魚病研究 Gyobyo Kenkyu, 27 (2), 103–108, 1992. 6

Pasteurella piscicida Infection in Hatchery-Reared Juvenile Striped Jack

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(Received February 17, 1992)

A mass mortality due to an infectious disease occurred in juvenile striped jack (*Pseudocaranx dentex*) reared at a station of the Japan Sea-Farming Association in Nagasaki Prefecture in February, 1991 and a total of about 10,000 juveniles (34%) were lost for about one month. Administrations of oxytetracycline and oxolinic acid with food were effective to decrease the mortality. One species of bacteria was purely or dominantly isolated from diseased juveniles and identified as *Pasteurella piscicida* based on its morphological, biochemical, genetical (G+C contents: 40.7–41.0 mol %), and serological characteristics. An experimental infection revealed that a selected isolate was pathogenic to juvenile striped jack and yound red sea bream, the LD_{50} by intraperitoneal injection for the two fish species being about 10^3 and 10^7 CFU/fish, respectively. It was also demonstrated that the extracellular products (ECP) of the isolate were lethal to both fishes when it was injected intraperitoneally.

In Japan, *Pasteurella piscicida* has been known as the causative bacterium of "pseudotuberculosis" in cultured yellowtail (*Seriola quinqueradiata*) for these two decades (Kimura and Kitao, 1971; Kusuda and Yamaoka, 1972). Yellowtail, the most imporatnt species in marine aquaculture in Japan, has suffered significant losses from the disease for many years, especially since chemotherapeutic treatments of the disease became difficult by the appearance of drug-resistant strains (Aoki and Kitao, 1985). For this reason, much efforts have been made on the development of vaccines against the disease. Although some successful results were reported in experimental vaccination trials (Fukuda and Kusuda, 1981, 1985; Kusuda and Hamaguchi, 1987; Kusuda *et al.*, 1988), these results were not reproducible in many experimental and field tests (Fisheries Agency, 1991*).

With a rapid increase in the variety of marine fishes for culture, *P. piscicida* infection has also been reported in other fishes such as juvenile black sea bream (*Mylio macrocephalus*=*Acanthopagrus schlegeli*) (Muroga *et al.*, 1977), red sea bream (*Pagrus major*) (Yasunaga *et al.*, 1983), juvenile redspotted grouper (*Epinephelus akaara*) (Ueki *et al.*, 1990), yatabe blenny (*Pictiblennius yatabei*) (Hamaguchi *et al.*, 1991), and juvenile ayu (*Plecoglossus altivelis*) reared in seawater (Matsuoka *et al.*, 1990). In addition, a mortality caused by the pathogen has been recorded in a wild population of oval filefish (*Navodan modestus*=*Thamnaconus modestus*) (Yasunaga *et al.*, 1984). Outside of Japan, *P. piscicida* infection has recently been recorded in juvenile gilthead seabream (*Sparus aurata*) in Spain (Toranzo *et al.*, 1991). Thus, the disease has been quickly spreading to various species of marine fishes, causing mass mortalities especially at their juvenile stages.

In the Japan Sea-Farming Association (JASFA), a seed production program for striped jack (*Pseu-*

* Fisheries Agency (1991): Studies on the development of inactivated vaccines against yellowtail pseudotuberculosis. Fisheries Agency of Japanese Government, Research Division, 346 pp. (in Japanese)

docaranx dentex) was started in 1978. With technical developements in both spawning and larval rearing procedures, mass production of juveniles became possible in 1988. However, heavy mortalities caused by known or unknown agents have occurred in larvae and juveniles since 1984. Among disease problems due to microbial agents, a disease called viral nervous necrosis (VNN) caused the most serious problem in the larval stage in recent years (Mori *et al.*, 1992). Although bacterial infections were not found in the seed production process of striped jack in JASFA before, a mass mortality by *Pasteurella piscicida* occurred in juveniles in 1991. This paper describes the present case of *P. piscicida* infection and chracteristics of the causative bacterium.

Materials and Methods

Culture Conditions and Disease Occurrence

Fertilized eggs were obtained from brood stocks (7-year-old) at Gotoh Station of JASFA in Nagasaki Prefecture (December 10, 1990). About two hundred thousands hatched larvae were reared in a 50 m³ tank and a total of 29,800 juveniles (51-day-old, average 32 mm in total length, 0.43 g in body weight) were transferred to another 80 m³ rearing tank (February 1, 1991). Mortality began in the fish group immediately after the transfer and the cumulative mortality reached about 26% after 15 days, when medication with oxytetracycline (OTC)-HCl was started. After administration of OTC-containing food for 8 days, oxolinic acid was given for 13 days, and daily mortality gradually decreased and ceased on March 8 (Fig. 1). The total cumulative mortality in this case was about 34% (10,100 juveniles) and the water temperature decreased from 22°C to 16°C during the period.

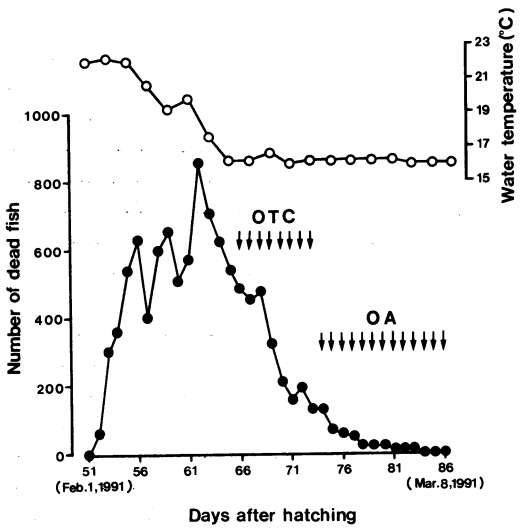

Fig. 1. Daily mortality of juvenile striped jack due to *Pasteurella piscicida* infection.
Arrows: administration of oxytetracycline-HCl(OTC) and oxolinic acid(OA).

There were no conspicuous external changes in the dead juveniles and no ectoparasites were found on the gills and body surface. Internally, the liver and spleen of some fish examined hemorrhaged; however, "white spots" in the kindney and spleen, the most characteristic sign of pseudotuberculosis in yellowtail, was not noticed. Bipolar-staining short rods were observed in smear specimens of the affected kidney or blood.

Bacterial Isolates

Bacterial isolation using brain heart infusion (BHI) agar (Eiken) supplemented with 1.5% NaCl (finally 2%) were made several times from dead juveniles during the epizootic. One colony type was obtained in pure culture from the liver and kidney of all the fish examined and randomly selected 7 isolates, designated SJ-9101 to -9107, were used in the following characterization and other experiments. These isolates were freeze-dried with 20% skim milk immediately after the establishment of pure culture and stored at −20°C until used. Five reference strains of *P. piscicida*, which had been isolated from various kinds of cultured marine fishes in Ehime Prefecture in 1991, were used in drug sensitivity and agglutination tests.

Characterization Test

Morphological and biochemical characteristics of the strains were examined by standard methods. A fresh culture of each strain grown on BHI agar (2% NaCl) at 25°C was inoculated in test media with 2% NaCl, and incubated at 25°C for a required period.

Determination of DNA Base Composition

The guanine-cytosine (G+C) contents of 2

isolates (SJ-9101 and SJ-9102) was examined by a high performance liquid chromatography (HPLC) method following Kumagai *et al.* (1988). DNA was extracted by the phenol method and hydrolysed into nucleotides with nuclease P1 (Yamasa Shoyu Co., Ltd.). The HPLC system consisted of an LC-5A Liquid Chromatograph (Japan Spectroscopic Co., Ltd.) and a Cosmosil 5C18-P column (Nakarai). The nucleotides were eluted by 2.5 mM KH_2PO_4–K_2HPO_4 buffer at a flow rate of 1 ml/min at room temperature. The G+C content (mol%) was estimated from the standard equimolar mixture of four dNMPs (Yamasa Shoyu Co. Ltd.).

Drug Sensitivity Test

The minimum inhibitory concentrations (MIC) of oxolinic acid and oxytetracycline-HCl were determined by a standard broth dilution method (Goto, 1979). Fresh bacterial cultures were inoculated in brain heart infusion broth (2% NaCl) containing drugs at various concentrations, ranging from 0.1 to 100 μg/ml. After incubation at 25°C for 48 h, the lowest concentration of the drugs inhibiting the bacterial growth was recorded as MIC.

Agglutination Test

Formalinized cells of one strain (SJ-9102) was mixed with Freund's complete adjuvant and injected hypodermically two times into a rabbit, followed by several booster injections without adjuvant into the marginal ear vein. Agglutinin titer of the antiserum obtained was 1: 64. Another antiserum against *P. piscicida* (yellowtail strain: K-III), which was distributed from Fish Disease Center of Japan Fisheries Resource Conservation Association, was also employed. Cross reactivity of 7 striped jack isolates and 5 *P. piscicida* reference strains with these antisera was examined by slide agglutination.

Pathogenicity Test

Experimental infections with an isolate SJ-9102 were carried out in healthy juvenile striped jack and young red sea bream, weighing 2.7 g and 34 g on average, respectively. Cells cultured on BHI agar at 25°C for 48 h were suspended in saline. Groups consisting of 10 striped jack were injected intraperitoneally with doses of $10^{5.2}$, $10^{4.2}$, and $10^{3.2}$ CFU/fish or immersed in $10^{4.9}$, $10^{3.9}$, and $10^{2.9}$ CFU/ml for 10 min. In the case of red sea bream, groups of 5 fish were intraperitoneally injected with doses of $10^{7.4}$, $10^{6.4}$, and $10^{5.4}$ CFU/fish. Sterile saline was given to control groups of both species. Fish were observed in 20 *l* sea water tanks at 24°C (striped jack) or 19°C (red sea bream). The daily mortality was recorded for 8 days and the dead fish were examined by bacterial isolation from the kidney in both challenge tests.

Toxicity of extracellular products (ECP) and sonicated cell extract of SJ-9102 was tested in young striped jack and red sea bream, weighing 25 g and 35 g on average, respectively. Cells were grown on cellophane-overlayed BHI agar at 25°C for 48 h and harvested with 4 ml of 0.01 M phosphate buffered saline (PBS, pH 7.0) per plate. After centrifugation at 10,000 rpm for 30 min, supernatant (ECP, ca. 9.4 mg protein/ml) was sterilized through a membrane filter (0.45 μm). Sedimented cells (50 mg/ml PBS) were washed 2 times with PBS, sonicated and filter-sterilized. Fish were injected intraperitoneally with 0.25 ml of the ECP or the sonicated cell extract and observed at 19–20°C for 5 days. The test was duplicated.

Results

Taxonomical Characteristics

The 7 isolates from diseased striped jack showed the same characteristics in the morphological and biochemical tests. The isolates were Gram-negative, non-motile, short rods with bipolar staining, and have biochemical characteristics shown in Table 1. The G+C contents were 41.0 (SJ-9101) and 40.7 (SJ-9102) mol %.

Drug Sensitivity

All tested strains except a reference strain (YT-9341) were highly sensitive to oxolinic acid and oxytetracycline-HCl (Table 2).

Agglutination Test

In slide agglutination, the 7 strains and 5 reference strains of *P. piscicida* were all positive to anti-SJ-9102 serum and anti-*P. piscicida* (K-III) serum, indicating the uniformity of serotype between striped jack and yellowtail *P. piscicida* strains.

Table 1. Biochemical characteristics of 7 isolates of *Pasteurella piscicida* from diseased juvenile striped jack

Character	Response
OF test	F
Gas from glucose	−
Production of:	
Catalase	+
Oxidase	+
Gluconate oxidase	−
Phenylalanine deaminase	−
Arginine dihydrolase	+
β-galactosidase	−
H_2S	−
Indole	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
2,3-butanediol dehydrogenase	+
Nitrate reduction	−
Methyl red test	+
Voges-Proskauer test	+(weak)
Degradation of:	
Arginine	+
Casein	−
Gelatin	−
Starch	+
Tween 80	+
Urea	−
Growth at	
0.5–3.0% NaCl	+
30°C/37°C	+/−
Growth on McConkey agar	−
Growth in potassium cyanide broth	−
Uniform turbidity in broth	+
Sensitivity to 0/129	+
Utilization of citrate	−
Acid from:	
Fuructose	+
Galactose	+
Glucose	+
Mannose	+
Other 22 sugars	−

Table 2. Drug sensitivity test of *Pasteurella piscicida* strains

Strain	Host fish	MIC (μg/ml)	
		OA	OTC
SJ–9101	Striped jack	<0.1	0.2
–9102	〃	<0.1	0.4
–9103	〃	<0.1	0.8
–9104	〃	<0.1	0.2
–9105	〃	<0.1	0.2
–9106	〃	<0.1	0.4
–9107	〃	<0.1	0.2
YT–9341	Yellowtail	3.1	50.0
RS–9137	Red sea bream	<0.1	0.2
SB–9188	Sea bass	<0.1	<0.1
OF–5001	Oval filefish	<0.1	0.4
PA–9103	Purplish amberjack	<0.1	0.2

OA: Oxolinic acid OTC: Oxytetracycline-HCl

Pathogenicity Test

The strain SJ-9102 was highly pathogenic to juvenile striped jack: the LD_{50} of intraperitoneal (IP) injection was less than $10^{3.2}$ CFU/fish and LC_{50} of bath challenge was less than $10^{2.9}$ CFU/ml (Table 3). In contrast, the LD_{50} for red sea bream by IP-injection was quite high ($10^{6.9}$ CFU/fish). The inoculated bacteria were recovered from all of the dead fish, but no conspicuous disease signs on the body surface or no white spots in the internal organs were observed as in naturally infected fish.

As shown in Table 4, the ECP of strain SJ-9102 was lethal to both striped jack and red sea bream, but the sonicated cell extract was not. The toxicity of the ECP was lost by a heat-treatment at 70°C for 30 min. The strain SJ-9102 cells were not recovered from any dead fish injected with the toxin, although different bacteria were isolated from a few dead fish.

Discussion

The morphological and biochemical characteristics of the 7 isolates from juvenile striped jack well agreed with those of *P. piscicida* from various marine fishes (Snieszko *et al.*, 1964; Kusuda and Yamaoka, 1972; Muroga *et al.*, 1977; Yasunaga *et al.*, 1983). The G+C contents of 2 selected isolates were 41.0 and 40.7 mol %. These values were almost the same as that of *P. piscicida* (41.5 mol %) reported in a previous work (Kusuda *et al.*, 1978) and fall into the range (40–45 mol %) of the genus *Pasteurella* (Krieg and Holt, 1984), though the methods employed were different. From these results, the present isolates were identified as *P. piscicida*. They could not be differenciated serologically from *P. piscicida* strains from yellowtail.

Multi-drug resistance induced by R-plasmids

Table 3. Pathogenicity of *Pasteurella piscicida* SJ-9102 strain in striped jack and red sea bream

Challenge method	Striped jack*		Red sea bream*	
	Dose (CFU)	Mortality (%)	Dose (CFU)	Mortality (%)
Injection (IP)	$10^{5.2}$/fish	100	$10^{7.4}$/fish	80
	$10^{4.2}$	100	$10^{6.4}$	20
	$10^{3.2}$	70	$10^{5.4}$	0
	Control	0	Control	0
Bathing in seawater	$10^{4.9}$/m*l*	100		
	$10^{3.9}$	100		
	$10^{2.9}$	80		
	Control	0		

* The average body weight of striped jack and red sea bream were 2.7 g and 34 g, respectively.

Table 4. Toxicity of extracellular products (ECP) of *Pasteurella piscicida* SJ-9102 strain in striped jack and red sea bream

Test fish	Experiment no. (fish weight)	Heat-treatment (70°C, 30 min)	Number of fish dead/tested	Time to death (days)
Striped jack	1 (27 g)	no	9/10	4–5
		yes	0/10	
	2 (23 g)	no	10/10	1–3
		yes	0/8	
Red sea bream	1 (31 g)	no	20/20	<1
	2 (38 g)	no	5/5	1–2
		yes	0/5	

has been found in *P. piscicida* strains from yellowtail (Aoki and Kitao, 1985). Kusuda *et al.* (1990) reported the drug sensitivity of *P. piscicida* strains isolated from yellowtail during the period from 1986 to 1988. According to their results, the MIC values of oxolinic acid for about one-third of the strains (110/306) were higher than 1.56 μg/m*l*. As shown in Table 2, the MIC values of the antibiotic for the strains isolated from other marine fishes than yellowtail were lower than 0.1 μg/m*l*. This difference seems to reflect the less frequency of medication in farms for these fishes. The high sensitivity of the isolates against oxolinic acid and oxytetracycline supports the effective control of the present case with these two drugs.

In the case of *P. piscicida* infection in yellowtail, the disease usually prevails in summer (June to September), when water temperature ranges from 20 to 28°C. In the present case, the mortality of striped jack broke out when the water temperature was 22–23°C and it gradually decreased when the water temperature went down below 19°C. Thus it seems that the treatment with antibiotics and the decline of water temperature acted synergistically on the control of the disease. In Ehime Prefecture, *P. piscicida* has been isolated sporadically from diseased young (0-year-old) striped jack but the disease usually did not result in mass mortality in any farms. Although the factors affecting the mortality due to *P. piscicida* infection have not been fully investigated, it can be said that the pathogen will cause significant mortality in this fish species only in juvenile stages reared at higher temperatures than 20°C.

Although *P. piscicida* is highly pathogenic not only to yellowtail but also to other marine fishes as demonstrated in experimental infections (Kusuda and Hamaguchi, 1987; Matsuoka *et al.*, 1990; Hamaguchi *et al.*, 1991). However, the pathogenicity mechanism has not been investigated at all even in yellowtail. The present study revealed that one strain from striped jack produced lethal ECP. The toxicity of the ECP was lost

by heating at 70°C for 30 min and it was retained in 65% ammonium sulfate precipitates (data not shown), suggesting that the toxic substance (s) in the ECP is a protein.

Acknowledgements

We are very grateful to Mr. T. Mekuchi, Hiroshima Prefectural Fisheries Experiment Station, for his kind support on the pathogenicity test of the present isolates.

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