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RESEARCH ARTICLE
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# The effect of heat stress on clinicopathological changes and immunolocalization of antigens in experimental *Streptococcus agalactiae* infection in Red hybrid tilapia (*Oreochromis* spp.)

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## Abstract

**Aim:** To understand the influence of environmental temperature on streptococcosis, heat stress associated pathological changes in acute streptococcosis in red tilapia using various routes of infection was investigated.

**Materials and Methods:** Red hybrid tilapia were inoculated with $10^9$ CFU/ml of *Streptococcus agalactiae* using intra-peritoneal (IP), immersion (IM), and immersion cut (IC) which were maintained at 34C for 24 hours while the positive control groups were infected using similar routes but maintained at 28C and the negative control was at 34C without infection. Samples from the gills, brain, eyes and kidney were taken for bacterial isolation, polymerase chain reaction (PCR), histopathology and immunohistochemistry (IHC).

**Results:** Diseased fish showed skin haemorrhage, fin haemorrhage and exophthalmia with more signs in IP route of infection followed by IC and lastly IM at 34C. The bacteria were isolated and detected with PCR in all the organs of fish from 4 hour post-challenge (hpc). The lesions were noticed earlier and severe in heat stressed groups from 4 hpc with 50% mortality in IP group while for all, the bacterium was isolated from 4 hpc in the brain. IHC test also detected the antigen in the tissues as early as 4 hpc with intense staining in the heat stressed group, IP followed by IC and IM routes. Positive immunostaining were observed in the thrombi, meningeal vessels, choroid and interstitial haemorrhagic areas.

**Conclusion:** The severe lesions observed in the brain, eye and kidney under heat stress reveals its contributory role to the mortality pattern and severe pathological changes associated with streptococcosis in fish.

**Keywords:** heat stress, red hybrid tilapia, *Streptococcus agalactiae*

## Introduction

Red hybrid tilapia (*Oreochromis* spp.) were first introduced in Malaysia in mid 1980's. Tilapia were considered to be resistant to diseases but in 1997, there was heavy mortality of about 300 to 400 g weight tilapia that were kept in floating net cages in Sungai Pahang [1]. Cages of Kenyir, Pedu and Pergau Lakes in Malaysia also experienced this problem in the mid year between April to July of 1997 and the laboratory tests revealed that the causative agent was Gram-positive bacteria, *Streptococcus agalactiae* [1]. Ever since in Malaysia, studies on *S. agalactiae* infection in fish had centred on the pathology and disease outbreaks [2, 3, 4] with very little attention on the possible role of environmental stressors especially temperature on the disease manifestation.

Most fish and aquatic animals are often subjected to a broad variety of stressors because their homeostatic mechanisms are dependent on the surroundings. In captive fish, the stress includes physical and mental trauma associated with capture, transport, handling, crowding,malnutrition, fluctuation in water temperature, low dissolved oxygen concentration, poor water quality (high ammonia or nitrite concentrations) salinity and alkalinity (pH>8) [5, 6]. Many researchers had implicated some of these stress factors like oxygen and nitrite levels [7] and water temperature [8] to influence the mortality pattern due to streptococcal infection especially that by *Streptococcus iniae* [9]. In Malaysia however, information on the effect of these environmental stressors on the mortality due to *S. agalactiae* had been inferences from epidemiological investigations that reported higher mortality during the high temperature seasons [10].

Previous studies had revealed that the sources of infection of *S. agalactiae* and *S. iniae* in most farms are mainly from newly introduced fish, faeces of infected fish and infection via contaminated water [11]. Other means of infection could be through wounds or abrasions of the skin [12] especially in high stock density farms. Since the pathogenesis of fish *S. agalactiae* infection is still poorly understood [13], the role of environmental stress and skin abrasions which are often encountered in the field need to be investigated. Therefore, this study investigates the heat stress associated pathological changes in acute *S. agalactiae* infection in Red hybrid tilapia using various routes of infection especially in the presence of skin abrasion.

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## Materials and Methods

**Fish:** A total of 120 Red hybrid tilapia (*Oreochromis* sp.) weighed approximately 100 - 150 g were obtained from the Aquaculture Extension Centre, Department of Fisheries Malaysia, Bukit Tinggi, Pahang. Prior to experiment, tanks were disinfected and cleaned. The source of water was dechlorinated and continuously aerated. Water quality was monitored prior to onset of the experiment. The fish used, were screened for bacteria particularly for *S. agalactiae* and external parasites in order to make sure that the fish are healthy. The fish were fed *ad libitum* with commercial feed. However throughout the experiment, the fish were off feed.

**Bacteria:** *S. agalactiae* isolated from outbreak case was used for the study [1]. The organism was maintained in stock agar and also in glycerol stock. The bacteria stocked in nutrient agar cultured onto blood agar plates and incubated in the incubator at 37C for 24 h. The brain heart infusion broth (BHIB) was used to subculture the colony and incubated in shaker incubator at 37C for 24 h. A serial dilution and standard plate count techniques were used in order to determine the bacteria concentration. 10 folds of dilution from highest dilution ($10^1$) to lowest dilution ($10^9$) where 1 ml of cultured broth *S. agalactiae* was serially added into 9 ml of peptone water respectively. Then 1 ml from the highest dilution was continuously diluted into another dilution till the lowest dilution. About 0.1 ml of each dilution was poured and spread onto the blood agar and incubated in normal incubator at 37C for 24 h. 25 to 250 colonies were counted before the concentration was expressed as colony forming unit per millilitre (CFU/ml). The last concentration of live *S. agalactiae* was recorded.

**Experimental design:** The experiment was conducted by challenging the fish with $10^9$ CFU/ml of live *S. agalactiae* using different routes of infection. The fish (n=120) were divided into 5 groups. Group 1 (n=24) were exposed to *S. agalactiae* through IP route and transferred into the 6 L tank, Group 2 (n=24) were exposed to *S. agalactiae* through IM bath (9 L of water + 1 L of *S. agalactiae* broth) for 10 min before transferred into the 6 L tank and Group 3 (n=24) were exposed to *S. agalactiae* through IM bath (9 L of water + 1 L of *S. agalactiae* broth) for 10 min with the body of fish being incised (0.5 cm) at the caudal part before being transferred into the 6 L tank. Group 4 (n=24) was the positive control which were challenged with similar dose but maintained at 28C and group 5 as the negative control group without challenge and maintained at 34C. The fish were gradually acclimatised to the higher temperature before being maintained at 34C for the bacterial challenge. The fish were kept for 24 h off fed and the clinical signs were observed continuously in the 24 h duration of the experiment. The brain, eyes and kidney were collected more than the gills (the major predilection tissues for *S. agalactiae* infection in fish) [3; 4] from three fish every 4 h within the 24 h. The tissues samples were subjected to bacterial culture, PCR, histopathology and IHC.

### Bacteria isolation and identification

**Bacteria culture:** The swab from the organs that were collected every 4 h were immediately streaked onto blood agar plates [14] and incubated at 30C for 18 h. The suspected colonies of *S. agalactiae* were subcultured onto a new blood agar and incubated at 30C for 18 h in order to obtain a pure culture. Gram stain test were performed to identify Gram-positive cocci in chain or paired and catalase test negative organisms. Finally, the colonies were further characterized using the commercialized test kit, API rapid ID 32 Strep® (bioMerieux SA, France).

**Polymerase chain reaction (PCR):** To confirm the *S. agalactiae*, total cellular DNA was extracted with Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's protocol. The samples were tested by the colonies that were subcultured every 4 h in the different routes of infection. The extracted DNA was then further evaluated by PCR for *S. agalactiae*-specific section of 16S-23S rRNA intergenic spacer region with primers STAUR 4 [ACG GAG TTA CAA AGG ACG AC] and STAUR 6 [AGC TCA GCC TTA ACG AGT AC], and cycling conditions were as follows; 1 cycle at 94C for 4 min, followed by 34 cycles at 94C for 1 min, 52C for 1 min, 72C for 1 min and finally elongation at 72C for 10 min [22].

**Histopathology:** The gills, brain, kidney and eyes that were collected every 4 h post-challenge (hpc) were routinely processed and stained with haematoxylin and eosin (H&E).

**Immunohistochemistry (IHC):** Serial 4 µm thick sections were cut from wax blocks onto silane-coated glass slides. The slides were dried for 15 min at 56-60C, dewaxed in xylene and rehydrated through a graded alcohol series. The slides were washed with phosphate-buffered saline-Tween 20 (PBST) for 10 min. Endogenous peroxidase activity was blocked with freshly prepared 3% hydrogen peroxide for 5 min in room temperature and rinsed and washed with PBST for 2 min. In enhancing the tissue to be immunoreactive, the heat-mediated antigen retrieval with citrate solution in microwave oven was used. Sections were blocked with blocking buffer 1% normal serum (bovine serum albumin) in PBST, and then sections were incubated with tilapia anti *S. agalactiae* with the dilution of 1:50 for at least 1 h at 37C in an incubator. Then, the procedure was followed by rinsing and washing with PBST for 5 min. Sections were incubated again at 37C for 30 min with secondary anti-tilapia IgM monoclonal-HRP antibody (Aquatic Diagnostic Ltd, UK) with the dilution of 1:100. The slides were rinsed and washed with PBST for 5 min before DAB was applied 1 ml diluents to a 1 drop DAB for the development of the color change. Once the sections

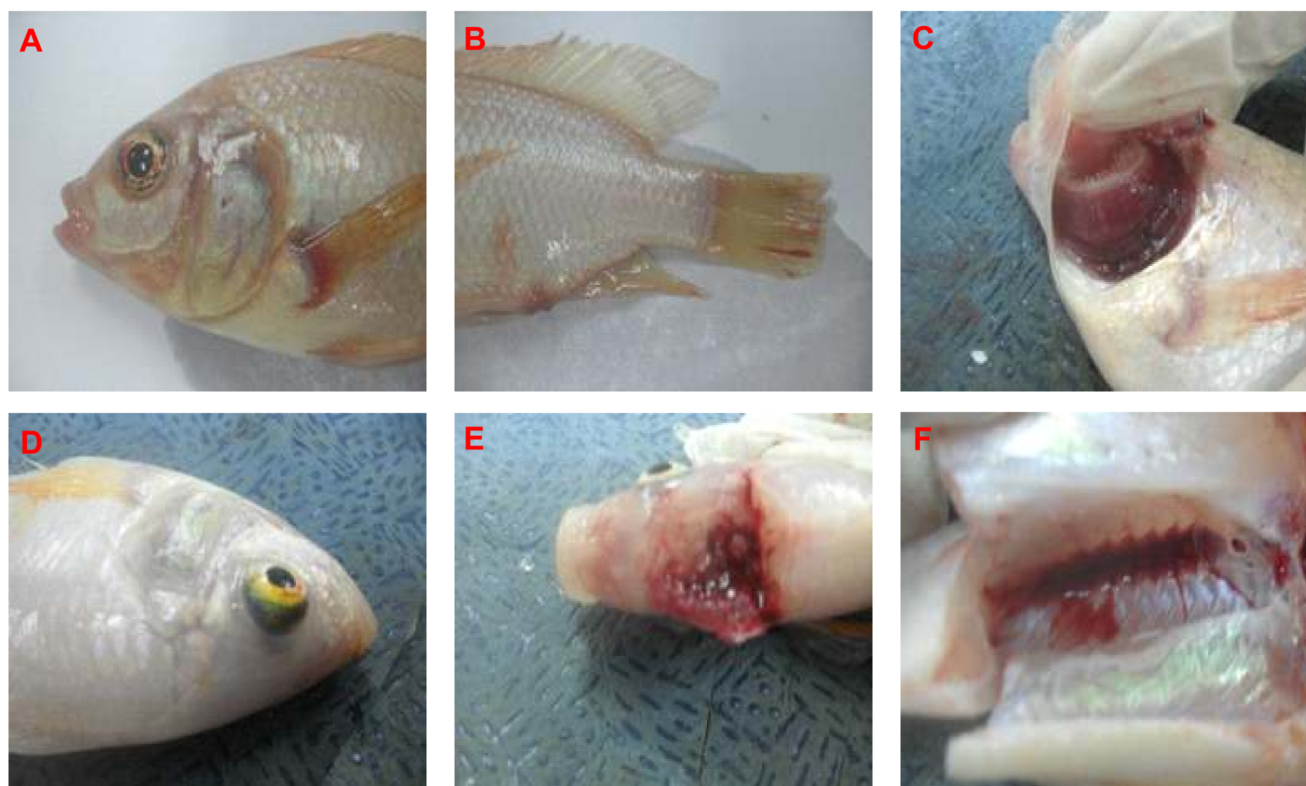


Figure-1. Lesions of Red hybrid tilapia challenged with *S. agalactiae*. A: Haemorrhage at the eyes and pectoral fin, B: Haemorrhage at the anal fin, dorsal fin, caudal fin and body. C: Congestion at the gills, D: Exophthalmia, E: Soft brain, F: Congestion and kidney enlargement.

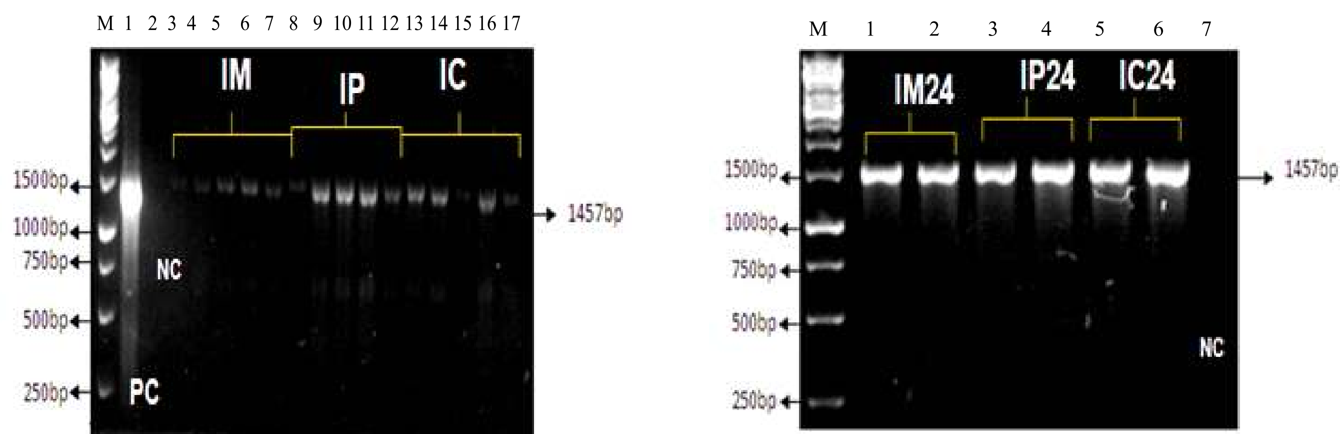


Figure-2. A: PCR at 4 hpc. M: GeneRuler 1-kb DNA ladder (Fermentas). Lane 1: positive control. Lane 2: negative control. Lane 3-7: positive for IM isolates. Lane 8-12: positive for IP isolates. Lane 13-17: positive for IC isolates. B: PCR at 24 hpc. M: GeneRuler 1-kb DNA ladder (Fermentas). Lane 1-2: positive for IM isolates. Lane 3-4: positive for IP isolates. Lane 5-6: positive for IC isolates. Lane 7: negative control.

became brown, the slides were immediately rinsed with distilled water and the slides were stained using Mayer's haematoxylin solution for the background colour. All the slides were analyzed and captured using image analyzer NIS-Elements D 3.2 (Nikon, Japan)

Statistical analysis: The lesion scoring data obtained was analysed using Kruskal-Wallis analysis of variance in Statistix ver. 9.0 (Analytical software, USA). Only values that were below the stated $P < 0.05$ were considered significant and all-pairwise comparisons tests were performed.

## Results

Clinical and macroscopic findings: The clinical signs observed during 24 hpc with *S. agalactiae* were haemorrhage eyes, operculum, fin and/or body, long mucoid faecal cast, erratic swimming, c-shaped body curvature, imbalance and dull. The clinical signs detected in the fish that were challenged by IP injection followed by IC and lastly by IM of live *S. agalactiae*. The clinical signs were observed as early as 2 hpc in most of the fish in the IP group while the IC and IM groups exhibited similar clinical signs at 4 h later. The internal lesions observed during post mortem every 4 hpc were enlarged and congested spleen, pale and haemorrhagic liver, empty gut with engorged gall bladder, congested kidney and soft brain (Fig. 1). The positive control group had similar pattern but the clinical features were observed at 4 hpc.

Mortality: Within 24 h, about 50% mortality was recorded in IP group only while other groups and the negative controls had no mortality within this period.

Table-1. Bacteria culture from the organs

| Time (hours) | Intraperitoneal (IP) | | | Immersion (IM) | | | Immersion cut (IC) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Brain | Eye | Kidney | Brain | Eye | Kidney | Brain | Eye | Kidney |
| 4 | / | / | / | / | X | X | / | / | X |
| 8 | / | / | / | / | / | X | / | / | / |
| 12 | / | / | / | / | / | / | / | / | / |
| 16 | / | / | / | / | / | / | / | / | / |
| 20 | / | / | / | / | / | / | / | / | / |
| 24 | / | / | / | / | / | / | / | / | / |
| Control | X | X | X | X | X | X | X | X | X |

X: No growth, /: Growth

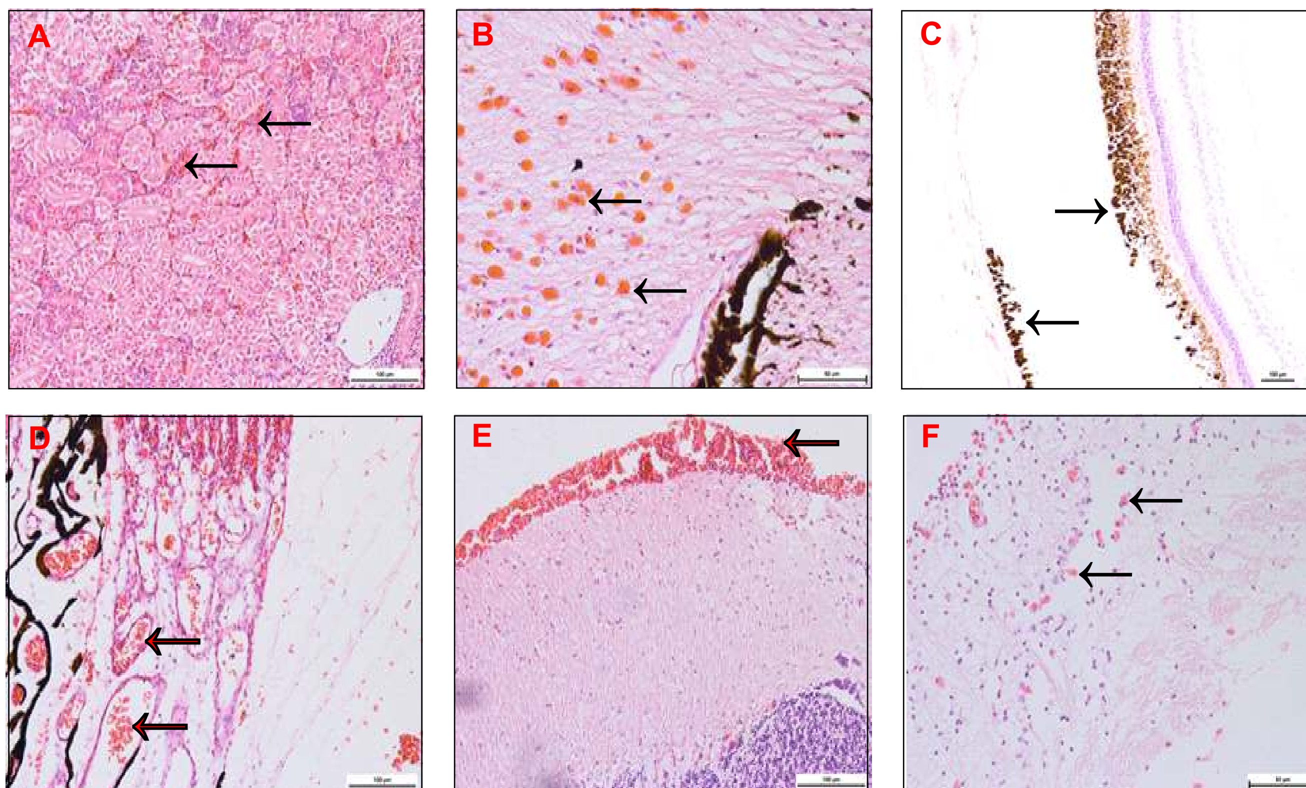


Figure-3. A: moderate haemorrhage of the kidney (arrows) (H&E, 100 μm). B: presence of eosinophilic granule cells in the eye (arrows) (H&E, 50 μm). C: Severe changes of the outer layer of the retina, the pigment cells (PiC) bases photoreceptors bodies were sloughed off (arrows) (H&E, 100 μm). D: congested blood vessel in the supportive tissue of eye (arrows) (H&E, 100 μm). E: congested meninges (arrow) (H&E, 100 μm). F: presence of eosinophilic granule cells in the meninges (arrows) (H&E, 50 μm).

Bacteria isolation and identification: Smooth transparent pin-point colonies were stained blue/purple (Gram-positive) cocci in chain or pairs and catalase negative further tested for other biochemical tests. API rapid ID 32 Strep® (bioMerieux SA, France) was used to confirm the identification and strain of the organism. Based on the API result, the bacterium was *S. agalactiae* with 99.9% identification. *S. agalactiae* was isolated in all the tissues examined in IP group from 4 hpc while in IM the isolation was 12 hpc. In IC the isolation was 8 hpc. In all the routes, the bacterium was isolated from 4 hpc in the brain. On the other hand, in the control group *S. agalactiae* was isolated in all the tissues examined in IP group from 4 hpc while in IM the isolation was 12 hpc in the kidney and the eye. In IC group the isolation was 8 hpc in the brain and eye (Table-1).

PCR: Based on Fig.2, all the colonies that were subcultured are positive for *S. agalactiae* at 1457-bp. PCR was used to confirm the bacterial isolation as it is a reliable method for detecting streptococcosis in tilapia.

Histopathology: *S. agalactiae* induced acute inflammation in the brain, eye and kidney while that observed in the gills were not remarkable. Histological lesions were more severe in IP followed by IC and lastly IM for every 4 hpc. The meninges of telecephalon and cerebellum were infiltrated with inflammatory cells, which in some cases were accompanied by haemorrhage and mild infiltration of eosinophilic granular cells, which is located dorsal to the fourth ventricle. The lesions were more severe in IP12 compared to IM12 and IC12. Kidney tissue showed various degree of degeneration. Large areas of cellular infiltration and numerous lesions were seen in kidney tissue. The lesions in the kidney included tubular necrosis, haemorrhage and infiltration of the inflammatory cells. Most of the kidney tissues that were infected showed haemorrhage in haemopoietic tissue and infiltrated by lymphocytes. The lesions were more severe in the IP12 where massive infiltration of inflammatory cells,

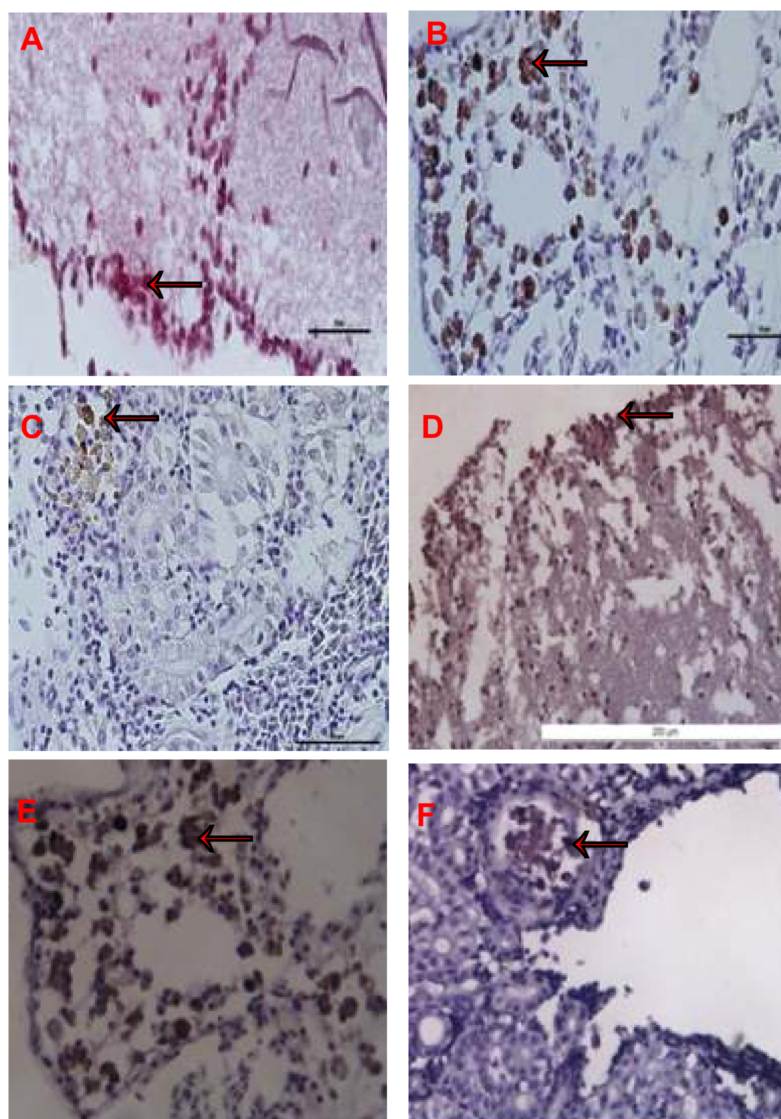


Figure-4. Immunostaining were found in A and D (IM20): the meninges and the meningeal blood vessel lumen and wall (arrow). B and E (IC16): blood vessels and capillaries in the choroid body containing eosinophilic granular cells and neutrophils (arrow). C and F (IP8): kidney; the immunostaining was observed in the blood vessels lumens (arrow); a thrombus in F (arrow).

Table-2. Results of IHC examination of different groups and organs of red tilapia for *S. agalactiae*

| Group | Brain | Eye | Kidney |
|---|---|---|---|
| C | - | - | - |
| IM4 | + | + | + |
| IM12 | + | + | + |
| Im16 | + | + | + |
| IC8 | + | + | + |
| IP4 | + | + | + |
| IP8 | + | + | + |
| IP12 | + | + | + |
| IP16 | + | + | + |
| IP20 | + | + | + |
| IP 24 | + | + | + |

-, negative brown staining; +, positive brown staining; C, control

tubular degeneration and haemorrhage can be seen compared to IM12 and IC12. Multiple granuloma-like lesions were observed with leukocytic infiltration around it. Eye lesions were characterized by a mild cellular infiltrate, composed of macrophages and eosinophilic granular cells. These findings were observed in the choroid tissues and also in periorbital tissues. The lesions in the eye can be seen at 12 hpc whereby some of the pigment cells surrounded the bases of the photoreceptors were sloughed off. The lesions could be seen clearly in IP16 followed by IC12 and lastly IM12. The meninges were congested and thickened with the presence of eosinophilic granular cells in the brain and it is associated with the loss of orientation and swimming abnormalities. There is significant difference ($P< 0.05$) in the lesions recorded between IP, IC routes when compared to IM route in most of the organs. However, there is no significant difference between IP to IC in most of the organs. On the other hand, there is no significant difference in most of the organs at $P > 0.05$ in different routes of infection in the negative control groups (Fig. 3).

IHC test: IHC test detected antigen in the brain, eye and kidney from 4 hpc with slight immunostaining within the capillaries in all the organs but more widely distributed in the kidney. There was no immuno-staining observed in the gills in all the routes. This immunostaining increased in intensity and distribution as the post-challenge period increased in the IP route followed by IC and IM. The immunostainings were observed in the thrombi, meningeal vessels, choroid and renal interstitial haemorrhagic areas. The presence of cocci-shaped structures with brown colour and/or a brown staining with a diffuse distribution was found within macrophages, associated with inflammatory reactions in the kidney, blood vessels lumen and wall and some within thrombi in the eye especially in choroid, there was also presence of the immunolabelling in the meningeal blood vessel both in the lumen and the wall (Fig.4). On the other hand, the immunostaining followed the same pattern but was observed as from 8 hpc (Table-2).

## Discussion

This investigation describes the effect of route and heat stress on the pathological changes in *S. agalactiae* infection in Red hybrid tilapia. The clinical features, pathology and detection of antigens were

earlier and more pronounced in heat stressed Red hybrid tilapia. The Red hybrid tilapia challenged with *S. agalac*tiae showed various clinical signs and lesions such as skin, fin and visceral organ haemorrhage/ congestion that are consistent with the disease while histopathological changes observed in the brain, kidney and eyes are highly suggestive of septicaemia [2,15,16,17] with detection of bacteria much earlier at 4 hpc in the brain and eye when heat stress was employed. This could explain the higher susceptibility accompanied by higher mortality and bacterial isolation usually observed during the hot season [10] and when the environmental temperature increases. This might aid the breaking of the skin natural defensive mechanisms to enhanced penetration and detection observed in IC and IM groups. The detection of this bacterium in the tissues examined except the gills soon after infection especially when heat stressed further suggested the three organs as the target organs of the organism in the order of the brain, eye and kidney [4].

The gross pathological changes seen in this study were typical of septicaemia associated with *S. agalactiae* infection. The meningitis and blood vessels congestion were associated with loss of orientation and abnormalities often observed in the disease [4,13]. The significant difference observed between the routes of infection in the brain, eye and kidney further lend credence to the fact that stress may increase the bacterial virulence [4].

The pathological changes observed in the blood vessel and the subsequent detection of antigens in blood vessels, capillaries in the kidney and meningeal capillaries further strengthen the observations of [3] that vasculitis and thrombosis are some of the features of streptococcosis in tilapia. The 50% mortality recorded in the stressed IP group was in consonance with the reports of some workers that mortality in fish challenged with $10^8$CFU using the IM route are often observed when the water temperature is above 26C [18, 19]. The observed mortality is very high and alarming especially when compared to the negative control where there was no mortality within 24 h despite pathological changes observed within this period. The mortality could be associated with the severe pathological changes in the IP stressed group. Comparing the mortality report in this experiment and that of [4] the 50% of the challenged fish in that experiment died on the 5$^{th}$ day post challenge (pc) while that of Raheed and Plumb 1984 reported such mortality 96 hpc. This showed that high water temperature could enhance the susceptibility to this infection and could result in very high mortality as experienced in the field [18, 19]. The possible pathogenesis of this influence may need further investigation but many researchers attributed this susceptibility to the immunosuppression caused by the heat stress [20, 21] while some workers suggested that haemolysin, a virulence factor as observed in human *Streptococcus* may be responsible for the pathogenicity associated with *Streptococcus* sp. in fish.

The fact that IP route had more lesion than IC and IM in this investigation showed that the pathogenicity of the bacteria depends on level of bacteraemia, with the brain being the primary target organ for *S. agalactiae* irrespective of the routes employed. The bacteria tropism at 4 hpc was uniform with wider distribution in IP routes as bacteria were found in the brain, eye and kidney. The wider coverage observed in IP showed that IP provides a direct route of infection into the body when compared to the IC which had cut as means of entry into the body system while IM has the natural skin defensive mechanisms to contend with.

The high temperature tends to be associated with more pronounced clinical signs, macroscopic and microscopic lesions in the fish challenged with *S. agalactiae* and this further confirm the epidemiological reports of higher mortality due to this infection in the hot season [10]. Additionally, the result showed that infection could be enhanced by abrasions especially when the water temperature is higher. In this study, there is a significant difference between different routes of infection (IP, IC and IM) in most of the organs especially in heat stressed group. This shows that, the infection via IC can be enhanced by heat stress implying that heat stress may play a major role in development of streptococcosis. This possible mechanism of this role needs further investigation especially as regards the effect of stress hormones and factors on the immune-competence of fish.

## Conclusion

In conclusion, *S. agalactiae* being recognized as pathogenic to fish especially tilapia, causes septicaemia and severe pathological changes in all different routes of infection (24 hpc) used in this study. The lesions observed are more in the brain, eye and kidney than gills and are more severe in the IP route, followed by IC and lastly IM especially when heat stress was employed. The higher temperature could have contributed to the higher percentage of mortality observed in IP route of infection however the possible mechanism that lead to this observation still needed to be verified.

## Authors' contributions

ON carried out the experiment and collecting samples. NAJ carried out the experiment and collecting samples. MYS is a project leader and supervised the project. BOE carried out the immunoperoxidase and pathology analyses. PNT carried out the immunoperoxidase and pathology analyses. MHL cut, trimmed and staining the tissues. SJ cut, trimmed and staining the tissues. All authors drafted and revised the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests.

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