

## Pathogenicity of *Streptococcus agalactiae* Isolated from a Fish Farm in Selangor to Juvenile Red Tilapia (*Oreochromis* sp.)

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**Abstract:** The rapid increases in global aquaculture industry have exposed many diseases that were not known in aquaculture fields. *Streptococcus agalactiae*, one of *Streptococci* species that infecting both terrestrial and aquatic animals. The organisms have been isolated from numerous fish species in natural disease outbreaks and showed to be pathogenic to several fish species. Recently, few streptococcosis outbreaks affecting cultured Red tilapia in Selangor were investigated and the *S. agalactiae* isolate was used to study its pathogenicity on juvenile Red tilapia. The 120 h 50% endpoint (LD<sub>50</sub>) value in juvenile tilapia injected Intraperitoneally (IP) was  $1.56 \times 10^5$  cfu mL<sup>-1</sup>. Experimental infections were carried out by bathing the fish for 30 min in water containing the bacteria and by IP injection. It was observed that IP route was more potent to cause mortality to juvenile Red tilapia and produced clear clinical signs within 5 days. It was noted that the mortality started to reduce after 5 days and fish recovered after 9 days post inoculation. In contrast, immersion route did not induce mortality but produced moderate clinical signs such as lethargy and loss of appetite and fish started to recover after 6 days. The findings of the current study would enable us to formulate a suitable measure to prevent and control future disease outbreaks.

**Key words:** Red tilapia, *Streptococcus agalactiae*, pathogenicity, measure, mortality, injection

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### INTRODUCTION

Streptococcal infection induced by *Streptococcus* sp. has become one of the most important fish diseases since, it was first reported in middle of the last century. Now-a-days, numerous terrestrial and aquatic animals including human were reported to be infected with *Streptococcus* sp. (Hernandez *et al.*, 2009; Evans *et al.*, 2002). *Streptococcus agalactiae* is a group B streptococcus, it was reported to cause neonatal pneumonia and meningitis in human (Brimil *et al.*, 2006; Johri *et al.*, 2006), mastitis in cows (Brochet *et al.*, 2006; Yildirim *et al.*, 2002) and streptococcal infection in fish (Toranzo *et al.*, 2005).

Since, it was first reported during the middle of the last century, *S. agalactiae* has been increasingly recognized as pathogenic to fish especially warm fresh water fish (Plumb, 1999; Pretto-Giordano *et al.*, 2010a). During the last decades, there were many reports of *S. agalactiae* outbreaks worldwide. The last few years saw numerous *S. agalactiae* infection outbreaks and were

documented in many fish farms especially tilapia farms in Asia including Malaysia (Musa *et al.*, 2009; Suanyuk *et al.*, 2005). Experimentally, *S. agalactiae* has shown to be more infectious than any other environmental bacteria and the mortality in fish may reach up to 100% (Ferguson *et al.*, 1994). Experimental study conducted by Rattanachaikunsopon and Phumkhachorn (2009) had shown that the mortality of Nile tilapia reached 94% in the first 2 weeks after intraperitoneal injection of different dilutions of *S. agalactiae* and the bacteria was re-isolated from liver and kidney of all dead fish.

Eldar *et al.* (1995) reported marked clinical signs such erratic swimming, decrease in feeding, lethargy, exophthalmia with intraocular hemorrhage and corneal opacity and ascites in the fishes infected by *Streptococcus* sp. Salvador *et al.* (2005) observed a high morbidity and mortality in cultivated tilapia infected with *S. agalactiae* which presented erratic swimming, loss appetite, exophthalmia and visceral cavity distension as main clinical signs. The same clinical signs and high mortality were first observed in Malaysian tilapia floating

cages in late 1990s (Siti-Zahrah *et al.*, 2008) and the laboratory tests revealed that *S. agalactiae* was the most common isolate. The pathogenicity and the virulence of the bacteria were not yet fully studied and described among the Malaysian farmed fish species. The *S. agalactiae* previously isolated and obtained from Aquatic Animal Health Unit, University Putra Malaysia was used in this study to calculate the 50% endpoint ( $LD_{50}$ ) and to determine the pathogenicity of *S. agalactiae* to juvenile Red tilapia by using Intraperitoneal (IP) and immersion routes.

## MATERIALS AND METHODS

**Experimental fish:** About 360, apparently healthy juvenile Red tilapia were obtained from a commercial hatchery in Serdang area. Fish were randomly tested and screened to ensure that they were disease and pathogen free. The fish average weight was  $9\pm 2$  g with average total body length of  $6\pm 1$  cm. All fish were maintained in 500 L fiberglass tank with aeration and acclimatized for 2 weeks before they were used for experiments. The fish were fed with 3% body weight commercial feed.

**Preparation of inoculums:** The stock bacteria were first passaged through healthy fish to potentiate its virulence and then grown on Brain Heart Infusion Agar (BHIA) at  $35^{\circ}\text{C}$  for 24 h. About 3-5 bacterial colonies were collected and then suspended in fresh 0.85% normal saline. The cultures were washed three times collected by centrifugation and re-suspended in fresh normal saline prior to use in the experiments proper.

**Calculation of 50% endpoint ( $LD_{50}$ ):** This was to determine the lowest bacterial dose which could causes 50% mortality in Red tilapia population. Fish were divided into 6 groups with 20 fishes in each group. The experiment was conducted in 50 L aquarium supplied with good aeration. The environmental condition were maintained as optimum as possible, the temperature was kept at  $28\pm 2^{\circ}\text{C}$ , the Dissolved Oxygen (DO) was  $4.4\pm 0.1$  mg  $\text{L}^{-1}$  and pH was  $7.2\pm 0.2$ .

The bacteria suspension that was previously prepared was adjusted to McFarland turbidity standard No. 1 which was equivalent to  $3\times 10^8$  cfu  $\text{mL}^{-1}$ . Ten folds serial dilutions were done to obtain *S. agalactiae* concentration of  $3\times 10^7$  to  $3\times 10^3$  cfu  $\text{mL}^{-1}$ . The fish in group number 1-5 were injected intraperitoneally with of  $3\times 10^7$ ,  $3\times 10^6$ ,  $3\times 10^5$ ,  $3\times 10^4$  and  $3\times 10^3$  cfu  $\text{fish}^{-1}$ , respectively. The 6th group was a control group and injected with normal saline. The experiment was run in duplicate. Fish mortality was recorded every 24 h for 5 days. The kidneys and brains of all dead fish were

collected for isolation of *S. agalactiae*. The  $LD_{50}$  value was calculated using Reed-muench (Specter *et al.*, 2000).

**Intraperitoneal and immersion infection trials:** This study was undertaken to determine the pathogenicity of *S. agalactiae* to juvenile Red tilapia. Fish were divided into 3 groups each group contained 22 fishes. Each group was moved to 50 L aquarium with good aeration. The temperature was maintained at  $28\pm 2^{\circ}\text{C}$ , DO at  $4.4\pm 0.1$  mg  $\text{L}^{-1}$  and pH was  $7.2\pm 0.2$ . Each Fish in the group 1 were IP injected with  $3\times 10^5$  cfu  $\text{fish}^{-1}$  of *S. agalactiae*.

Fish in the group 2 were immersed in 10 L bath containing  $3\times 10^5$  cfu  $\text{mL}^{-1}$  *S. agalactiae* for 30 min and then returned back to the 50 L aquarium. The group 3 acted as control group. The experiment was run in duplicate. Clinical signs and mortality were monitored and recorded daily. About 2 fish were collected from each group every 2 days post inoculation (dpi) and blood samples, livers, kidneys, spleens and brains were collected for bacteriological and histological examinations.

## RESULTS AND DISCUSSION

In the  $LD_{50}$  trial, fish in groups 1-4 showed mortalities and clinical signs such as lethargy and off feed during 24 h post inoculation (hpi), some fish died without any clinical signs. Mortality started after 48 hpi in group 5 (Table 1). The highest mortality in most groups occurred on the second day with 40% accumulated mortality (Fig. 1). Clinical signs such as lethargy, erratic swimming, corneal opacity and caudal fin rot were observed during the experiment.

*Streptococcus agalactiae* was re-isolated from kidneys and brains of all dead fish. After 120 hpi the fish injected with  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$  and  $10^3$  cfu  $\text{mL}^{-1}$  bacteria, showed 80, 75, 50, 30 and 15% mortality, respectively (Fig. 2). The calculated  $LD_{50}$  of *S. agalactiae* for juvenile Red tilapia was  $1.6\times 10^5$  cfu  $\text{mL}^{-1}$ . The fish from control group were active during the whole experiment and showed neither mortality nor clinical signs. In the  $LD_{50}$  determination, Rasheed and Plumb (1984) and Rattanachaikunsopon and Phumkhachorn (2009), respectively recorded  $1.4\times 10^4$  and  $3.79\times 10^5$  cfu  $\text{mL}^{-1}$  which were similar to  $1.6\times 10^5$  cfu  $\text{mL}^{-1}$   $LD_{50}$  observed in this study.

The dose was able to kill about 50% of challenged fish in 5 days. *S. agalactiae* isolate used in this study was significantly pathogenic to challenged fish and produced disease in the fish supporting its pathogenicity evidence. The inoculation routes may have affect on the mortality of experimentally inoculated fish. Robinson and

Table 1: Total cumulative mortality recorded for juvenile Red tilapia intraperitoneally inoculated with live *S. agalactiae*

Group	cfu mL <sup>-1</sup>	No. of dead fish (hpi)					Total no. dead	Mortality (%)
		24	48	72	96	120		
1	10 <sup>7</sup>	4	7	3	1	1	16/20	80
2	10 <sup>6</sup>	4	6	3	1	1	15/20	75
3	10 <sup>5</sup>	3	3	2	1	1	10/20	50
4	10 <sup>4</sup>	1	2	2	1	0	6/20	30
5	10 <sup>3</sup>	0	2	1	0	0	3/20	15

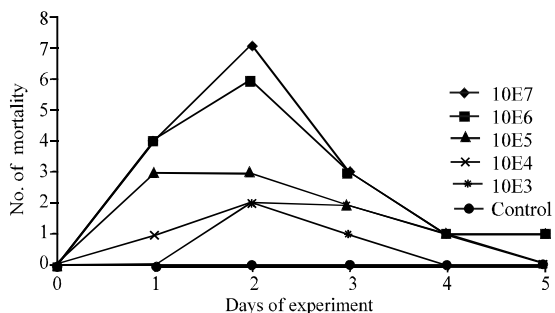


Fig. 1: Total mortality versus days of experiment

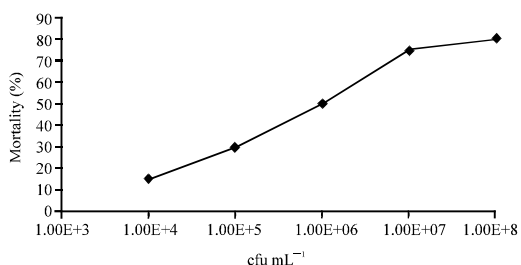


Fig. 2: Percentage of mortality versus bacterial concentration

Meyer (1966) were able to establish disease through water-borne challenge by dipping fish for 10 min in a bacteria concentration of 10<sup>6</sup> cfu mL<sup>-1</sup>. Rasheed and Plumb (1984) using group B *Streptococcus* sp. observed up to 50% mortality after 96 h via IP inoculation of 1.4×10<sup>4</sup> cfu mL<sup>-1</sup>. They also observed that the stress increased the bacterial virulence. Perera *et al.* (1997) observed mortality rate of 50, 34 and 95% when the tilapia were infected with *Streptococcus iniae* by oral route, immersion and IP injection, respectively.

In IP trials, *S. agalactiae* caused 55% mortality within 10 days. The affected fish showed behavioral changes such as lethargy, loss of appetite, grouping at the aquarium bottom and abnormal swimming. At the 3rd dpi some fish showed exophthalmia, corneal opacity (Fig. 3) and tail erosion. *S. agalactiae* was recovered from kidneys and brains of dead fish and from spleens, kidneys, livers, blood and brains of sampled live fish. The post-mortem inspection showed spleen and gall bladder



Fig. 3: Red tilapia infected with *S. agalactiae* showing bilateral corneal opacity (left) and bilateral exophthalmia (right)



Fig. 4: Red tilapia infected with *S. agalactiae* via IP route showing enlargement of Spleen (S) and Gall bladder (G)

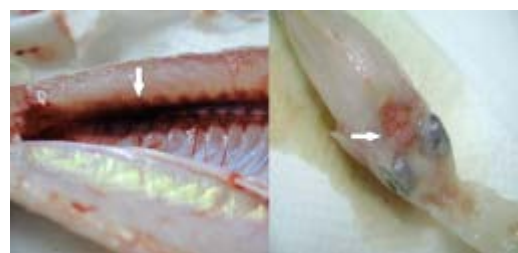


Fig. 5: Red tilapia infected with *S. agalactiae* via IP route showing congested kidney (left) and brain softening (right)

enlargement (Fig. 4), brain softening and congestion of kidney (Fig. 5). No clinical signs appeared after the 8th dpi and no *S. agalactiae* were isolated on the 10th dpi. No mortality was observed in fish group infected by immersion method. Only lethargy and loss of appetite were observed. Internally, most of organs did not show significant changes but *S. agalactiae* was able to be re-isolated from spleens, kidneys and brains of some fish. No clinical signs were observed after 5th dpi and fish

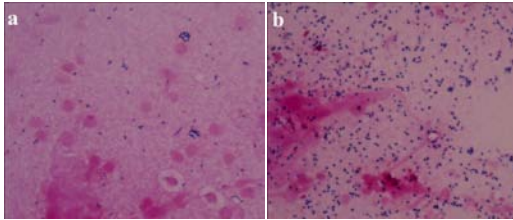


Fig. 6: Tissues of Red tilapia infected with *S. agalactiae* showing gram positive cocci in a) kidney and b) spleen and (Gram stain, 1000x)

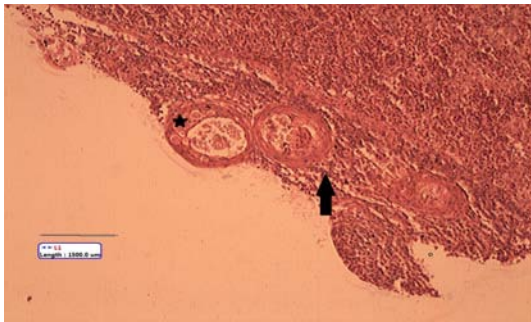


Fig. 7: Spleen of Red tilapia infected with *S. agalactiae* showing granulomas (star) and mononuclear cells infiltration (arrow) (H and E, 100x)

started to recover. No *S. agalactiae* were isolated after 6th dpi from all the fish samples. In the current study, possible pathogen entry into fish through water was established while the bacteria were highly pathogenic if it reached the host blood stream via IP inoculation. With suitable infective dose, *S. agalactiae* can cause mortality up to 40% during 24 hpi and cause 80% mortality within 5 days. Similar results of the highest mortality during 24 hpi were described by Preto-Giordano *et al.* (2010b). The infected fish in this study showed clinical signs such as lethargy, anorexia, erratic swimming, unilateral or bilateral exophthalmia and high mortality.

Similar clinical signs to those observed in this study were reported in natural and experimental streptococcal infection by Evans *et al.* (2002) in Kuwait, Salvador *et al.* (2005) in the Brazil, Suanyuk *et al.* (2005) in Thailand and Musa *et al.* (2009) in Malaysia. However in the present study, clinical signs disappeared after the 8th dpi in the IP challenge and the 5th dpi in immersion challenge. This condition could be related to the ability of the immune system to resolve the infection in juvenile Red tilapia. The squash smears of the organs stained with gram stain showed numerous gram positive cocci arranged singly or in chains between tissues (Fig. 6a, b). The spleen tissue showed red pulp degeneration, hemosiderosis and the

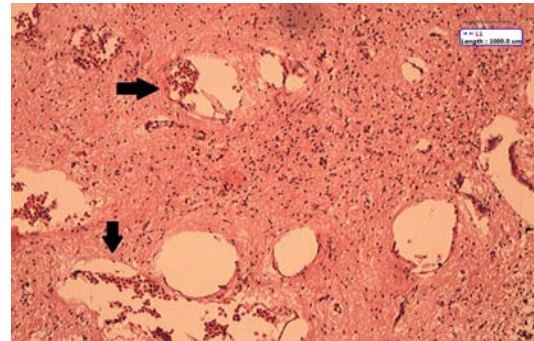


Fig. 8: Brain of Red tilapia infected with *S. agalactiae* showing dilatation and detachment of blood capillaries (arrow) (H and E, 100x)

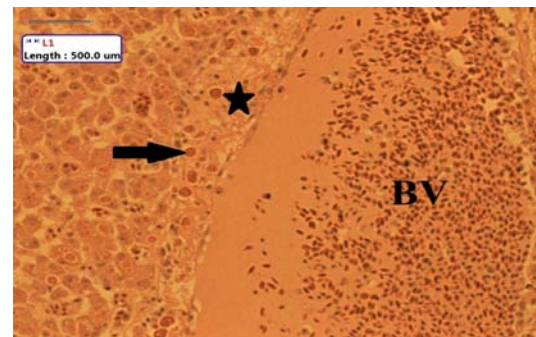


Fig. 9: Liver of Red tilapia infected with *S. agalactiae* showing hepatic necrosis (star) and eosinophilic granular inflammatory cells infiltration (arrow), Blood Vessels (BV) (H and E, 400x)

splenic blood vessels were markedly congested. In addition to that granulomas with lymphocytic infiltration were found in splenic tissue (Fig. 7). Brain blood vessels were congested and some exhibited dilatation and detachment of blood vessels wall (Fig. 8). Meningitis and thickening of meninges were also observed in most brain samples. The histological sections of liver tissue showed loss of hepatic parenchyma structure, hepatocytes vacuolation and degeneration, hepatopancreas cells degeneration and necrosis and severe blood vessels congestion. Infiltration of eosinophilic granular inflammatory cells around the blood vessels was also observed (Fig. 9).

Kidney hematopoietic tissue showed various degree of degeneration. *Streptococcus agalactiae* infection characterized by septicemia and meningoencephalitis (Mian *et al.*, 2009), hepatocyte vacuolization and necrosis and splenic necrosis and congestion (Evans *et al.*, 2002). In addition, Suanyuk *et al.* (2005) observed granulomas in the brain. The gross pathological changes seen in this

study were typical of a septicemic infection and the observed lesions and clinical abnormalities corresponded to histopathological findings. The brain meningitis and blood vessels congestion were associated with loss of orientation and swimming abnormalities. These findings supported that *S. agalactiae* was neurotropic as described earlier by Eldar *et al.* (1995). The increased in Melanomacrophages Centers (MMC) in the liver and spleen in the infected tilapia was possibly related with the cellular effective immune response of fish. Filho *et al.* (2009) described the relevant role of MMC and *S. agalactiae* infection in Nile tilapia and suggested that the macrophages were associated with lymphocytes for antigen trapping. The results of this experiment showed that the *S. agalactiae* were pathogenic to tilapia causing septicemia and meningitis.

### CONCLUSION

*Streptococcus agalactiae* strain isolated from the naturally infected tilapia was able to reproduce the illness in the juvenile Red tilapia experimentally inoculated with different infective doses of bacteria via IP and immersion route of infection. The LD<sub>50</sub> was calculated to be 1.56×10<sup>5</sup> cfu mL<sup>-1</sup>. Clinical signs, gross pathologies and microscopical findings were similar to those described in natural disease infection.

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### REFERENCES

- Brimil, N., E. Barthell, U. Heindrichs, M. Kuhn, R. Luttkien and B. Spellerberg, 2006. Epidemiology of *Streptococcus agalactiae* colonization in Germany. Int. J. Med. Microbiol., 296: 39-44.
- Brochet, M., E. Couve, M. Zouine, T. Vallaeys and C. Rusniok *et al.*, 2006. Genomic diversity and evolution within the species *Streptococcus agalactiae*. Microbes Infect., 8: 1227-1243.
- Eldar, A., Y. Bejerano, A. Livoff, A. Horovitz and H. Bercovier, 1995. Experimental *Streptococcal meningo-encephalitis* in cultured fish. Vet. Microbiol., 43: 33-40.
- Evans, J., P. Klesius, P. Gilbert, C. Shoemaker and M. Al-Sarawi *et al.*, 2002. Characterization of B-haemolytic group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L. and wild mullet, *Liza klunzingeri* (Day) in Kuwait. J. Fish Dis., 25: 505-513.
- Ferguson, H.W., J.A. Morales and V.E. Ostland, 1994. *Streptococcosis* in aquarium fish. Dis. Aquatic Organisms, 19: 1-6.
- Filho, C.I., E.E. Muller, L.G. Pretto-Giordano and A.F. Bracarense, 2009. Histological findings of experimental *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). Brazilian J. Vet. Pathol., 2: 12-15.
- Hernandez, E., J. Figueroa and C. Iregui, 2009. Streptococcosis on a red tilapia, *Oreochromis sp.*, farm: A case study. J. Fish Dis., 32: 247-252.
- Johri, A.K., L.C. Paoletti, P. Glaser, M. Dua and P.K. Sharma *et al.*, 2006. Group B Streptococcus: Global incidence and vaccine development. Nature Rev. Microbiol., 4: 932-942.
- Mian, G.F., D.T. Godoy, C.A.G. Lea, Y.T. Yuhara, G.M. Costa and H.C.P. Figueiredo, 2009. Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. Vet. Microbiol., 136: 180-183.
- Musa, N., L.S. Wei, N. Musa, R.H. Hamdan and L.K. Leong *et al.*, 2009. Short communication: Streptococcosis in red tilapia (*Oreochromis niloticus*) commercial farm in Malaysia. Aquac. Res., 40: 630-632.
- Perera, R.P., S.K. Johnson and D.H. Lewis, 1997. Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas. Aquaculture, 152: 25-33.
- Plumb, J.A., 1999. Health Maintenance and Principal Microbial Diseases of Cultured Fishes. Iowa State University Press, Ames.
- Pretto-Giordano, L.G., E.E. Muller, J.C. de Freitas and V.G. da Silva, 2010a. Evaluation on the Pathogenesis of *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*). Brazilian Arch. Biol. Technol., 53: 87-92.
- Pretto-Giordano, L.G., E.E. Muller, P. Klesius and V.G.D. Silva, 2010b. Efficacy of an experimentally inactivated *Streptococcus agalactiae* vaccine in Nile tilapia (*Oreochromis niloticus*) reared in Brazil. Aquacult. Res., 41: 1539-1544.
- Rasheed, V. and J.A. Plumb, 1984. Pathogenicity of a non-haemolytic group B *Streptococcus* sp. in gulf killifish (*Fundulus grandis* Baird and Girard). Aquaculture, 37: 97-105.
- Rattanachaikunsopon, P. and P. Phumkhachorn, 2009. Prophylactic effect of *Andrographis paniculata* extracts against *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). J. Biosci. Bioeng., 107: 579-582.

- Robinson, J.A. and F.P. Meyer, 1966. Streptococcal fish pathogen. *J. Bacteriol.*, 92: 512-512.
- Salvador, R., E.E. Muller, J.C. Freitas, J.H. Leonhardt, L.G. Preto-Giordano and J.A. Dias, 2005. Isolation and characterization of *Streptococcus* spp. group B in Nile tilapias (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northern region of Parana State, Brazil. *Ciencia Rural*, 35: 1374-1378.
- Siti-Zahrah, A., B. Padilah, A. Azila, R. Rimatulhana and R. Shahidan, 2008. Multiple Streptococcal Species Infection in Cage-Cultured Red Tilapia but Showing Similar Clinical Signs. In: *Diseases in Asian Aquaculture VI*, Bondad-Reantaso, M., C. Mohan, M. Crumlish and R. Subasinghe (Eds.). Asian Fisheries Society, Manila, pp: 313-320.
- Specter, S., R.L. Hodinka and S.A. Young, 2000. *Clinical Virology Manual*. 3rd Edn., ASM Press, Washington DC. USA., pp: 623.
- Suanyuk, N., H. Kanghear, R. Khongpradit and K. Supamattaya, 2005. *Streptococcus agalactiae* infection in tilapia (*Oreochromis niloticus*). *Songklanakarin J. Sci. Technol.*, 27: 307-319.
- Toranzo, A.E., B. Magarinos and J.L. Romalde, 2005. A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, 246: 37-61.
- Yildirim, A.O., C. Lammler and R. Weib, 2002. Identification and characterization of *Streptococcus agalactiae* isolated from horses. *Vet. Microbiol.*, 85: 31-35.