在来マス及びアユに対するYersinia ruckeriの病原性

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Virulence of *Yersinia ruckeri* for Four Indigenous Fish Species in Japan

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**ABSTRACT**—Enteric redmouth disease (ERM), which is caused by *Yersinia ruckeri*, has not been reported in Japan. In the present study, virulence of the bacterium were assessed by experimental challenges with the rainbow trout *Oncorhynchus mykiss* and 4 indigenous fish species in Japan: yamame (masu salmon parr) *O. masou masou*, amago *O. masou macrostomus*, whitespotted char *Salvelinus leucomaenis* and ayu *Plecoglossus altivelis*. When intraperitoneally (ip) injected with the bacterium at a dose of 7.1 × 10^5 CFU/g fish body weight (BW), rainbow trout, char, yamame, and amago showed the cumulative mortalities of 100%, 60%, 30% and 30%, respectively. The cumulative mortalities of ip-injected ayu with doses of 1.5 × 10^5 , 1.5 × 10^6 and 1.5 × 10^7 CFU/g BW were 87%, 47%, 13% and 0%, respectively. In addition, the cumulative mortalities of ayu immersed in water containing 1.7 × 10^9 and 1.7 × 10^10 CFU/mL of the bacterium were 60% and 6.7%, respectively. Typical clinical signs of ERM, such as reddening of the wall of the oral cavity, tongue, upper jaw, lower jaw, operculum, bases of fins, or skin around the anus, were observed in all dead salmonids but not in ayu. Instead, exophthalmos, hemorrhage in the eyes, ascites or hypertrophy of the kidney were often observed in dead ayu. These results indicate that *Yersinia ruckeri* is a potentially dangerous bacterium to freshwater aquaculture in Japan.

**Key words:** *Yersinia ruckeri*, enteric redmouth disease, *Oncorhynchus mykiss*, *Oncorhynchus masou masou*, *Oncorhynchus masou macrostomus*, *Salvelinus leucomaenis*, *Plecoglossus altivelis*

*Yersinia ruckeri* is the causative agent of enteric redmouth disease (ERM) in salmonids (Furones et al., 1993). In farmed fish, particularly for rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar*, the disease has caused high mortalities and severe economic losses (Ewing et al., 1978). The bacterium was first isolated from rainbow trout in North America (Rucker, 1966). The bacterium has a wide host range, having been isolated from many salmonids (*O. kisutch*, *O. mykiss*, *O. nerka*, *O. tshawytscha*, *O. clarkii*, *S. salar*, *S. trutta*, *Salvelinus alpinus*, *Salv. fontinalis* and *Salv. maima*), as well as from other fish species, such as sturgeon *Acipenser baeri* Brandt, European eel *Anguilla anguilla*, gold fish *Carassius auratus* and carp *Cyprinus carpio* (Furones et al., 1993). The bacterium has been isolated also from higher vertebrates such as birds and human (Willumsen, 1989; Furones et al., 1993). Although the disease has occurred in various regions including North America, Venezuela, Chile, Australia, Europe, and South Africa (Furones et al., 1993), the occurrence of the disease has not been reported in Japan. Therefore, according to the law to protect aquaculture industry in Japan, the Japanese government has designated ERM as one of the diseases that needs strict watch for their invasions. However, little is known about the virulence of *Y. ruckeri* against most of the indigenous fish species in Japan. In the present study, we tested the virulence of the bacterium in 4 indigenous fishes; yamame (landlocked masu salmon) *O. masou masou*, amago *O. masou macrostomus*, whitespotted char *Salv. leucomaenis leucomaenis*, and ayu *Plecoglossus altivelis*. These fishes are widely cultured in freshwater fish farms in this country. In particular, ayu comprises one third of the total value of the production of freshwater fisheries. In addition, we included rainbow trout, which are also widely cultured in this country, in the experiments as the control to assess virulence of the bacterium. Handling of the live bacterium and all experimental infections were conducted in the research facilities designed and built to study exotic diseases in the National Research...
Institute of Aquaculture (NRIA) so that the pathogen is strictly contained.

Materials and Methods

Rainbow trout, amago and yamame used in the present experiments were bred from broodstocks kept in the Inland Station of the NRIA. Fry of whitespotted char were obtained from Miyagi Prefecture Fisheries Technology Institute. Juvenile ayu were obtained from a private hatchery (Nisshin Marine Tech Co.). These fishes were reared in aquaria filled with 400 L of running water, aerated well water at 17°C by feeding commercial pellet diet until the experiments.

Yersinia ruckeri FPC443 isolated from rainbow trout in USA was used for the experimental infections. This strain was kindly donated to the NRIA by Emeritus Professor H. Wakabayashi, the University of Tokyo. We confirmed the bacterium as Y. ruckeri by polymerase chain reaction (PCR) according to LeJeune and Rurangirwa (2000). In addition, the bacterium was classified as the serotype I. Although there are varieties of serotypes in the bacterium, most of the ERM incidents are associated with serotype I (Bullock et al., 1978; McCarthy and Johnson, 1982; Davies, 1991). Prior to each experiment, the bacterium was cultured at 25°C in triplicate soy (TS) broth (Becton Dickinson) for 24 h and suspended in 10 mL phosphate-buffered saline (PBS, pH 7.2). Furthermore, the bacterial cells were harvested by centrifugation (8,000 xg, 10 min, 4°C), washed twice with PBS, and suspended in PBS for experimental challenges. The number of viable cells was estimated according to the methods of Miles and Misra (1938). Serial 10-fold dilutions of the bacterium were prepared with PBS.

To confirm the virulence of the bacterium, the median lethal dose (LD$_{50}$) was examined using 4 groups of rainbow trout (n = 10) by intraperitoneal (ip) injections with the bacterium at doses of 7.0 x 10$^7$, 7.0 x 10$^8$, 7.0 x 10$^9$ CFU/g fish body weight (BW), or 0 (PBS). The LD$_{50}$ was calculated by the method of Reed and Muench (1938). Following the determination of the LD$_{50}$, which was 2.4 x 10$^3$ CFU/g BW the bacterium at the dose of 7.1 x 10$^2$ CFU/g BW (approximately 30-fold higher than the LD$_{50}$ for the rainbow trout) was injected intraperitoneally to rainbow trout (n = 20), whitespotted char (n = 20), amago (n = 20) and yamame (n = 20). The control fish (n = 20) for each species were similarly injected with PBS. Also, 5 groups of ayu (n = 15/group) were injected intraperitoneally with the bacterium at doses of 1.5 x 10$^3$, 1.5 x 10$^4$, 1.5 x 10$^5$, 1.5 x 10$^6$ and 1.5 x 10$^7$ CFU/g BW, and PBS, respectively. Mortality of each group was recorded for 14 days after the bacterial injections. The pathogenicity of the bacterium against ayu was also tested with an immersion challenge. For this challenge, three groups of ayu (n = 15/group) were immersed in 20 L of water containing either 1.7 x 10$^8$ CFU/mL of the bacterium, 1.7 x 10$^7$ CFU/mL of the bacterium, or 20 mL of vehicle (PBS) for 90 min. Mortality was recorded for 21 days for these groups.

At the time of the experimental challenges, mean body weight of rainbow trout, amago, yamame, whitespotted char and ayu were 20.6 g, 19.4 g, 24.1 g, 5.5 g and 1.3 g, respectively. Each experimental group was reared in a 30 L aquarium filled with 20 L of running, aerated well water at 17°C with a flow rate of 0.5 L/min. During the experiments, fish were fed commercial pellet diet daily ad libitum.

Dead fish and survivors of all the experiments were autopsied, and the liver, kidney and spleen were subjected to bacterial isolation on TS agar to confirm the cause of death. The cultivation was performed for 48 h at 25°C. The identification of Y. ruckeri was conducted by PCR according to LeJeune and Rurangirwa (2000).

For histopathological observation, 2 groups of ayu (n = 10/group) were immersed either in water containing 1.7 x 10$^5$ CFU/mL of the bacterium or water without the bacterium for 90 min as described above. Three days after the challenge, 5 fish were sampled at random from each of these groups. The eye, brain, heart, gills, liver, spleen, kidney, muscle and digestive tract of each specimen were removed, fixed in Davidson's fixative (330 mL of 95% ethanol, 220 mL of commercial formaldehyde solution, 115 mL of glacial acetic acid, and 335 mL of distilled water), transferred through a series of ethanol and xylene, and embedded in paraffin. Two sets of sections were cut at 3 μm from each tissue and stained with hematoxylin and eosin or May-Grunwald and Giemsa.

Results and Discussion

The bacterial strain used in the present study was highly virulent for rainbow trout. The cumulative mortalities of rainbow trout injected with Y. ruckeri FPC443 at the doses of 7.0 x 10$^5$, 7.0 x 10$^6$, 7.0 x 10$^7$, 7.0 x 10$^8$, and 0 CFU/g BW were 100, 65, 30, 0 and 0%, respectively. The LD$_{50}$ based on these results was 2.4 x 10$^3$ CFU/g BW.

The experimental challenges clearly showed the potential of the bacterium to induce disease in the indigenous salmonid species tested, although the lower mortalities of the three species probably suggest that these salmonids are less susceptible to Y. ruckeri than the rainbow trout (Fig. 1). Yersinia ruckeri was reisolated from all dead fish but not from survivors. The clinical signs exhibited by challenged salmonids are principally the same as those of ERM reported previously (Rucker, 1966; Furones et al., 1993; Tobbback et al., 2007). All dead fish showed reddening of the oral cavi-
ty, tongue, jaw, operculum, base of fins, and anus (Fig. 2). In the autopsies, reddening was observed on the liver, pyloric caeca, swim bladder or in the lateral muscle. Hypertrophy of the spleen and kidney was also noticed.

Reddening of base of fins, anus or abdominal organ has been found in indigenous salmonids suffering from vibriosis or streptococciosis (Hatai and Ogawa ed., 2005). However, no disease with similar reddening of the oral cavity, tongue, jaw and operculum as ERM has been found so far in Japan. Therefore, these clinical signs observed in the present challenge tests would be useful for the presumptive diagnosis of Y. ruckeri infections in these indigenous salmonids.

Fig. 1. Cumulative mortalities of amago, yamame, whitespotted char and rainbow trout after intraperitoneal injections with Yersinia ruckeri FPC443. Fish of each experimental group were injected with the bacterium at $7.1 \times 10^2$ CFU/g fish body weight. Fish of each control group were injected with the vehicle (PBS). $n = 20$ for each group.

Fig. 2. A dead specimen of whitespotted char after the challenge with Y. ruckeri FPC443. Arrowheads indicate the reddened sites. Hypertrophy of the spleen (arrow) and kidney (asterisk) are observed.
The bacterium caused dose-dependent mortalities of ayu both in the injection and immersion challenges, indicating that \textit{Y. ruckeri} is also pathogenic to this species (Figs. 3 & 4). The LD$_{50}$ calculated on the basis of the injection challenges, however, was $2.0 \times 10^5$ CFU/g BW, which was much higher than that for rainbow trout. \textit{Y. ruckeri} was reisolated from all dead fish in both challenge tests. In addition, the bacterium was reisolated from approximately 50% of the survivors. On the other hand, the affected ayu did not show typical clinical signs of ERM found in salmonid fish, such as reddening of the oral cavity, tongue, jaws or operculum. Pale coloration of the gills was observed in all dead fish. In addition, most of the dead fish showed exophthalmos or hemorrhage in the eyes (Fig. 5). Also, either ascites or kidney hypertrophy was observed in 50% of the dead fish in challenged groups. In each control group, there was no fish that showed any signs of a disease and the bacterium was not isolated. In carp infected by \textit{Y. ruckeri}, the exophthalmos has also been observed whereas reddening around the mouth has not been noticed (Berc \textit{et al.}, 1999). Thus the clinical sign of “red mouth” by \textit{Y. ruckeri} may be unique to salmonid fishes. It would be difficult to suspect \textit{Y. ruckeri} infection in ayu from the clinical signs because affected ayu do not exhibit the typical clinical signs found in ERM of salmonid fishes. Observed clinical signs such as exophthalmos or ascites has also been found in the fish infected with \textit{B}-hemolytic \textit{Streptococcus} (Ohnishi and Jo, 1981) or \textit{Edwardsiella ictaluri} (Sakai \textit{et al.}, 2008). Hence, bacterial isolation and identification would be necessary for the diagnosis of \textit{Y. ruckeri} infection in ayu. \textit{Yersinia ruckeri} can be distinguished from \textit{Streptococcus} by Gram-differentiation test (Ryu, 1940). However, biochemical profiles of \textit{Y. ruckeri} are very similar to those of \textit{E. ictaluri}. PCR tests for the identification of \textit{Y. ruckeri} have been reported by LeJeune and Rurangirwa (2000), Altinok \textit{et al.} (2001), or Del Cerro \textit{et al.} (2002). These PCR tests can identify \textit{Y. ruckeri} among Gram-negative, pathogenic bacteria (Sakai \textit{et al.}, 2006). Therefore, PCR would be useful for rapid and confirmed diagnosis of \textit{Y. ruckeri} infection in ayu and salmonids.

For histopathology, short, rod-shaped bacterial cells were observed in the lumen of many blood vessels including capillaries of the choroid coat or gills in the five fish subjected to histological observations (Fig. 6A & B). In addition, the bacterial cells were found more frequently in arteries than in veins (Fig. 6C). A particularly large number of bacterial cells were found in one fish, in which many bacterial cells were observed in the glomerular capillaries of the kidney. In this fish, the lumen of renal tubules was often dilated and the epithelial cells were flattened (Fig. 7), and the tubules were frequently encircled by a fibrous tissue. In another fish, infiltration of inflammatory cells was observed in the gills. It is not certain, however, whether these pathological changes without the association of the bacterium had
be cea ed by the bacterial in ection. Rainbow trout infected with Y. ruckeri shows septicemia with an inflammatory response in virtually all tissues (Rucker, 1966). The bacterial colonization occurs in the capillaries of highly vascularized tissues such as the gills, muscle, kidney, liver, spleen, and heart (Furones et al., 1993). Hemorrhages or systemic tissue edema has been observed in these sites. The disease of ayu infected with Y. ruckeri seems also caused through the colonization of the bacterium in blood vessels. However, no marked inflammatory response was observed against the bacterium in ayu, which may be related to the lower virulence of the bacterium to this fish.

Although there is no doubt that the prevention of the entry of Y. ruckeri into Japan should be our foremost consideration, we also have to assume the invasion of the bacterium and the occurrence of Y. ruckeri infections in Japanese trout or ayu. The spread of Y. ruckeri infection is attributed to transfer of the carrier fish or fish-eating birds (Willumsen, 1989) and, at present, it is impossible to stop importing fish into Japan from foreign countries completely. Therefore, the prompt and accurate diagnosis of Y. ruckeri infection is very important and the ability of the diagnosis should be maintained to protect Japanese freshwater fish industry.

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References

Virulence of *Y. ruckeri* in salmonids and ayu

161–172.


海生甲殻類から分離した病原卵菌類のITS1 領域の塩基配列による同定とアルテミア卵化卵生に対する病原性

感染実験からみた魚病細菌のサケ科魚類卵内感染機序

小原昌和・笠井久会・吉本 守

ニジマスおよびアマゴ卵を用いて Flavobacterium psychrophilum, Renibacterium salmoninarum および Aeromonas salmonicida の感染実験を行い、卵内感染機序を検討した。F. psychrophilum は卵の吸水時に卵門から侵入すると考えられた。F. psychrophilum 感染症は、汚染水吸水卵よりも卵表面汚染後に吸水させた卵に有意に高く、成立条件は 10^6 CFU/mL 以上であった。また、高濃度の R. salmoninarum で表面汚染卵において卵内感染がみられた。F. psychrophilum または A. salmonicida 汚染卵を吸水させたところ、F. psychrophilum は卵内侵入後に増殖したが、A. salmonicida は侵入後数日で消減した。

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タイのテラピア養殖場から分離された Aeromonas hydrophila 多剤耐性株

N. Tipmongkolsip ・ C. S. del Castillo ・引間順一
T-S. Jung ・近藤秀裕・原野育生・寺 由

タイのテラピア養殖場の感染魚より55株の A. hydrophila を分離し、11薬剤の最小発育阻止濃度を調べた。その結果、全ての分離株が 1 ～ 3 剤の組合せの耐性を示し、5 剤以上の耐性価が約半数を占めた。これらの薬剤耐性株中、1 剤から ABPC、CP、SM、SMMX および TC の 5 剤に対して耐性を示す伝達性 R プラスマドを検出した。このプラスミドは、耐性遺伝子として blaOXA-35, cat2, aadA1, sul1 および tetA を含んでいた。

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在来マス及びアユに対する Yersinia ruckeri の病原性

坂井賞光・中島千早・伊東貴史・三輪 理
大迫進久・飯田賢次

ニジマス、イワナ、アマゴ及びヤマメの腹腔内に Y. ruckeri を 7.1×10^5 CFU/魚体重 (g) 接種して攻撃した。各魚種の累積死亡率は、100％、80％、60％、50％とあり、すべての死亡魚がレッドマウス病の症状を示した。1.5×10^5～1.5×10^6 CFU/魚体重 (g) で腹腔内接種したアユの累積死亡率は、0％～87％であった。また、浸漬攻撃でも高い累積死亡率が観察された。死亡したアユにサケ科魚類と同様のレッドマウス病の症状は見られず、眼球の突出や出血、腹水の貯留が観察された。

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ポルトガルで養殖ターポットに発生した Streptococcus parauberis 感染症

M. F. Ramos ・ J. F. Marques ・ J. V. Neves ・ T. Barandela
J. A. Sousa ・ A. Saravia ・ P. N. Rodrigues

2004年の5月から8月にかけてポルトガル北部の1養殖場でターポット（体重 90-1,020 g 水温12-14℃）に、眼球突出、背部や軟骨部の出血と浮腫、また病理組織学的には乾固膜を特徴とする大量死亡が発生した。病魚の内臓諸器官からグラム陽性の α 溶血性球菌が分離され、それらは生化学的・血清学的性状および遺伝学的性状（16S rDNA を標的とした PCR）から S. parauberis に同定された。これはポルトガルにおける本菌感染症の初報告である。

魚病研究. 47 (2), 80-82 (2012)