Short communication

**Bactericidal Effects of Disinfectants
on *Yersinia ruckeri***Masatoshi Yamasaki^{1*}, Takamitsu Sakai¹,
Takafumi Ito¹ and Koh-ichiro Mori²¹Tamaki Laboratory, Research Center for Fish Disease,
National Research Institute of Aquaculture, Japan
Fisheries Research and Education Agency,
Mie 519-0423, Japan²Research Center for Fish Disease, National Research
Institute of Aquaculture, Japan Fisheries Research and
Education Agency, Mie 516-0193, Japan

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ABSTRACT—Enteric redmouth disease caused by *Yersinia ruckeri*, is one of the most important aquaculture diseases worldwide. In this study, the effects of 11 disinfectants on *Y. ruckeri* ATCC29473 (2.8×10^8 CFU/mL) and *Y. ruckeri* FPC1215 (2.9×10^8 CFU/mL), were examined at 20°C. Nine disinfectants exhibited minimum bactericidal concentrations against both strains after 30 s exposure: 40% ethanol, 30% isopropanol, 4 mg/L povidone iodine, 1.25 mg/L sodium hypochlorite, 1:15,000 Rontect®, 1:3,840 Acecide®, 1:100 Hibiten®, 1:1,000 Osuban® and 1:1,000 Hyamine®. However, no disinfection effects were observed for Saponated cresol solution® or Pyceze®.

Key words: enteric redmouth disease, *Yersinia ruckeri*, disinfection, phenol coefficient

Enteric redmouth disease (ERM, yersiniosis) caused by *Yersinia ruckeri* is one of the most important diseases of salmonids and often leads to significant economic losses (Horne and Barnes, 1999). Despite the availability of a commercial vaccine routinely used to protect salmonids against *Y. ruckeri*, outbreaks attributable to nonmotile and lipase-negative strains (serotype O1 biotype 2) and other uncommon serotypes still occur (Cascales *et al.*, 2017). The bacterium was first isolated from rainbow trout *Onchorhynchus mykiss* in North America (Rucker, 1966), and has a wide host range, having been isolated from many salmonids (coho salmon *O. kisutch*; rainbow trout *O. mykiss*; sockeye salmon *O. nerka*; Chinook salmon *O. tshawytscha*; cutthroat trout *O. clarkii*; Atlantic salmon *Salmo salar*; brown trout *Salmo trutta*; Arctic char *Salvelinus alpinus*;

brook trout *Salvelinus fontinalis*; and Dolly Varden *Salvelinus malma*) and other fish species (Furones *et al.*, 1993). The disease is prevalent in North America, Venezuela, Chile, Australia, Europe, and South Africa (Furones *et al.*, 1993). In February 2015, ERM was first observed in Japan in chum salmon *O. keta* hatchery**. To prevent the spread of infection in aquaculture, it is necessary to employ the appropriate disinfection methods for equipment, tanks, aquaculture ponds, and clothing. In this regard, there is fragmentary information available on the effects of certain disinfectants, including iodophor, chlorine, and peracetic acid on *Y. ruckeri* (Ross and Smith, 1972; Wedemeyer and Nelson, 1977; Verner-Jeffreys *et al.*, 2009). Although these studies have examined by different test methods, and the effective concentrations and reaction times of the disinfectant against pathogens have been reported, it would be preferable to employ a single standardized test method. The diagnosis manual for specific diseases describes the effectiveness of alcoholic disinfectants and cationic surfactants against *Y. ruckeri* (the Japan Fisheries Resource Conservation Association, 2016). However, detailed information, such as the minimum effective concentration for each exposure time and non-effectiveness disinfectants, remains to be clarified.

The phenol coefficient method is a general test method used as a bactericidal efficacy test of a disinfectant against bacteria (Tilley, 1921; Akiyama *et al.*, 1989). The phenol coefficient is a measure of bactericidal power of a disinfectant in relation to phenol. To calculate phenol coefficient, the value indicating the degree of the greatest dilution of the test disinfectant in which it kills the bacteria within a given time (i.e. in 10 minutes but not in 5 minutes) is divided by the value indicating the degree of the greatest dilution of phenol. Because of this calculation method, the phenol coefficient is not recommended for alcoholic or halogenic disinfectants that work in a short time. The experimental method does, however, enable evaluation of the minimum effective concentration of these types of disinfectant by setting a short reaction time (Tsuji *et al.*, 1979; Shibata *et al.*, 1984). In addition, Tanaka *et al.* (1981) reported that the phenol coefficient method is suitable for evaluating the efficacy of disinfectants against bacteria causing fish diseases bacteria, as well as against other general bacteria.

In this study, we examined the effectiveness of 11 disinfectants using a method similar to that used for determining the phenol coefficient. In addition, we reported the minimum effective concentration for each exposure period of each disinfectant.

* Corresponding author
E-mail: myamasaki@affrc.go.jp

** Mano *et al.* (2015), Book of Abstracts of the Autumn Annual Meeting of The Japanese Society of Fish Pathology, p. 7.

Table 1. Disinfectants examined in this study

Group	Disinfectant	Component	Conventional concentration
Alcohols	Ethanol	Ethyl alcohol (99.5%)	76.9–81.4 v/v%
	Isopropanol	Isopropyl alcohol (99.7%)	50–70 vol%
Phenols	Saponated cresol solution [®]	Cresol (42–52 vol.%)	1:33–100
Biguanide	Hibiten [®]	5 w/v% Chlorhexidine gluconate	1:10–100
Cationic surfactant	Osuban [®]	10 w/v% Benzalkonium chlorite	1:100–1,000
	Hyamine [®]	10 w/v% Benzethonium chloride	1:50–1,000
	Rontect [®]	10 w/v% Didecylmethylammonium chloride	1:50–8,000
Halogens	Isodine [®]	10% Povidone iodine	50 mg/L ^{*1}
	Sodium hypochlorite	Available chlorine (Cl): 5.0%	100–200 mg/L ^{*2}
Bronopol	Pyceze [®]	Bronopol (500 g/L)	1:5,000–10,000
Peracetic acid	Acecide [®]	6 w/v% Peracetic acid (PAA)	0.2–0.3 w/v%

^{*1}; Povidone iodine concentration. ^{*2}; Effective chlorine concentration.

Materials and Methods

Two strains of *Y. ruckeri*, ATCC29473 and FPC1215, were used in the present study. *Y. ruckeri* FPC1215 was isolated from the kidney of a naturally infected chum salmon fry in Japan. The strains were incubated for 48 h at 25°C on Trypticase™ Soy Agar (TSA, Becton Dickinson) before use in disinfection tests. The bacteria were suspended in sterilized phosphate buffered saline. For use in disinfection tests, each bacterial suspension was adopted a concentration ranging from 10⁸ to 10¹⁰ CFU/mL according to the phenol coefficient method of Akiyama *et al.* (1989). Bacterial number was then determined according to the method of Miles *et al.* (1938) with TSA.

The disinfectants examined in this study were as follows: ethanol (Wako Pure Chemical Industries), isopropanol (Wako Pure Chemical Industries), Hibiten[®] (Sumitomo Dainippon Pharma), Osuban[®] (Nihon Pharmaceutical), Hyamine[®] (Daiichi Sankyo Espha), Rontect[®] (Scientific Feed Laboratory), Saponated cresol solution[®] (Taiyo Pharmaceutical), povidone iodine, Isodine[®] (DS Pharma Animal Health), sodium hypochlorite (Wako Pure Chemical Industries), Pyceze[®] (Novartis Animal Vaccines), and Acecide[®] (Haginoya). The 11 disinfectants are summarized in Table 1. The bactericidal effects of disinfection were determined using the method of Tanaka *et al.* (1981) with slight modifications. In brief, each disinfectant was diluted with sterilized distilled water to the concentrations ranging from 20% to 50% for ethanol and isopropanol, 1:50 to 1:800 for Saponated cresol solution[®], 1:100 to 1:8,000 for Hibiten[®], 1:1,000 to 1:8,000 for Osuban[®] and Hyamine[®], 1:10,000 to 1:40,000 for Rontect[®], 0.06–8.00 mg/L for povidone iodine, 0.3–10.0 mg/L for sodium hypochlorite, 1:5,000 to 1:60,000 for Pyceze[®], and 1:960 to 1:122,880 for Acecide[®]. Similarly, an 80–120 times dilution series was prepared for phenol (Wako Ltd.). An aliquot of bacterial suspension (100 µL) was mixed with 1 mL of

each disinfectant dilution in a deep, 96-well plate. After 0.5, 2.5, 5.0, 10.0, 15.0, and 20.0 min of incubation at 20°C, 10 µL of reaction mixture was added to 300 µL Mueller-Hinton II (MH2) Broth (Becton Dickinson) to determine the bactericidal activities of the disinfectant. To measure the effect of halogenic disinfectants, 10 µL of reaction mixture were combined with 20 µL of 0.01 M sterilized sodium thiosulfate solution to terminate the action of the disinfectant before adding it to the MH2 broth. After 2 days of incubation at 25°C, *Y. ruckeri* growth was evaluated based on the turbidity of bacterial suspension observed with the naked eye. In addition, the phenol coefficients of Hibiten[®], Osuban[®], and Hyamine[®] were calculated as previously described (Tanaka *et al.*, 1981).

Results and Discussion

The disinfection effect of each disinfectant tested is shown in Table 2. The minimum effective concentration for a 30 s exposure was 40% for ethanol, and 30% for isopropanol. These results indicate that ethanol and isopropanol would be effective for hand and equipment disinfection. Efficacy of sodium hypochlorite against *Y. ruckeri* was observed at 1.25 mg/L, and the bactericidal effect at this effective chlorine concentration did not change from 0.5–5 min. Povidone iodine exhibited a disinfection effect at concentrations greater than 4 mg/L at all reaction times. In contrast, Ross and Smith (1972) reported that 25 ppm of iodophor killed *Y. ruckeri* in less than 15 s, indicating that iodophor may be able to kill *Y. ruckeri* in a shorter reaction time. Recently, Glenn *et al.* (2015) reported the possibility that *Y. ruckeri* can be vertically transmitted in Chinook salmon and indicated the importance of egg disinfection in preventing vertical *Y. ruckeri* infection. In the present study, povidone iodine had a satisfactory disinfecting effect on *Y. ruckeri*, and is therefore expected to be suitable for egg surface disinfection to prevent the vertical transmission

Table 2. Effective minimum concentrations of disinfectants against *Yersinia ruckeri*

Disinfectant	Strain ^{*1}	Reaction time (min.)						PC ^{*3}
		0.5	2.5	5	10	15	20	
Ethanol	ATCC29473	40%	40%	40%	30%	30%	30%	N.R. ^{*6}
	FPC1215	40%	40%	40%	40%	40%	40%	
Isopropanol	ATCC29473	30%	30%	30%	20%	20%	20%	N.R.
	FPC1215	30%	30%	30%	30%	30%	30%	
Saponated cresol solution [®]	ATCC29473	— ^{*2}	—	—	—	—	—	N.C. ^{*7}
	FPC1215	—	—	—	—	—	—	N.C.
Hibiten [®]	ATCC29473	1:1,000	1:3,500	1:5,000	1:6,500	1:7,000	1:7,000	62
	FPC1215	1:100	1:100	1:100	1:1,000	1:2,000	1:3,000	16
Osuban [®]	ATCC29473	1:3,000	1:4,000	1:5,500	1:7,000	1:7,000	1:8,000	67
	FPC1215	1:1,000	1:2,000	1:3,000	1:5,000	1:6,000	1:7,000	53
Hyamine [®]	ATCC29473	1:2,000	1:4,000	1:4,500	1:6,500	1:6,500	1:8,000	62
	FPC1215	1:1,000	1:2,000	1:2,000	1:3,000	1:3,000	1:4,000	32
Rontect [®]	ATCC29473	1:15,000	1:25,000	1:25,000	1:25,000	1:25,000	1:30,000	N.C.
	FPC1215	1:15,000	1:25,000	1:30,000	1:30,000	1:30,000	1:30,000	N.C.
Isodine [®]	ATCC29473	4 mg/L ^{*4}	4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L	N.R.
	FPC1215	4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L	
Sodium hypochlorite	ATCC29473	1.25 mg/L ^{*5}	1.25 mg/L	1.25 mg/L	0.63 mg/L	0.63 mg/L	0.63 mg/L	N.R.
	FPC1215	1.25 mg/L	1.25 mg/L	1.25 mg/L	1.25 mg/L	1.25 mg/L	1.25 mg/L	
Pyceze [®]	ATCC29473	— ^{*2}	—	—	—	—	—	N.C.
	FPC1215	—	—	—	—	—	—	N.C.
Acecide [®]	ATCC29473	1:3,840	1:7,680	1:7,680	1:7,680	1:7,680	1:15,360	N.R.
	FPC1215	1:3,840	1:3,840	1:7,680	1:7,680	1:7,680	1:15,360	

^{*1}; Bacterial number of ATCC29473 and FPC1215 in 2 mg/mL of bacterial suspension was 2.8×10^9 CFU/mL and 2.9×10^9 CFU/mL, respectively. ^{*2}; No effect on the tested concentrations. Cresol[®] was tested at dilution concentrations from 1:50 to 1:800. Pyceze[®] was tested at dilution concentrations from 1:5,000 to 1:60,000. ^{*3}; PC: Phenol coefficient. ^{*4}; Available iodine concentration. ^{*5}; Effective chlorine concentration. ^{*6}; Evaluation by phenol coefficient is not recommended. ^{*7}; N.C.: not calculated.

of *Y. ruckeri*. Hibiten[®], Osuban[®], and Hyamine[®] showed bactericidal effects against *Y. ruckeri* within the range of concentrations described in the instruction manuals. The phenol coefficients for Hibiten[®], Osuban[®], and Hyamine[®] against *Aeromonas salmonicida* ATCC14174 and *Staphylococcus aureus* ATCC6538P reported by Tanaka *et al.* (1981) tended to be similar to those determined in the present study. These results indicated that Hibiten[®], Osuban[®], and Hyamine[®] may have the same disinfection ability against *Y. ruckeri* as against *A. salmonicida* and *S. aureus*. The disinfectant effect of Rontect[®] was effective at approximately 4 times the dilution of the maximum dilution ratio described in the instruction manual, and it exhibited no difference in its effect between the two *Y. ruckeri* strains examined. The disinfection effect of peracetic acid (PAA) against *Y. ruckeri* has been previously determined (Verner-Jeffreys *et al.*, 2009). In this study, the minimum effective concentration of Acecide[®] for a reaction time of 30 s was 1:3,840 (PAA: 0.001563%) indicating that Acecide[®] exhibits a sufficient disinfecting effect against *Y. ruckeri*. However, the concentration of PAA differs between commercial products, and when the PAA content is low, the disinfecting effect against *Y. ruckeri* decreases (Meinelt *et al.*, 2015). Therefore, it may be necessary to independently confirm the disinfecting effect of other PAA

products.

Saponated cresol solution[®] was tested at 50 times dilution for 20 min; however, no disinfecting effect was observed. Additionally, Pyceze[®] was also ineffective against *Y. ruckeri*, although it was tested at a dilution of 5,000 times for 20 min. Moreover, when the reaction time was extended to 30 min for the FPC1215 strain, as described in the manual (data not shown), we still observed no disinfection effect. These results indicate that these two disinfectants, Saponated cresol solution[®] and Pyceze[®] are not effective as disinfectants against *Y. ruckeri* when use as recommended in the instruction manuals.

In conclusion, nine disinfectants (ethanol, isopropanol, sodium hypochlorite, Isodine[®], Osuban[®], Hyamine[®], Rontect[®], Hibiten[®] and Acecide[®]) exhibited bactericidal effects against both the *Y. ruckeri* ATCC29473 and FPC1215 strains examined in the present study. Further, the results indicated that these disinfectants may have maximum efficacy against *Y. ruckeri* when used according to the manufacturer's recommendations.

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