Short communication

Therapeutic Efficacy of Oral Administration of Fosfomycin on Experimentally Induced Edwardsiellosis in Japanese Flounder

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ABSTRACT—Juvenile Japanese flounder *Paralichthys olivaceus* were immersion-challenged (2.0×10^6 CFU/mL, 60 min) with *Edwardsiella tarda* NUF806 strain. From the following day, diets containing fosfomycin (FOM) calcium were administered orally at concentrations of 20, 40 or 80 mg potency/kg body weight/day for 6 consecutive days. Cumulative mortality of fish during the observation period of 21 days after the drug administration was significantly lower in all groups of FOM-treated fish than that in the control group. In addition, NUF806 strain and recent clinical isolates ($n = 30$) of *E. tarda* examined were all susceptible to FOM, with MIC of 2–4 $\mu\text{g/mL}$ when estimated using the glucose-6-phosphate-containing Mueller-Hinton broth. The results suggest that FOM is a promising candidate for the treatment of edwardsiellosis in cultured Japanese flounder.

Key words: fosfomycin, *Paralichthys olivaceus*, *Edwardsiella tarda*, chemotherapeutic efficacy, MIC

Edwardsiella tarda infects a variety of marine and freshwater fish species, including at least 40 species that have been recorded as hosts of this bacterium (Evans *et al.*, 2011). Among the economically important aquaculture species in Japan, Japanese flounder

Paralichthys olivaceus (Nakatsugawa, 1983), red sea bream *Pagrus major* (Yasunaga *et al.*, 1982) and the Japanese eel *Anguilla japonica* (Hoshina, 1962) are most severely affected by *E. tarda* infections.

Most of the research for the chemotherapeutic treatment of *E. tarda* infection has been focused on the Japanese eel so far. Kashiwagi *et al.* (1980) reported that immersion in 1 ppm furazolidone (FZD) was highly effective against an FZD-sensitive strain and effective at > 4 ppm against an FZD-intermediately sensitive strain. Oral administration of a potentiated sulfonamide, sulfisoxazole-trimethoprim, was relatively effective against experimentally infected *E. tarda* in eels (Fujiwara *et al.*, 1984). Florfenicol (FF) was very effective against *E. tarda* infection in eels when given orally, and its efficacy was superior to that of thiamphenicol, chloramphenicol (CP) and oxytetracycline (OTC) (Fukui *et al.*, 1987). Oral administration of a combination of ormetoprim-sulfamonomethoxine (OMP–SMMX), oxolinic acid (OA) and miloxacin was effective against a strain of *E. tarda* that were resistant to CP, tetracycline and sulfonamide (Aoki *et al.*, 1989). To date, four antibiotics (OA, FF, OMP–SMM and OTC) have been approved specifically for the treatment of edwardsiellosis in farmed eels in Japan. In other species including red sea bream, only fosfomycin (FOM) calcium has been approved for edwardsiellosis. Yamashita *et al.* (2014) demonstrated that oral administration of FOM was therapeutically effective against experimentally induced or naturally occurring edwardsiellosis in red sea bream. However, no chemotherapeutic agents are currently approved for the treatment of edwardsiellosis in the Japanese flounder.

In this study, we report the efficacy of oral administration of FOM on experimentally induced edwardsiellosis in the flounder. The minimum inhibitory concentrations (MIC) of FOM against recent *E. tarda* isolates from the diseased Japanese flounder were also determined.

Materials and Methods

Bacterial strains

Edwardsiella tarda NUF806 (kindly provided by Professor Kinya Kanai, Nagasaki University) isolated from diseased Japanese flounder in 1997 was used for the experimental infection. Prior to the challenge, the bacterium was inoculated in brain heart infusion (BHI) broth (BD Biosciences), supplemented with NaCl to a final concentration of 2.0%, and incubated at 25°C for 24 h. In addition to NUF806, thirty recent clinical isolates of *E. tarda*, which were isolated from flounder farms in Ehime or Oita Prefecture in 2008 (Oita, $n = 2$), 2009 (Oita, $n = 3$) and 2012 (Oita, $n = 4$; Ehime, $n = 21$), were subjected to a brief survey for FOM-resistance. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as

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quality control strains for the determination of MIC. These bacteria were cultured on Mueller-Hinton agar (MHA) (Becton Dickinson) prior to the MIC testing.

Chemotherapy with FOM

Juvenile Japanese flounder were obtained from a commercial supplier (Marinotech). Chemotherapy with FOM was conducted in four 60-L tanks supplied with each 45 L of flow-through, sand-filtered seawater (25°C). Each tank contained 18 fish (mean body weight = 55.3 g), and the fish were acclimated to the tanks for 10 days without feeding prior to the immersion challenge. For the challenge, inflow of water was stopped and water was drained from each tank to a volume of 20 L. *Edwardsiella tarda* NUF806 was then added to the tanks to a final concentration of 2.0×10^6 colony-forming unit (CFU)/mL. After 60 min, the water flow was resumed and the tank volume was allowed to return to 45 L. The gruel-like diet was prepared by mixing a powdery commercial diet for eel (Seiman-yo N21; Marubeni Nissin Feed) with distilled water. An appropriate amount of fosfomycin (FOM) calcium (Fosmicin; Meiji Seika Pharma) was then added to the diet. For drug administration, fish were anesthetized with 2-phenoxyethanol, and a 1-mL aliquot of the diet was administered directly into the stomach of each fish using a 10-mL plastic syringe (Terumo) fitted with an animal feeding needle (Fuchigami). From the following day of the challenge, the diet containing FOM were administered to the fish in the experimental tanks at doses of 0, 20, 40 and 80 mg potency/kg BW/day, respectively, once a day for 6 consecutive days. The day after the final FOM administration, five fish from each group were sampled for the estimation of bacterial load ($n = 5$) and remaining fish were kept for monitoring mortality. The mortality was recorded up to 21 days after the sampling. Tissues of trunk kidney of all dead fish were subjected to bacterial isolation to confirm the infection of NUF806.

Estimation of E. tarda NUF806 load in trunk-kidney

The day after the final FOM administration, five fish of each treatment group were anesthetized with 2-phenoxyethanol, and the trunk kidney was excised to estimate the load of live *E. tarda* in the fish. The tissue (20–90 mg) from each individual was mixed with 9 volumes of phosphate-buffered saline containing 1% Triton X-100 and immediately homogenized. Insoluble materials in the homogenates were removed by centrifugation at 100 g for 10 min. The supernatant was then serially diluted with phosphate-buffered saline and plated on *Salmonella Shigella* (SS) agar (Becton Dickinson) to count the number of viable bacterium. The detection limit for this assay was 1 CFU per 10 mg trunk-kidney tissue.

MIC test for FOM

The MIC of FOM sodium (Wako) was determined for *E. tarda* isolates. This study was designed in accordance with the guideline described in the revised standard method of the Japanese Society of Antimicrobials for Animals in 2003. *E. tarda* isolates and the control bacteria were inoculated into cation-adjusted Mueller-Hinton broth (CAMHB) (Becton Dickinson), and incubated at 25°C for 24 h. Each bacterium was cultured to give a turbidity equivalent to a 0.5 McFarland standard and diluted 1:10 (v/v) in CAMHB. Then, 5 μ L aliquots of the diluted inoculum was added to the 0.1 mL CAMHB containing serially diluted concentrations of FOM in a 96-well microplate. Plates were incubated at 25°C for 24 h and the MIC was recorded as the lowest concentration of FOM that macroscopically inhibited bacterial growth compared with the medium of negative control, which does not contain FOM. The MIC of FOM for each isolate in the presence of glucose-6-phosphate (G6P) at a concentration of 25 μ g/mL was also determined with the same procedure.

Minimum bactericidal concentration of FOM

The minimum bactericidal concentration (MBC) was also determined for *E. tarda* NUF806 with the material collected from the 96-well microplate used to establish the MIC of FOM. Aliquots of 100 μ L were plated on the Mueller-Hinton agar and incubated for 24 h at 25°C. The MBC was defined as the lowest FOM concentration that killed more than 99.9% of the initial bacterial count.

Results and Discussion

Chemotherapeutic effect of FOM

The chemotherapeutic administration of FOM against experimental infection with *E. tarda* NUF806 in the flounder was highly effective (Table 1), and no apparent side effects were noted during the experiment. The cumulative mortality in the control group was significantly higher (67%) than those in the FOM-treated groups (0–15%) (Fisher's exact probability test, $P < 0.015$). The application of FOM at 40 mg potency/kg BW/day for 6 consecutive days is the approved chemotherapeutic protocol for edwardsiellosis of fishes that belong to the order Perciformes in Japan (instructed by the guideline of pharmaceutical agents in aquaculture issued by the Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries). This protocol is usually applied to the disease of red sea bream (Yamashita *et al.*, 2014). Although *E. tarda* isolates causing edwardsiellosis in the red sea bream are designated atypical and their genotypic and phenotypic traits are different from those causing the disease in the flounder, which is called typical (Matsuyama *et al.*, 2005; Nakamura *et al.*, 2013), the results of this

Table 1. *In vivo* evaluation of the chemotherapeutic efficacy of fosfomycin calcium against *E. tarda* NUF806

Bacterial dosage of bath challenge (CFU/mL)	FOM administration (mg/kg BW)	Number of dead fish/total number of fish in the tank	Chemotherapeutic efficacy		Number of <i>E. tarda</i> positive fish* ¹	Number of the fish of the range of viable counts* ¹ (CFU/10 mg of kidney tissue)			
			Cumulative mortality (%)	Two-sided <i>p</i> value (Fisher's exact probability test)		0	1–100	101–1000	≥ 1001
2.0 × 10 ⁶	0 (control)	8/12* ²	67	–	4/5	1	3	0	1
	20	2/13	15	< 0.015	0/5	5	–	–	–
	40	0/13	0	< 0.01	0/5	5	–	–	–
	80	0/13	0	< 0.01	0/5	5	–	–	–

*¹ Examined at the day after final FOM administration. Viable count was estimated from the homogenate of trunk kidney plating on *Salmonella Shigella* (SS) agar.

*² One fish in the control tank was dead during FOM treatment (at 6 days post challenge) due to *E. tarda* NUF806 infection.

study suggest that oral administration of FOM is similarly effective against typical-*E. tarda* infections in the flounder.

Bacterial load after administration of FOM

Viable *E. tarda* cells were detected in four of five fish (80%) in the control group, whereas no bacterium was isolated from FOM-treated groups after the final FOM administration (Table 1). This difference between the control and FOM-treated groups was, however, not statistically different ($n = 5$; analysis of variance, $P = 0.34$) due to the large variation in the bacterial counts in the control specimens (1 to 1,530 CFU/10 mg of tissue). Further, viable *E. tarda* NUF806 cells were detected in 20% of survived fish in the control group, while no bacterial cells were detected in survivors of the FOM-treated fish at 21 days after the final FOM administration (data not shown). *Edwardsiella tarda* prevents the activation of reactive oxygen species generation system during phagocytes (Srinivasa Rao *et al.*, 2001; Ishibe *et al.*, 2008). This property enables *E. tarda* to multiply in the host's phagocytic cells and be distributed throughout the body (Evans *et al.*, 2011), resulting in chronic and severe systemic infection. Thus, these observations suggest that the oral administration of FOM prevented the growth of the bacterium and efficiently eliminated them from fish body.

Susceptibility of *E. tarda* isolates to FOM

Fosfomycin is transported into microorganisms through a mechanism called hexose phosphate trans-

port system, which is induced by G6P (Kahan *et al.*, 1974). As a consequence, the presence of G6P potentiates the activity of FOM against a wide range of bacteria (Grimm, 1979). The enhancement of the efficacy of FOM by G6P against *E. tarda* isolates was also observed in this study. The MICs of FOM for recent clinical isolates in CAMHB range from 8 to 32 µg/mL, while the MICs were decreased to a narrow range of 2–4 µg/mL with the addition of G6P to the medium (Table 2). Similarly, the MIC of FOM against *E. tarda* NUF806 was decreased from 16 to 2 µg/mL. MBC of FOM for *E. tarda* NUF806 in the G6P-containing CAMHB was 8 µg/mL, and the MBC/MIC ratio for this bacterium is accordingly estimated to be 4. Thus, FOM is regarded as bactericidal against NUF806. MICs of FOM for the quality control bacteria, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were > 128 and 16 µg/mL in CAMHB respectively, while the MICs were decreased to 4 and 2 µg/mL with the G6P-containing medium. Fosfomycin has been used for two decades to treat pseudotuberculosis, caused by *Photobacterium damsela* subsp. *piscicida*, in cultured yellowtail *Seriola quinqueradiata* in Japan. Despite such a long period of use, few FOM-resistant strains of *P. damsela* subsp. *piscicida* have been reported, even though the multidrug-resistant strains against other antibiotics have appeared frequently (Fukuda, 2003; Kawanishi *et al.*, 2006). Kijima-Tanaka *et al.* (2007) detected R-plasmids encoding drug resistance genes, except for FOM resistance, from 74 field isolates of *P. damsela* subsp.

Table 2. Minimum inhibitory concentration (MIC) of fosfomycin calcium for 30 recent clinical isolates of *E. tarda*

Medium	No. of strains with MIC (µg/mL) of:										
	0.13	0.25	0.5	1	2	4	8	16	32	64	128
CAMHB	–	–	–	–	–	–	20	9	1	–	–
CAMHB + G6P (25 µg/mL)	–	–	–	–	16	14	–	–	–	–	–

CAMHB = cation-adjusted Mueller-Hinton broth ; G6P = glucose-6-phosphate.

piscicida. Similar to the case of *P. damselae* subsp. *piscicida*, no resistant bacterium against FOM were found in the 67 isolates of *E. tarda* from cultured red sea bream during the period from 2009 to 2012 (Yamashita *et al.*, 2014). Further, in this study, no FOM-resistance was detected in the 30 recent isolates of *E. tarda*, which were obtained from the flounder farms that were widely located geographically. Therefore, it seems safe to assume that FOM-resistant *E. tarda*, if any, are not widely distributed in the flounder farms in Japan, and FOM is a strong candidate for the therapeutic antibiotics against edwardsiellosis in the Japanese flounder.

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