-PAGE gel allowed the two protein fractions (MPs and CPs) to be distinguished, demonstrating the efficiency of the method for separating these proteins. Individual assessment of IgY specificity for the two fractions by Western blotting revealed several immunolabeled proteins—eight for MPs and 10 for CPs—indicating the multi-specific potential of IgY (Fig. 2).

3.3. IgY purification and characterization

The pectin precipitation purification method was effective; the SDS-PAGE gel of the purified yolk extract showed a 20-kDa band for the light chain (Fab) and a second 75-kDa band for the heavy chain (Fc). The Fc-positive immunolabeling also demonstrated the presence of IgY in the purified extract (Fig. 3).

3.4. Potential for *in vitro* sensitivity of IgY

The sensitivity of IgY was tested by ELISA (Fig. 4), and a concentration of 5 µg/mL of specific IgY was effective for detecting 5, 10, 15 and 20 µg/mL of both the *Sta* MPs and CPs bacterial fractions ($p < 0.05$). Non-specific IgY did not show significant reactivity when compared to specific IgY. The detection curve increased exponentially and increased relative to the increase in the specific IgY concentration. However, the equivalence zone was observed at a specific IgY concentration of 15 µg/mL, while a 20 µg/mL concentration appears to have saturated the antigen-binding sites.

3.5. Immunohistochemistry and histopathology

Anti-*Sta* IgY was efficacious in the detection of intact cocci in *Sta* culture (Fig. 5), diffuse and phagocyte-internalized in cardiac and cerebral tissue (Fig. 6). The detection sensitivity was similar for both IMF and IHC. In cardiac tissue, immunolabeled cocci were associated with hemorrhagic lesions in the cardiac fibers with the presence of leukocyte infiltrate. In nerve tissue, *Sta* was associated with suppurative meningitis, which was accompanied by intense inflammation, as shown in the histopathological analysis (Fig. 7).

4. Discussion

Several studies have provided evidence for the effectiveness of IgY, which is extracted from egg yolks of laying hens, in the diagnosis and treatment of bacterial infections by *Streptococcus mutans* [21], *Salmonella* sp. [22], *Yersinia* sp. [23], *Staphylococcus* sp. [24], and *Helicobacter pylori* [25], and infections by parasites such as *Trichinella spiralis* [26] and *Schistosoma japonicum* [27]. In addition, IgY has been shown to be effective in the diagnosis of tropical diseases such as dengue [28], in the

detection of neoplastic cells from human mammary tumors [29,30] and when applied as serotherapy for snake poisoning [31] and as an agent to block bacterial toxins [32].

Therefore, in the present study, we produced a polyclonal IgY by immunizing laying hens with *Sta* bacterial proteins isolated from tilapia. The humoral immune response of the birds, with only three doses at a concentration of 200 µg/mL each, produced specific IgY for the two bacterial fractions from day 15, and the antibody titers were constant during the 150 days of the immunization protocol. This enabled the acquisition of large amounts of specific antibodies. In this work, the IgY purification from egg yolk has a low production cost and does not involve bleeding or sacrifice of the bird, as in the production of poly/monoclonal IgG in mammals [33]. Because eggs contain 100 mg of antibodies in the yolk, on average, and laying hens produce on average of 20 eggs per month, 30 days of IgY production can easily reach values greater than 2 g per bird, making it economically feasible to use hens for antibody production [34].

In addition, progressive affinity/avidity maturation and the multi-specific potential of specific IgY observed in the ELISA and Western blot assay, respectively, demonstrate the efficacy of the immunization method and the ability of the humoral response of the bird to recognize distinct antigenic epitopes between the two bacterial fractions. Another relevant fact is that the method for inactivating *Sta* by ultrasound preserved the structural and chemical integrity of the bacterial proteins, neutralizing the pathogen by rupture without altering its structure by physical denaturation, heat, or chemical inactivation by aldehydes [35]. Similarly, ultracentrifugation maintained the structural and chemical integrity of the proteins during the separation process [36]. This allowed the recognition of the antigenic epitopes in their native form, producing antibodies of high affinity and avidity, thus increasing the sensitivity of detection of the pathogen. This was demonstrated by the sensitivity test, which suggests a possible application of anti-*Sta* IgY in the detection of bacteria and their bacterial products, such as exo/endotoxins in biological fluids such as serum, tears, cerebrospinal fluid and other bodily secretions. In fact, preliminary results obtained in our laboratory are based on this application (unpublished data).

The IgY of birds is intrinsically biochemically divergent from mammalian IgG [37]. Due to the phylogenetic distance between the vertebrates, the constant fraction (Fc) of IgY does not have a binding affinity for the C1q molecule of the complement system and the rheumatoid factors present in biological samples of heterologous species, reducing the false-positive reactions for immunological tests [38]. In addition, the heritability of specificity genes, derived from Jurassic ancestors, enables IgY to recognize antigenic epitopes present in mammals and other phylogenetically distinct species with equal or greater affinity than IgG produced in mammals [33].

In this study, the IgY produced was sensitive enough to detect *Sta* by direct IMF and indirect IHC in pure colonies and in bacterial cells processed in cardiac and brain tissue. This allowed the distinction of the extra/intracellular location of *S. agalactiae* in host tissue, allowing observation of the two stages of the pathogen's infectious cycle. Another important factor is that the detection sensitivity was similar for both tests in paraffinized tissue. In addition, the ability of the specific IgY to detect the pathogen in paraffin-embedded tissue allows the sample to be fixed and embedded in paraffin, preserving the structural morphology of the tissue as well as the antigenic epitopes and, by consequence, the reliability of the results. This validated the use of IgY for the diagnosis and pathophysiological study of *Sta* infections in Nile tilapia, as well as the use of this species as a pathophysiological model for this disease and its application in veterinary and human neonatal medicine.

Ethics statement

All handling of fish an avian was, conducted in accordance with the National Council for Control of Animal Experimentation (CONCEA),

approved by the ethics council number: 17209/15.

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