***In Vivo* Analysis on the Adherence and Infection Route of *Photobacterium damsela* subsp. *piscicida* in Yellowtail**

Ichiro Nagano¹, Syun-ichirou Oshima¹ and Kenji Kawai^{2*}

¹Graduate School of Kuroshio Science, Kochi University, Kochi 783-8502, Japan

²Fish Disease Laboratory, Department of Aquaculture, Kochi University, Kochi 783-8502, Japan

(Received February 10, 2011)

ABSTRACT—Pseudotuberculosis caused by *Photobacterium damsela* subsp. *piscicida* is a serious problem in fish culture industry. The initial stage of host-pathogen interaction is primarily important to understand infectious disease. However, the mode of transmission and route of infection of the agent are not fully studied. In this study, changes in the distribution of the bacterium on and in yellowtail *Seriola quinqueradiata* body during infection process was investigated using immersion challenge. The result revealed that external regions such as the lower jaw, pectoral fin, skin and gills permitted bacterial adherence soon after immersion. Viable bacterial counts increased in the gills soon after infection, then they increased in the blood, spleen and kidney. Moreover, fish were exposed to bacteria at the gills or posterior part of body surface to elucidate the primary infection route. The fish group challenged at gills showed higher mortality than the other group. These results reveal that the gills are the primary regions for *P. damsela* subsp. *piscicida* to invade and proliferate in yellowtail.

Key words: bacterial adherence, gill, infection route, *in vivo* analysis, *Photobacterium damsela* subsp. *piscicida*

Pseudotuberculosis, also referred to as pasteurellosis, exhibits whitish tubercle-like tissue structures consisting of bacterial accumulations and surrounding epithelial cell layers in the internal organs of diseased fish (Kubota *et al.*, 1970a; 1970b; 1972). The aetiological agent of pseudotuberculosis is a Gram-negative bacterium *Photobacterium damsela* subsp. *piscicida* (Truper and DeClari, 1997). The disease was first described in wild populations of white perch *Morone americanus* and striped bass *M. saxatilis* in 1963 during a massive epizootic in the upper region of Chesapeake Bay, USA (Snieszko *et al.*, 1964). A few years later, pseudotuberculosis emerged in yellowtail *Seriola quinqueradiata* culture in Japan (Kimura and Kitao, 1971). Soon thereafter, the disease spread to other fish species in Japan, and several epizootics were described in red sea bream *Pagrus major* (Yasunaga *et al.*, 1983), red grouper *Epinephelus akaara* (Ueki *et al.*, 1990), Japanese flounder *Paralichthys olivaceus* (Fukuda *et al.*, 1996) and so on. The use of the disease name “pseudotuberculosis” is restricted only for infection in yellowtail as proposed by the Japanese Society of Fish Pathology (2010), however, in the present paper the disease name is applied also to infection in

other fish species (pasteurellosis) to avoid confusion. Toranzo *et al.* (1991) described the first outbreak of the disease in Spain, which affected juvenile gilthead sea bream *Sparus aurata* cultures in the north-western region of Spain. Simultaneously, outbreaks occurred also in south-western Spain, France, Italy, and Portugal, mainly in populations of sea bream and sea bass *Dicentrarchus labrax* (Magariños *et al.*, 1996). The hosts of the pathogen have been reported in various species of marine fishes (Austin and Austin, 1999). Pseudotuberculosis has affected aquaculture industry mainly yellowtail culture in Japan and the Mediterranean aquaculture industry of sea bream and sea bass.

Although the pathology of pseudotuberculosis is well documented (Noya *et al.*, 1995), little information is available on the initial stages of host-pathogen interaction. The first step, attachment to the host, plays an important role in initiating infection with pathogens (Marques *et al.*, 1984; Oldham and Rodgers, 1985). It was indicated that an *in vitro* assay for adhesive ability was useful to evaluate bacterial invasiveness (Oldham and Rodgers, 1985; Sansonetti *et al.*, 1986; Jande *et al.*, 1991). In the case of pseudotuberculosis, it was shown that the gill is the important organ in the process of pseudotuberculosis (Nagano *et al.*, 2011) and *P. damsela* subsp. *piscicida* has the ability to adhere to and invade fish epithelial cells (Yoshida *et al.*,

* Corresponding author
E-mail: kenkawai@kochi-u.ac.jp

1997; Lopez-Doriga *et al.*, 2000). Moreover, Nagano *et al.* (2009) indicated that experimental immersion infection induced moderate mortality and clinical signs similar to natural infection. These results suggest that the possible pathways of entry for the bacterium into fish body are external organs such as the skin, gills and oral cavity which contact with outer environment.

In this study, the distribution of *P. damsela* subsp. *piscicida* on and in fish body at the early stage of immersion-infection was investigated. Adhering bacteria on the external areas of yellowtail were determined qualitatively, then change in bacterial count in various internal tissues was determined quantitatively. Furthermore, body part-limited infection to yellowtail was performed, and the mortality of fish was examined. Thus, infection route was analyzed.

Materials and Methods

Bacterial strain and culture

O-82352 strain identified as *P. damsela* subsp. *piscicida* was used. The strain was originally isolated from the kidney of yellowtail during an outbreak of pseudotuberculosis at a culture farm in Oita Prefecture, Japan in 1998 (Nagano *et al.*, 2009). The strain was stored at -80°C . After thawed, the bacterium was inoculated into 7-mL brain heart infusion broth (Difco) supplemented with 1.5% NaCl (BHI), and pre-cultured at 25°C with shaking at 100 rpm for 16 h. Then, 0.75 mL of the suspension was inoculated into a 300 mL of BHI broth, which was incubated at 25°C with shaking at 100 rpm until middle to late logarithmic culture phase for 15–17 h.

Experimental fish

Yellowtail, mean body weight was 4 g, were obtained from an aquaculture farm, which had been originally captured off the coast of Tosa Bay, Kochi Prefecture and had no history of pseudotuberculosis. The fish were kept in 1,100-L fiberglass-reinforced plastic (FRP) tanks being supplied with flowing, sand-filtered and aerated seawater. The fish were fed daily with commercial pellets (Nissui) except for the day before and on the day of transfer and experimental treatments. At least three days before experimental infection, fish were transferred into enclosed circulation FRP tanks (infection tanks), and water temperature was adjusted at 24°C – 25°C . Before transfer, some samples of fish were examined to verify the absence of *P. damsela* subsp. *piscicida* by bacterial isolation and microscopic observation on the slide glass imprints of the spleen, kidney and gills. Just before experimental infections, all the experimental fish were anaesthetized with seawater containing ethylene glycol monophenyl ether (1/5,000, 3 min).

Adherence of bacteria to external tissues

Three independent experiments were performed. In every experiment, the fish were immersed in tanks with aerated seawater containing 17-h cultured O-82352 strain at 25°C for 10 min. The concentration of bacteria in the seawater and the body weight of fish were shown in Table 1. After immersion, each fish group was transferred to another tank containing same volume seawater as immersion treatment, where fish were left swimming in the seawater freely for 5 min so that non-adherent bacteria should be washed out. The fish were then anesthetized in 1 L of seawater containing the lethal dose of ethylene glycol monophenyl ether. The whole lower jaw (LJ), whole pectoral fin (PF), trunk skin (S) and whole outer most gill filaments (G) were cut off from the fish. The tissue specimens were put into sterilized seawater (1 mL for 6-g fish or 5 mL for 13-, 11- and 161-g fish) and shaken strongly. The bacteria-containing seawater were ten-fold diluted using sterilized seawater, and then cultured on BHI agar at 25°C for 30 h. *P. damsela* subsp. *piscicida* colonies were identified by agglutination reaction using an anti-*P. damsela* subsp. *piscicida* O-82352 strain rabbit serum prepared in our laboratory (Nagano *et al.*, 2009) and counted.

Change in the distribution of bacteria in infected yellowtail

Yellowtail, mean body weight was 110 g, were used. Three groups of fish (36 fish for group A and 35 fish each for groups B and C) were immersed in 35 L of aerated seawater containing 15-h cultured O-82352 strain at a concentration of 9.0×10^6 CFU/mL at 25°C for 10 min. For the control group, 35 fish were immersed in 35 L of seawater for 10 min. After immersion infection, fish groups were reared in 800-L infection tanks separately. Group A was used for the mortality record. From groups B and C, one living fish each was taken periodically (total 43 times, 0–3 h interval from 0 to 75 h after immersion, 1–48 h interval from 75 to 240 h after immersion), and viable bacterial count in the blood, gills, spleen and kidney was determined according to the method below. Blood was drawn from the blood vessel near the tail using a syringe. The gills, spleen and kidney were put into 1 mL of sterilized seawater and homogenized using a homogenizer pestle (1 mL scale, AS ONE). After strongly shaken, samples were placed for 30 s to precipitate tissue debris. The upper layer of the supernatant was subjected to bacterial count (CFU/g). The blood samples were subjected directly to bacterial count (CFU/mL). The tissue homogenates were ten-fold diluted using sterilized seawater, and then cultured on BHI agar at 25°C for 30 h. The identification of colonies was performed by agglutination reaction using an anti-*P. damsela* subsp. *piscicida* O-82352 strain rabbit serum and counted. From the control group, two fish were taken up and bac-

terial count in the blood, gills, spleen and kidney were determined at 48, 114 and 240 h after immersion.

Comparison of immersion and oral infection

As shown in Table 2, three independent experimental infections using 17-h cultured O-82352 strain were conducted to compare mortalities between immersion and oral infection. Immersion infection was carried out by immersing fish in aerated seawater (5 L for 7-g fish or 30 L for 18- and 29-g fish) containing bacteria for 10 min at 25°C. Control fish in each experiment were immersed in seawater for 10 min. Oral infection was carried out by compulsory administration. The fish were administered with 0.1 mL of bacterial suspension into the stomach using cannulae. Control fish were administered 0.1 mL of sterile phosphate-buffered saline (PBS). All the fish groups were reared in 200-L infection tanks and mortality was recorded for 240 h.

Experimental infection by body-regional exposure to bacteria

Yellowtail, mean body weight was 37 g, were used. The 16-h culture of O-82352 strain at a concentration of 1.4×10^7 CFU/mL was dropped onto the gill filament of 16 fish individually (gill-specific infection group). The infection dose was calculated as 2.8×10^6 CFU/0.2 mL/gill. The rear half-body part (including dorsal, anal and caudal fins) of another 19 fish was dipped into similar bacterial suspension at a concentration of 1.4×10^7 CFU/mL for 5 s individually (skin-specific infection group). For control, seawater was dropped onto the gill lamellae of 15 fish. After treatment, individual fish were soon (within 3 s) put in three different tanks according to treatments. Mortality was recorded for 240 h. Difference in mortality was analyzed using Fisher's exact probability test (*F*-test).

Results

Bacterial adherence to external tissues of yellowtail

The results are shown in Table 1. Because external organs examined were different in size and surface

structure, the data given in Table 1 are qualitative rather than quantitative. There was no clear relation between adhering bacterial number and fish size, but higher number of adhering bacteria was detected in the gills in all experiments.

Distribution of bacteria in infected yellowtail

The mortality results are shown in Fig. 1a. Mortality started 69 h after infection and continued until 138 h after infection. Final mortalities at 240 h after infection in infected fish group and control group were 72% and 0%, respectively.

In the sampling groups (B and C), totally 27 fish were dead before sampling. Totally 43 living fish were taken up and bacterial count in/on the blood, gills, spleen and kidney were determined. Throughout experimental period, bacteria were detected more frequently in the gills (37/43, 86%) than in the blood (11/43, 26%), spleen (17/43, 40%) and kidney (18/43, 42%). Figure 1b shows bacterial count in the blood of infected yellowtail. No bacterium was detected between 0 and 39 h after infection. At 40 h after infection, bacteria were detected for the first time, when the bacterial count was 1.3×10^2 CFU/mL. Bacteria were detected with high frequency between 40 and 91 h after infection; the maximum number of bacteria was 1.7×10^6 CFU/mL at 72 h after infection. After 91 h, detection frequency decreased.

Figure 1c shows bacterial count in/on the gills. Bacteria were detected with a high frequency throughout 240 h experimental period. The maximum bacterial count was 3.9×10^5 CFU/g at 72 h after infection.

Figure 1d and 1e show bacterial count in the spleen and kidney, respectively. No bacterium was detected for 15 h after infection. At 18 h after infection, bacteria were detected for the first time when bacterial count was 2.1×10^3 CFU/g in the spleen and 1.1×10^3 CFU/g in the kidney. High detection frequency was shown from 34 to 139 h after infection with the maximum bacterial count of 7.8×10^6 CFU/g in the spleen and 9.2×10^6 CFU/g in the kidney at 72 h after infection. After 139 h, no bacterium was detected in both organs.

No bacterium was detected in the blood, gills,

Table 1. Relative adherence of *Photobacterium damsela* subsp. *piscicida* on the lower jaw (LJ), pectoral fin (PF), skin (S) and gill filament (G) of immersion-infected yellowtail

Fish group, weight $\pm$ SD	Infection dose (CFU/mL)	Relative adherence ^a of bacteria at			
		whole LJ	whole PF	S ^b	G ^c
6 $\pm$ 0.8 g (n = 5)	1.7×10^7	+ (1), ++ (4)	++ (5)	++ (5)	++ (3), +++ (2)
13 $\pm$ 1.6 g (n = 4)	4.1×10^6	++ (4)	NE ^d	– (3), + (1)	++ (4)
161 $\pm$ 11.0 g (n = 3)	8.0×10^6	++ (2), +++ (1)	++ (3)	– (1), ++ (2)	++ (1), +++ (2)
11 $\pm$ 0.5 g (n = 3)	8.0×10^6	++ (3)	++ (3)	– (1), + (2)	++ (3)

^a –, below detection limit; +, 10^2 order; ++, 10^3 order; +++, 10^4 order. Figures in parentheses indicate number of fish.

^b 12 mm $\times$ 12 mm for 161 g fish and 5 mm $\times$ 5 mm for other size fish.

^c Per one gill filament.

^d Not examined.

spleen and kidney of control fish at 48, 144 and 240 h after infection (data not shown).

Comparison of immersion and oral infection

The results of mortality at 240 h after immersion and oral infection are shown in Table 2. In experiment using the 7-g fish, mortality in the immersion-infected groups began from 48 h after infection. All the infected fish died in two to 96 h after infection. In the control group, mortality was not observed. In the orally-infected groups, only one fish died with the highest dose infection.

In experiment using the 18-g fish, mortality in the immersion-infected groups began from 48 or 72 h after infection and lasted until 168 h after infection. In all immersion-infected groups, mortalities were over 90%. In the orally-infected groups, totally two fish died; one fish in the 10^6 CFU infected group and one fish in the 10^7 CFU infected group. One dead fish occurred in the control group. However, *P. damsela* subsp. *piscicida* was not detected from this fish.

Fig. 1. Cumulative mortality (a) and bacterial count in/on the blood (b), gill filament (c), spleen (d) and kidney (e) in infected fish group. In Fig. 2a, black circles and white circles indicate infected fish and uninfected control fish, respectively. Dashed lines represent detection limit.

Table 2. Mortality in immersion- and orally-infected yellowtail with *Photobacterium damsela* subsp. *piscicida*

Fish group, weight $\pm$ SD	Immersion infection		Oral infection	
	Infection dose (CFU/mL)	Dead/used fish	Infection dose (CFU/fish)	Dead/used fish
7 $\pm$ 0.4 g	1.6×10^7	5/5	1.7×10^7	1/10
	1.8×10^6	5/5	1.7×10^6	0/10
	1.8×10^5	5/5	1.7×10^5	0/10
	Control ^a	0/5	Control ^b	0/10
18 $\pm$ 2.1 g	2.8×10^7	11/11	3.0×10^7	1/14
	3.0×10^6	18/20	3.0×10^6	1/14
	3.1×10^5	20/20	3.0×10^5	0/14
	3.1×10^4	15/15	3.0×10^4	0/14
	Control ^a	0/20	Control ^b	1/14
29 $\pm$ 4.9 g	8.7×10^6	15/15	8.8×10^6	0/10
	8.7×10^5	14/14	8.8×10^5	0/10
	8.7×10^4	10/15	8.8×10^4	0/10
	8.7×10^3	2/15	8.8×10^3	0/10
	8.7×10^2	0/15	Control ^b	0/10
	Control ^a	0/15		

^a As control for immersion infection, fish were immersed in seawater.

^b As control for oral infection, fish were administrated PBS.

In experiment using the 29-g fish, mortality in the immersion infected groups began from 48 to 120 h after infection and lasted until 144 h after infection. Final mortality showed a correlation with infection dose. In the orally-infected groups including control group, mortality was not observed throughout experimental period.

Experimental infection by body-regional exposure to bacteria

The results of gill-specific and skin-specific infections are shown in Fig. 2. In the gill-specific infection group, mortality occurred from 96 h after infection and continued until 168 h after infection. In the skin-specific infection group, only two fish died at 96 h after infection. Final mortalities after 240 h for the gill-spe-

Fig. 2. Cumulative mortalities in yellowtail by body-regional exposure to bacteria. Black circles, gill-specific infection; black squares, skin-specific infection; white circles, control.

cific infection group, skin-specific infection group and control group were 7/16 (44%), 2/19 (11%) and 0/15 (0%), respectively. Significant difference was detected between mortalities of the two infection groups ($P < 0.05$).

Discussion

In vitro assay showed the possibility of *P. damsela* subsp. *piscicida* to attach onto cultured fish cells or fish tissues (Magariños *et al.*, 1996; Yoshida *et al.*, 1997; Lopez-Doriga *et al.*, 2000). However, *in vivo* assay using living fish has not yet generalized. One reason for the difficulty of *in vivo* assay is that there are two or more possible pathways for bacterial entry (Kawahara *et al.*, 1989). Another reason is that mortality or infectivity is variable by infection methods or experimental conditions (Kimura and Kitao, 1971; Wakabayashi *et al.*, 1977; Fukuda and Kusuda, 1981; Magariños *et al.*, 1996). Concerning the latter difficulty, authors have established a repeatable and appropriate experimental infection method by immersion infection recently (Nagano *et al.*, 2009). In the present study, the immersion infection method was applied to investigate the early process of infection by *in vivo* assay.

In the first step, adherence of *P. damsela* subsp. *piscicida* was compared among different regions on yellowtail body. The experimental results showed that the lower jaw, pectoral fin, skin and gills of fish permitted bacterial adhesion. Kawahara *et al.* (1989) suggested that the infection of *P. damsela* subsp. *piscicida* can occur through plural tissues from observations by fluorescent antibody technique. If adhering sites were identical to the entry route of the bacterium into its host, *P. damsela* subsp. *piscicida* may have multiple routes for infection. As adherence experiment in present study contained washing processes to remove weakly attaching bacteria, body regions where high level of adherence occurred was confirmed. The result demonstrated that *P. damsela* subsp. *piscicida* tightly adhered to above external tissues of host fish.

In our previous study, it was shown that the gills are the key tissue of this disease (Nagano *et al.*, 2009, 2011). Kawahara *et al.* (1989) observed that when fish were infected by immersion, bacteria were found in the gills, and then rapidly spread to various internal organs. However, the observation was not quantified. In present study, infection process was monitored quantitatively in the gills and internal organs. Bacterial count increased in the gills soon after infection, however, the bacterium was not detected in the blood, spleen or kidney until 18 h (spleen and kidney) and 40 h (blood) after infection. The high bacterial count in the gills, even if it is considered that the surface area of the gills is large, indicates a possibility that this pathogen selectively invade to the gills because this tissue is generally

protected by non-specific defense factors. The result demonstrates that the gills are the possible portal route and the suitable organ for bacterial proliferation. Bacteria were detected more frequently in the gills than in the blood, spleen and kidney until the later period of infection. However, the maximum viable bacterial count in the gill (3.9×10^5 CFU/g) was lower than those in the blood (1.7×10^6 CFU/mL), spleen (7.8×10^6 CFU/g) and kidney (9.2×10^6 CFU/g). These data indicate that the gills are the "bridgehead" tissue to initiate infection, and the spleen and kidney are suitable final tissues for bacterial proliferation to accomplish it. It is also interesting that the first detection of bacteria in the blood was later than in the spleen and kidney, and bacterial detection rate in the blood was obviously low throughout experimental period. The results suggest that the blood is a pathway rather than the place where this bacterium proliferates. In conclusion, the gills are the starting and principal sites of early infection process. Bacteria comfortably proliferate in this organ then spread to internal organs via the blood.

Experimental immersion infection method has similar processes with natural infection from a view point that pathogens existing in environmental water invade into fish body. This process involves the attaching of bacteria to external tissues as well as penetrating through gastric and/or intestinal tissues. The results of present study that compared immersion infection and oral infection denied the latter provability. Oral entry route was approved in previous study (Wakabayashi *et al.*, 1977), however, the authors also suggested that the oral infection experiment was carried out with too much infection dose. Kawahara *et al.* (1989) showed increase in bacterial count in orally infected fish, however, it was not clearly shown if the infected fish died or not after infection experiment. Present study demonstrates the possibility that external tissues such as gills, skin and fins are the entry routes of *P. damsela* subsp. *piscicida*. Further, the contribution of the gills for bacterial entry was demonstrated by an experimental result that gill-specifically infected fish had higher mortality than skin-specifically infected fish. This indicates that the gills are the main entry route, and that the skin is the secondary possible entry route. As discussed in Galan (1994), Leung *et al.* (1996), Tan *et al.* (1998) and Wang *et al.* (1998), the invading ability of pathogens into host body is a key determinant for their virulence. The invading process of *P. damsela* subsp. *piscicida* in the gill of host fish is the issue of proof to elucidate the infection mechanism of pseudotuberculosis.

References

- Austin, B. and D. A. Austin (1999): 2 Characteristics of the diseases. In "Bacterial fish pathogens: diseases of farmed and wild fish (third edition)" (ed. by Austin, B. and

- D. A. Austin). Springer-Verlag, Berlin, Heidelberg, New York, pp. 13–124.
- Fukuda, Y. and R. Kusuda (1981): Efficacy of vaccination for pseudotuberculosis in cultured yellowtail by various routes of administration. *Nippon Suisan Gakkaishi*, **47**, 147–150. (in Japanese with English abstract)
- Fukuda, Y., M. Matsuoka, Y. Mizuno and K. Narita (1996): *Pasteurella piscicida* infection in cultured juvenile Japanese flounder. *Fish Pathol.*, **31**, 33–38. (in Japanese with English abstract)
- Galan, J. E. (1994): Interaction of bacteria with non-phagocytic cells. *Curr. Opin. Immunol.*, **6**, 590–595.
- Jande, J. M., S. L. Abbot and L. S. Oshiro (1991): Penetration and replication of *Edwardsiella* spp. in HEp-2 cells. *Infect. Immun.*, **59**, 154–161.
- Kawahara, E., K. Kawai and R. Kusuda (1989): Invasion of *Pasteurella piscicida* in tissues of experimentally infected yellowtail *Seriola quinqueradiata*. *Nippon Suisan Gakkaishi*, **55**, 499–501.
- Kimura, M. and T. Kitao (1971): On the causative agent of tuberculoidosis of yellowtail. *Fish Pathol.*, **6**, 8–14. (in Japanese with English abstract)
- Kubota, S., M. Kimura and S. Egusa (1970a): Studies of a bacterial tuberculoidosis of the yellowtail–1. Symptomatology and histopathology. *Fish Pathol.*, **4**, 111–118. (in Japanese)
- Kubota, S., M. Kimura and S. Egusa (1970b): Studies on a bacterial tuberculoidosis of the yellowtail–2. Mechanism of nodule formation. *Fish Pathol.*, **5**, 31–34. (in Japanese)
- Kubota, S., M. Kimura and S. Egusa (1972): Studies on a bacterial tuberculoidosis of the yellowtail–3. Findings on nodules and bacterial colonies in tissues. *Fish Pathol.*, **6**, 69–72. (in Japanese)
- Leung, K. Y., T. M. Lim, T. J. Lam and Y. M. Sin (1996): Morphological changes in carp epithelial cells infected with *Aeromonas hydrophila*. *J. Fish. Dis.*, **19**, 167–174.
- Lopez-Doriga, M. V., A. C. Barnes, N. M. S. Santos and A. E. Elis (2000): Invasion of fish epithelial cells by *Photobacterium damsela* subsp. *piscicida*: evidence for receptor specificity, and effect of capsule and serum. *Microbiol.*, **146**, 21–30.
- Magariños, B., A. E. Toranzo and J. L. Romalde (1996) Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. *Ann. Rev. Fish. Dis.*, **6**, 41–64.
- Marques, L. R. M., M. R. F. Toledo, N. P. Silva, M. Magalhaes and L. R. Trabulsi (1984): Invasion of HeLa cells by *Edwardsiella tarda*. *Curr. Microbiol.*, **10**, 129–132.
- Nagano, I., S. Inoue, K. Kawai and S. Oshima (2009): Repeatable immersion infection with *Photobacterium damsela* subsp. *piscicida* reproducing clinical signs and moderate mortality. *Fish. Sci.*, **75**, 707–714.
- Nagano, I., S. Oshima and K. Kawai (2011): Importance of gills for development of pseudotuberculosis at early phase of infection in amberjack. *Fish Pathol.*, **46**, 31–33.
- Noya, M., B. Magariños, A. E. Toranzo and J. Lamas (1995): Sequential pathology of experimental pasteurellosis in gilthead seabream *Sparus aurata*. A light-and electron-microscopic study. *Dis. Aquat. Org.*, **21**, 177–186.
- Oldham, L. J. and F. G. Rodgers (1985): Adhesion, penetration and intracellular replication of *Legionella pneumophila*, an *in vitro* model of pathogenesis. *J. Gen. Microbiol.*, **131**, 697–706.
- Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli and J. Mounier (1986): Multiplication of *Shigella flexneri* within HeLa cells: lysis of the pathocytic vacuole and plasmid-mediated contact hemolysis. *Infect. Immun.*, **51**, 461–469.
- Snieszko, S. F., G. L. Bullock, E. Hollis and J. G. Boone (1964): *Pasteurella* sp. from an epizootic of white perch (*Roccus americanus*) in Chesapeake Bay tidewater areas. *J. Bacteriol.*, **88**, 1814–1815.
- Tan, E., K. W. Low, W. S. F. Wong and K. Y. Leung (1998): Internalization of *Aeromonas hydrophila* by fish epithelial cells can be inhibited with a tyrosine kinase inhibitor. *Microbiol.*, **144**, 299–307.
- The Japanese Society of Fish Pathology (2010): Selected name of fish diseases. *Fish Pathol.*, **45**, 186–197. (in Japanese)
- Toranzo, A. E., S. Barreiro, J. F. Casal, A. Figueras, B. Magariños and J. L. Barja (1991): Pasteurellosis in cultured gilthead seabream (*Sparus aurata*): first report in Spain. *Aquaculture*, **99**, 1–15.
- Truper, H. G. and L. DeClari (1997): Taxonomic note: necessary correction of epithets formed as substantives (nouns) “in apposition”. *Int. J. Syst. Bacteriol.*, **47**, 908–909.
- Ueki, N., Y. Kayano and K. Muroga (1990): *Pasteurella piscicida* infection in juvenile red grouper (*Epinephelus akaara*). *Fish Pathol.*, **25**, 43–44. (in Japanese)
- Wakabayashi, H., H. Toyota and S. Egusa (1977): Artificial infection of yellowtail with a gastral administration of cultured *Pasteurella piscicida* cells. *Fish Pathol.*, **11**, 207–211. (in Japanese with English abstract)
- Wang, X. H., H. L. Oon, G. W. P. Ho, W. S. F. Wong, T. M. Lim and K. Y. Leung (1998): Internalization and cytotoxicity are important virulence mechanisms in vibrio-fish epithelial cell interactions. *Microbiol.*, **144**, 2987–3002.
- Yasunaga, N., K. Hatai and J. Tsukahara (1983): *Pasteurella piscicida* from an epizootic of cultured red sea bream. *Fish Pathol.*, **18**, 107–110. (in Japanese with English abstract)
- Yoshida, M., V. Inglis, N. Misawa, R. Kruger and M. Sakai (1997): *In vitro* adhesion of *Pasteurella piscicida* to cultured fish cells. *J. Fish. Dis.*, **20**, 77–80.