Distribution of *Edwardsiella tarda* Antigens and IgM Containing Cells in Tilapia Immune Organs during Septicemia: an Immunohistochemical Study

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Abstract

An immunohistochemical study describes the localization of IgM containing cells involved in the primary immune organs of tilapia and the fate of *E. tarda* antigen distributed in the tissues during a course of intraperitoneal challenge. The *E. tarda* antigen was diffusely detected in various organs except the brain and spinal cord. The positive signals were identified within various cell types, including phagocytic cells, serosal mesothelia, myocardia, intestinal and gill epithelia and glomerular capillary endothelia. No extracellular deposition of bacterial antigen was observed. The bacterial antigen can persist up to 1 month in the granuloma and melano-macrophage center, confirming the intracellular invasive ability of *E. tarda* disseminated in the tissues during septicemia. The granuloma-participating phagocytic and IgM containing cells play an important role in the specific immune response against *E. tarda* during septicemia. The IgM containing cells uniformly distributed as a peri-granuloma arrangement at 7-14 DPI. *E. tarda* antigen decreased significantly after the appearance of IgM response, suggesting the phagocytic enhancement of granuloma-participating cells to eliminate *E. tarda* bacteria was influenced by these IgM cells.

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บทคัดย่อ

การกระจายตัวของ *Edwardsiella tarda* แอนติเจนและเซลล์สร้าง IgM ในอวัยวะสร้างภูมิคุ้มกันปลานิลระหว่างติดเชื้อในกระแสเลือด: การศึกษาทางอิมมูนโนฮิสโตเคมี

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ศึกษาทางอิมมูนโนฮิสโตเคมีเพื่ออธิบายลักษณะตำแหน่งที่พบ และการกระจายตัวของเซลล์สร้าง IgM ในอวัยวะที่เป็นแหล่งสร้างภูมิคุ้มกันชนิดปฐมภูมิของปลานิล และอธิบายการกระจายตัวของเชื้อ *Edwardsiella tarda* ในปลานิลหลังจากฉีดเชื้อเข้าทางช่องท้อง ผลการศึกษา พบการปรากฏของเชื้อในทุกอวัยวะยกเว้นสมองและไขสันหลัง สามารถพบเชื้อได้ภายในเซลล์แมคโครฟาจ กล้ามเนื้อหัวใจ เซลล์เยื่อบุ serosa เซลล์เยื่อบุผิว และเซลล์เยื่อบุผนังหลอดเลือด ไม่พบการปรากฏของเชื้อภายนอกเซลล์ แอนติเจนของเชื้อยังคงสามารถตรวจพบได้ภายในก้อนแกรนูโลมาตลอดการทดลอง แต่มีปริมาณลดลง 14 วันหลังจากติดเชื้อ แสดงให้เห็นถึงความสามารถของแบคทีเรียในการรุกรานเซลล์และเนื้อเยื่อสูง เซลล์แมคโครฟาจในก้อนแกรนูโลมา (granuloma-participating phagocytic cell) และ IgM มีบทบาทสำคัญในการสร้างระบบภูมิคุ้มกันในช่วงที่มีการติดเชื้อในกระแสโลหิต เซลล์ชนิดที่สร้าง IgM จำนวนมากจะกระจายตัวล้อมรอบบริเวณก้อนแกรนูโลมา ในช่วง 7-14 วันหลังจากฉีดเชื้อ เชื้อแบคทีเรีย *E. tarda* ลดจำนวนลงอย่างมีนัยสำคัญทางสถิติในช่วงท้ายหลังจากที่พบการกระจายตัวของเซลล์สร้าง IgM ผลการทดลองชี้ให้เห็นประสิทธิภาพการทำงานร่วมกันของเซลล์สร้าง IgM และเซลล์แมคโครฟาจที่เป็นส่วนประกอบของแกรนูโลมาในการเก็บกินและทำลายเชื้อแบคทีเรียชนิดนี้ได้

คำสำคัญ : *Edwardsiella tarda* IgM อิมมูนโนฮิสโตเคมี ปลานิล

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Introduction

Edwardsiella tarda is the causative agent of septicemic disease in freshwater and marine fish (Salati et al., 1987; Miyazaki et al., 1992; Darwish et al., 2000; Padros et al., 2006). It has been reported in humans as the cause of gastroenteritis and generalized infections mainly among individuals who are immunocompromised (Ling et al., 2000). This bacterium has the ability to escape from the serum- and phagocyte-mediated killing activity of the host, to invade host cell and tissue and to produce dermatotoxins and haemolysin causing disseminated septicemic condition (Srinivasa et al., 2001). The bacteria can survive and intracellularly replicate in phagocytic cells during septicemia. Induction of apoptosis by this bacterium prior to the inflammatory response is the critical step of generalized septicemia (Pirarat et al., 2007). Several attempts to study immune responses, including innate and specific response, against this bacterium have been made in a number of fish species. A high level of antibody titer during vaccination or challenge has been shown to be an effective protective mechanism against *E. tarda* (Salati et al., 1984; Salati et al., 1987; Gutierrez and Miyazaki, 1994). However, the specific distribution and the localization of antibody-secreting cells against this bacterium have never been shown and no study has yet been conducted to analyse the relationship between the fate of *E. tarda* and the specific immune response in vivo. The objectives of the present study were to assess the invasive ability of *E. tarda* in tilapia organs and to study the host immune response against *E. tarda* and the distribution of specific IgM response during *E. tarda* septicemia.

Materials and Method

Experimental fish and *E. tarda* infection

Forty tilapias, *Oreochromis niloticus*, 90-110 g in body weight, were placed in two 60-L tanks (control and infected group) under recycled water system at 25°C, with 5.5-6 ppm of dissolved oxygen (DO) and 6.5-7.0 in pH. The fish were acclimatized for 7 days before being separated into two groups. All fish were fed with commercial dry pellets throughout the experimental period. Fish were fed approximately 1% of body weight once a day. *E. tarda* E381 was given intraperitoneally at a concentration of 1×10^8 CFU/fish. The control group was injected with sterile phosphate buffer solution. Two weeks after infection, fish were sampled at the different time points for immunohistochemical study.

Histopathology

Tissues (brain, heart, gill, spleen, head and trunk kidney, liver, pancreas and intestine) were collected at 1, 3, 7, 14 and 30 days post infection (DPI) (six fish each) and fixed in 10% buffered formalin for histopathology. The fixed tissues were processed according to standard histological techniques and tissue sections were stained with Hematoxylin and Eosin (H&E).

Immunohistochemistry

Tissues (brain, heart, gill, spleen, head and trunk kidney, liver, pancreas and intestine) were collected at 1, 3, 7, 14 and 30 DPI and fixed in 10% buffered formalin. Tissues were routinely processed according to standard techniques and tissue sections were stained with H&E. Tissue samples were processed for immuno-histochemical study using rabbit polyclonal anti-*E. tarda* antibody (courtesy by Dr. Fukuda) (1:1000 dilution) as the primary antibody. Tissues sections were rehydrated in alcohol series, treated with 3% hydrogen peroxide in absolute methanol for 20 mins. and incubated with 1% bovine serum albumin for 30 mins. Slides were incubated at 4°C overnight after incubation with primary antibody. The tissue sections were incubated with the secondary antibody conjugated universal immuno-enzyme polymer using Histofine MAX PO kit (Nichirei, Japan) for 30 mins at room temperature, washed in PBS and either diaminobenzidine(DAB) or 3-amino, 9-ethyl-carbazole (AEC) substrate was applied. For head kidney and spleen, The interpretation of the immuno-histochemical stains was based on counting the number of positive *E. tarda* stainings in ten concentrated area at x40 (HPF) and obtaining an average number of *E. tarda* antigens per HPF for statistic analysis. To study the immune response against this bacterium, the antibody against tilapia IgM (1: 500) (Aquatic diagnostics, Scotland) was also applied as a primary antibody. The positive IgM immunoreactivity level was scored based on the frequency and intensity of positive signals per section on a scale of 0-3, with 0 representing no reactivity, 1 representing 1-50 positive signals, 2 representing 50-100 positive signals, 3 representing 100-200 positive signals.

Statistic analysis: For immunohistochemical scores, data were evaluated to analyze variance by using a non-parametric Mann-Whitney test. All tests used a significant difference level of $p< 0.05$.

Results

During *E. tarda* challenge, the remarkable histo-pahological change was granulomatous inflammation. This inflammatory response was initially observed after 3 DPI and widespread diffusion in tilapia organs, generating the septicemic condition (Fig.1). The *E. tarda* antigen was diffusely detected in various organs except the brain. The positive signals were identified within various cell types, including phagocytic cells, serosal mesothelia, myocardia, intestinal and gill epithelia and glomerular capillary endothelia. Immunolabeling was predominantly distributed in the head kidney, spleen, peritoneal tissues, pancreas, trunk kidney, gills, heart and liver respectively. The positive reaction in intestinal tissues was scantly observed in lamina propia and

mucosal epithelium throughout the experiment. Most positive reactions were intensely observed along the serosal surface of intestine at 1 to 3 DPI. In the trunk kidney, a positive reaction was clearly seen in glomeruli and interstitial tissues and rarely presented in the renal tubular epithelium. Immunolabeling in liver was randomly found in serosal surface at the early stage of infection (1 to 3 DPI). The gills showed positive immunolabeling in primary lamellar cells and occasionally in the secondary lamellar epithelium. The distribution of the positive reaction in the heart was mostly seen in myocardium, epicardium and pericardium respectively. At 1 DPI, the immunolabeling in the spleen was diffusely distributed especially in the peri-sinusoidal and ellipsoidal tissue. The distribution pattern of positive reaction in the head kidney and spleen was detectable within the independent phagocytic cells at 1 to 3 DPI. Positive signals were clearly observed within granuloma-participaing phagocytic cells at 7 and 14 DPI. The number of positive bacteria distributed within phagocytic cells of spleen and head kidney was highly observed at 3 DPI and this gradually decreased within granuloma participating cells at 7 to 14 DPI (Fig. 2). The immunolabeling antigen was significantly lower at 14 DPI than at other experimental periods. However, the immunolabeling bacteria could be detected within granuloma and melano-macrophage centers up to 30 DPI but at a much lower intensity and quantity (data not shown).

Immunohistochemical study on the distribution of IgM producing cells revealed the positive cells were mostly present in tilapia immune organs such as, the head kidney and spleen at 7 and 14 DPI (Fig. 3). The positive reaction was hardly detectable during the early stage of infection (1 to 3 DPI). The IgM producing cell was initially observable at 7 DPI. They distributed uniformly at the marginal zone of granuloma as a peri-granuloma arrangement. Most of positive IgM responses were clearly seen adjacent to granulomatous reaction at 14 DPI and some IgM producing cells were also incorporated into granulomatous nodules, forming rigid specific immune response against this bacterium. There was no statistical difference of immunolabeling IgM distribution between the head kidney and spleen throughout the experiment. IgM producing cells were also detectable adjacent to granuloma in other organs including, the liver, serosal peritoneum, trunk kidney and pancreas in the later stage of infection (7 to 14 DPI).

Discussion

An inflammatory response is one of the morphological evidences to study host immune response against infectious agents in fish. Several authors have described the inflammatory response of Japanese eel and Japanese flounder to *E. tarda* as suppurative. In contrast, they described the response of red sea bream, Pagrus major, and tilapia, *Tilapia nilotica*, as granulomatous (Miyazaki et al., 1992; Darwish et al., 2000; Padros et al., 2006). Our study confirmed that the granulomatous response was present after 3 Days-PI and persisted up to 1 month-PI in tilapia tissues during *E. tarda* septicemia.

In spite of many studies on the in vitro effects of *Edwardsiella tarda* on immune cells, the fate of *E. tarda* in vivo has not been studied. The ability of the body to eliminate *E. tarda* may determine how long this bacterium can exert its effects on the immune system. The bacteria can persist up to 1 month in granuloma and in melano-macrophage center and no extra-cellular positive reaction was found throughout the experiment. The study confirmed the intracellular invasive ability of *E. tarda* disseminated in the tissues during septicemia. Macrophage was permissive for *E. tarda* replication. The granuloma-participating phagocytic cells and the melano-macrophage centres were the site of *E. tarda* persistence in fish.

IgM is a major component of the teleost humoral immune system against bacteria, parasites and viruses (Ellis, 1986). Immunohistochemical studies using antibody raised against IgM have been used to describe the ontogeny of B cells in fish (Castillo et al., 1993; Schroder et al., 1998; Press et al., 1999; Uchida et al., 2000).
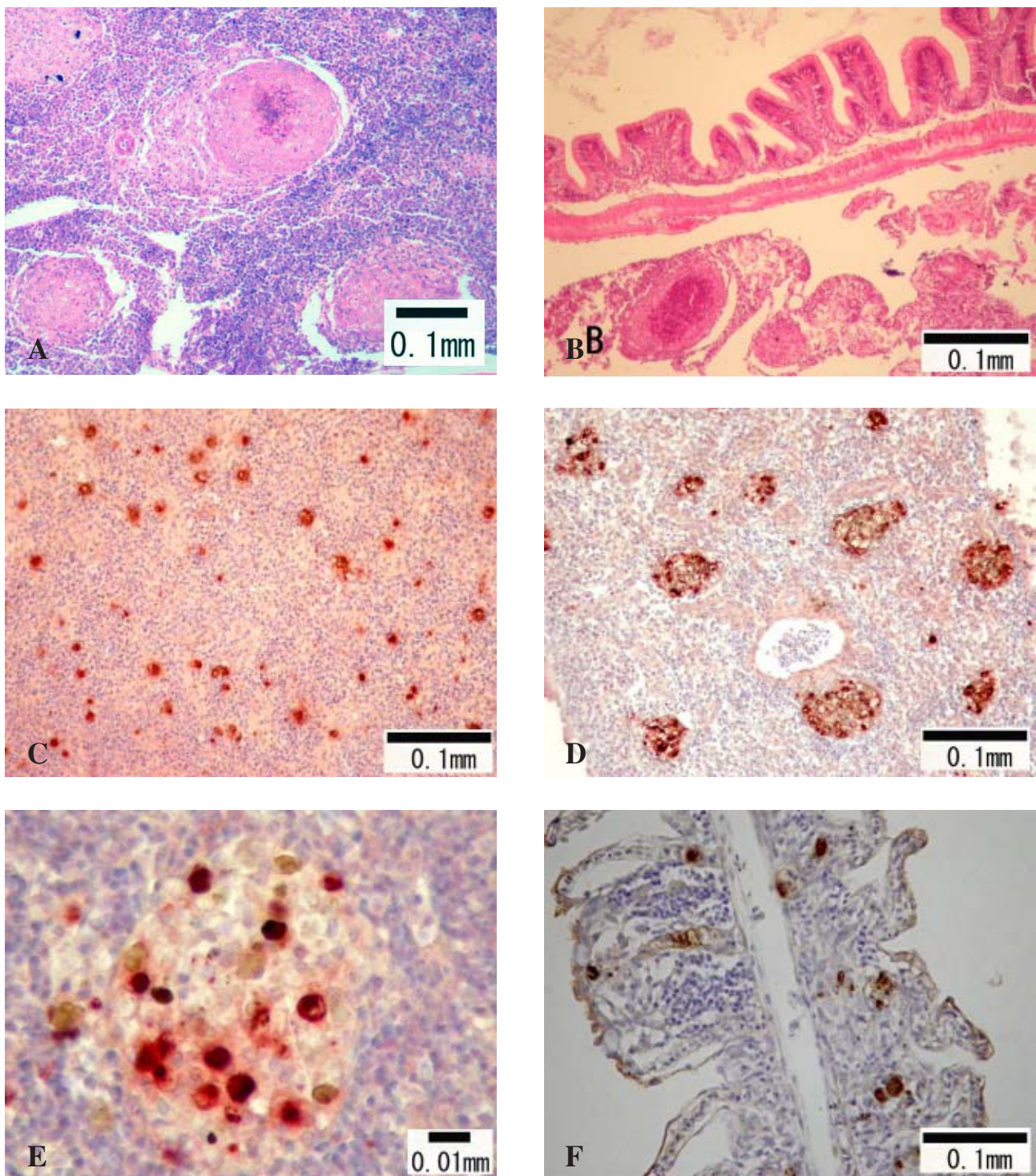
Figure 1 Histopathology of tilapia after intraperitoneally challenged with *Edwardsiella tarda*. In head kidneys, necrotic formation was detectable in 1 DPI and gradually shifted to granuloma formation at the late stage (A). Severe acute peritonitis with early stage of granulomatous formation was seen at 3 DPI (B). In head kidney and spleen, an immunohistochemistry using polyclonal antibody against *E. tarda* revealed the positive antigens localized in the independent phagocytic cells at the early stage (C) and within granuloma at the late stage (D&E). Bacterial antigens widely distributed in internal organs including secondary gill lamellae (F).
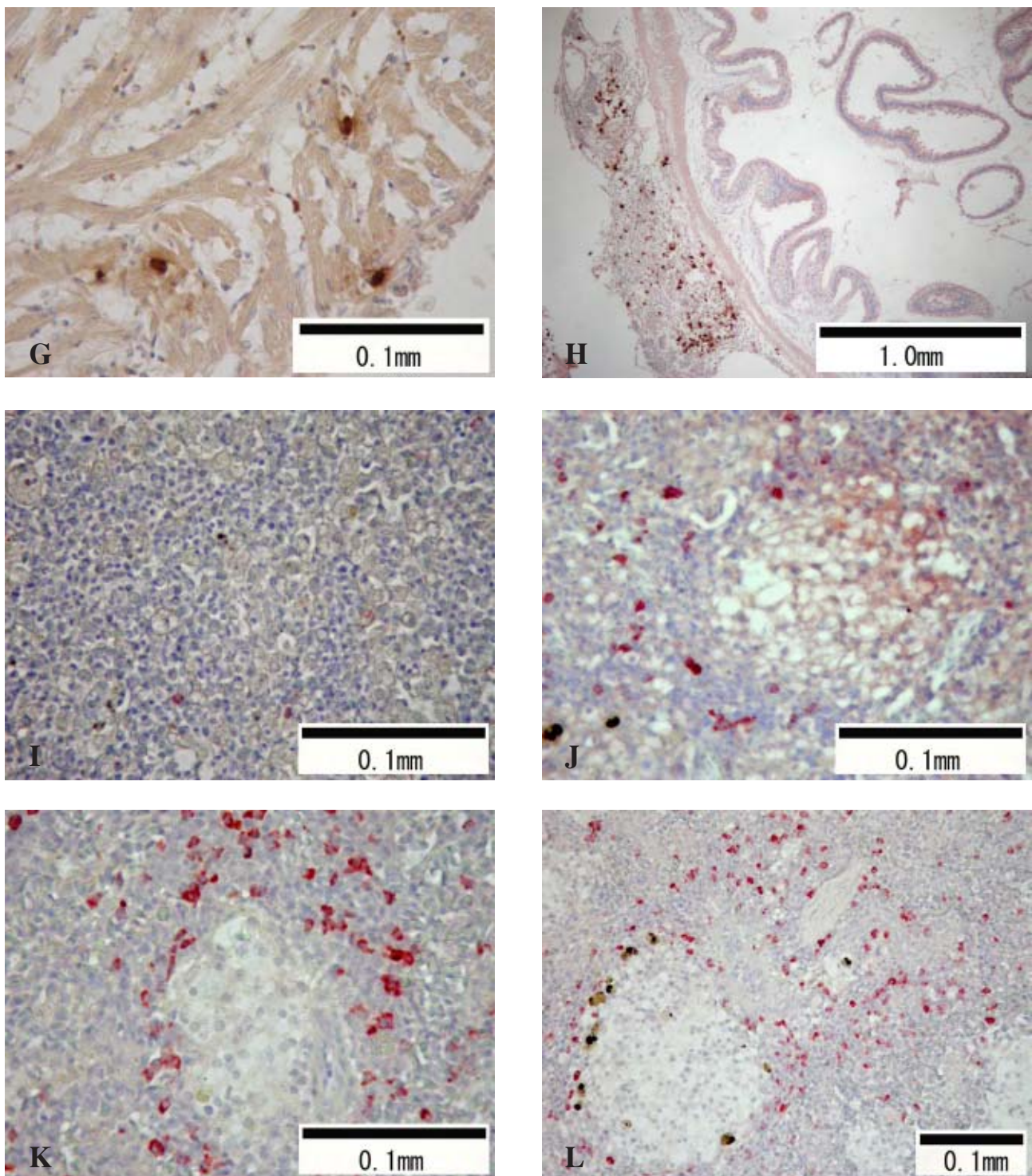
Figure 1 Histopathology of tilapia after intraperitoneally challenged with *Edwardsiella tarda*. In head kidneys, necrotic formation was detectable in 1 DPI and gradually shifted to granuloma formation at the late stag. Bacterial antigens widely distributed in myocardium (G) and serosal peritoneum (H). The antibody response against this bacterium was scantly seen at 3 DPI (I). IgM p roducing cells were clearly detected at 7 DPI (J) and remarkably observable in the marginal zone of granulomatous lesions at 14 DPI (K&L).
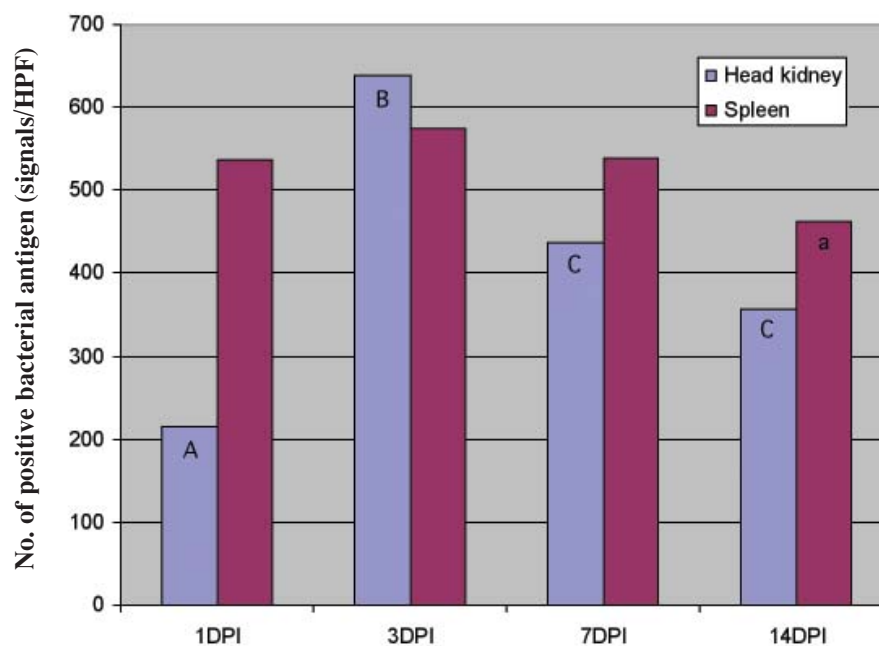
Figure 2. Average number of immunolabeling *E. tarda* antigens in the head kidney and spleen of infected tilapias. Different capital and small letters differ significantly at *p*< 0.05 among trial periods in each organ(n=6).
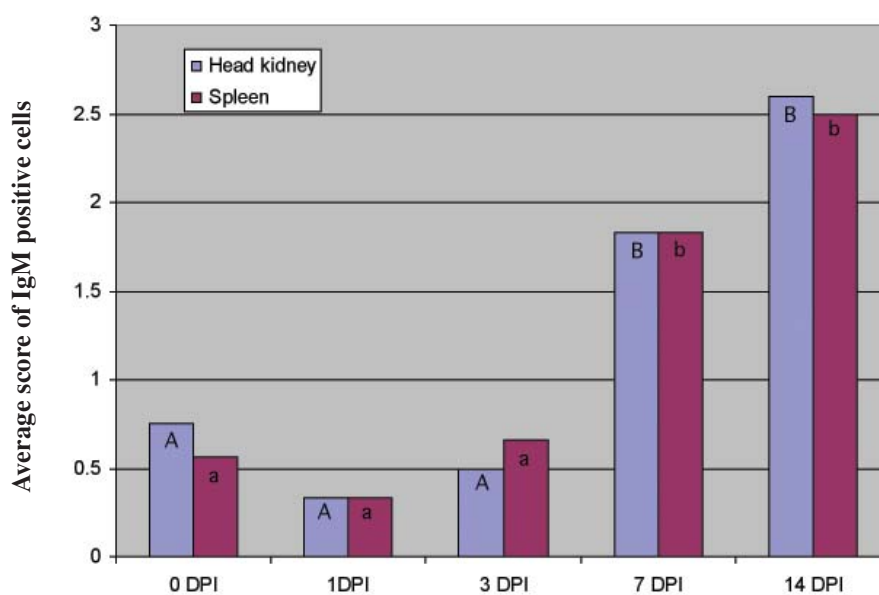

Figure 3. Average score of IgM positive cells in the head kidney and spleen of tilapias infected with *E. tarda* at different times post-infection(n=6). Significant statistic differences in each organ at different times are indicated using capital and small letters(n=6).

The present paper describes the localization of IgM containing cells involved in the primary immune response during *E. tarda* septicemia. These IgM producing cells are mostly distributed into the marginal zone of granuloma area and were believed to be a specific antibody response against *E. tarda*. Interestingly, some IgM producing cells obviously participated in granulomatous formation, confirming that the B cell is also the major cell component in the granulomatous reaction as seen in other higher vertebrates. The result also showed that *E. tarda* antigens were significantly reduced during the appearance of the IgM producing cells at 7 to 14 DPI, suggesting the accumulation of IgM at the site of infection was the crucial step in eliminating

E. tarda antigen. The enhancement in phagocytic activity of granuloma-participating cells was influenced by these IgM cells. This synergistic action showed the effective response in the clearance and the elimination of *E. tarda* in vivo according to several reports which have examined the higher protective antibody response after immunization with various extracts of *E. tarda* preparations (Salati et al., 1984; Salati et al., 1987; Gutierrez and Miyazaki, 1994).

In conclusion, this immunohistochemical study allows the visualization of the site of *E. tarda* antigen distribution correlating with histopathological changes and the better understanding of the specific tilapia immune response against *E. tarda*. Granuloma-participating phagocytic and IgM containing cells showed themselves to be a superb defensive mechanism against this intracellular pathogen during generalized septicemia.

Acknowledgements

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