In Vitro* and *In Vivo* Activities of Drugs against *Mycobacterium marinum* in Yellowtail *Seriola quinqueradiata

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ABSTRACT—*Mycobacterium marinum* (NJB 0419, MY 0401) isolated from yellowtail *Seriola quinqueradiata* showed drug susceptibility to rifampin, streptomycin (SM), ethambutol, kanamycin, sulfamethoxazole, amikacin, ciprofloxacin and ofloxacin by the broth dilution method. Furthermore, SM, kanamycin and sulfamonomethoxine activities were tested using the proportion method, and *M. marinum* was most susceptible to SM. Therefore, SM was used for the treatment of yellowtail (mean body weight 236 g) intramuscularly injected with NJB 0419 at a dose of 5.6×10^4 CFU/fish. The diet medicated with SM was orally administered at doses of 25 and 50 mg/kg body weight/day for 7 consecutive days after 4 h or 10 days following injection. Fish receiving 50 mg SM had lower mortality than the controls when medication started 4 h after injection. Therefore, SM may be an effective drug to control *M. marinum* infection. The timing for initiating oral medication with SM requires further study.

Key words: *Mycobacterium marinum*, *Seriola quinqueradiata*, yellowtail, drug susceptibility, treatment, mycobacteriosis

Recently, *Mycobacterium marinum* infections have been shown to occur in cultured yellowtail *Seriola quinqueradiata* and damaged to the health of the fish (Weerakhun *et al.*, 2007). The use of drugs for the treatment of *M. marinum* infections in humans are well known (Edelstein, 1994; Harth *et al.*, 1994; Lam *et al.*, 2006). However, chemotherapeutics against *Mycobacterium* infection in fish and drug sensitivities of *M. marinum* are not well known (Decostere *et al.*, 2004). Thus, the selection of effective chemotherapeutics *in vitro* was first examined following guidance from the National Committee for Clinical Laboratory Standards (NCCLS), which included some information on antimicrobial agents for *Mycobacterium* spp. infection in aquatic animals (Heifets, 1991a).

In vitro activities of drugs against *M. marinum* are estimated by the proportion method and broth microdilution method. Until now, the classical proportion method has been used for drug susceptibility testing

against *Mycobacterium* spp. If the proportion of resistant colonies is less than or equal to 1%, the organism is considered susceptible (Wallace *et al.*, 1986; Heifets, 1991c). The major advantage of this system is that susceptibilities can be tested directly. However, problems are that an agar dilution is cumbersome for the testing of drugs at various concentrations and the evaluation of drugs in combination at various concentrations. Recently, it was reported that the microdilution test method for *M. tuberculosis* is a practical, rapid, quantitative and non-radiometric alternative for the determination of minimum inhibitory concentrations (MICs) (Wallace *et al.*, 1986; Leite *et al.*, 2000) and is suitable for the early determination of multidrug-resistant *M. tuberculosis* (Coban *et al.*, 2004). Based on previous success with rapidly growing mycobacteria, a microdilution MIC system was devised for slow-growing mycobacterial species using Middlebrook 7H9 broth (Wallace *et al.*, 1986).

In this paper, we examined the drug sensitivity of *M. marinum* using broth dilution method and the proportion method, then the selected antibiotics was used for *in vivo* trial for chemotherapy against *M. marinum* infection.

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Materials and Methods

Drug sensitivity test by the broth microdilution method

Preparation of bacterial suspension

Two isolates of *M. marinum*, NJB 0419 and MY 0401, from yellowtail were used (Table 1). Each bacterium was inoculated onto 1% Ogawa medium and incubated for 2 wk at 25°C. They were then suspended with sterilized distilled water, and the concentration was adjusted with 0.5 McFarland standard. The bacterial suspension was inoculated onto Middlebrook 7H10 agar with OADC enrichment (Becton Dickinson and Company) and the number of colony forming units (CFU) per mL was counted.

Table 1. Sources of *Mycobacterium marinum* used in this study

Strain	Host	Prefecture	Date of isolation
NJB 0419	Yellowtail	Kagoshima	Oct. 18, 2004
MY 0401	Yellowtail	Ehime	Sept. 6, 2004

Drugs

Thirteen anti-tuberculosis and antimicrobial drugs were used for sensitivity tests. The original drug solution was diluted with sterilized distilled water. Dilutions of antibiotics were performed, based on critical concentrations of each drug for conventional drug sensitivity testing with other *Mycobacterium* spp. (Cynamon and Klemens, 1991; Heifets, 1991c).

Antibiotics and the concentrations used in this study were isoniazid (INH; 0.045–200 µg/mL), rifampin (RFP; 0.045–200 µg/mL), streptomycin (SM; 0.045–200 µg/mL), ethambutol (EMB; 0.045–50 µg/mL), ethionamide (ETH; 0.045–50 µg/mL), kanamycin (KM; 0.045–200 µg/mL), cycloserine (CS; 0.045–200 µg/mL), amikacin (AMK; 0.045–200 µg/mL), ciprofloxacin (CPFX; 0.045–50 µg/mL), ofloxacin (OFLX; 0.045–200 µg/mL), doxycycline (DOXY; 0.045–200 µg/mL), sulfamethoxazole (SMX; 0.045–200 µg/mL) and erythromycin (EM; 0.045–200 µg/mL) and erythromycin (EM; 0.045–200 µg/mL) and sulfamethoxazole (SMX; 0.045–200 µg/mL).

Sensitivity testing

The broth microdilution method was performed in U-shaped 96-well microtitre plates following a modification of Coban *et al.* (2004). The wells were filled with 0.1 mL Middlebrook 7H9 broth with OADC, and serial dilutions were performed for each drug. Each plate was kept at 25°C until used. The first well was filled with medium without drug as a control. Each well was inoculated with 5 µL of bacterial suspension containing 1.25×10^4 CFU/mL. The plate was sealed and incubated at 25°C for 1–2 mo until bacteria grew in the control medium. In addition, growth of the control was also confirmed on Middlebrook 7H10 agar with OADC. The

minimal inhibitory concentration (MIC) was defined as the lowest concentration that exhibited no growth by visual reading under the microscope. The isolates were considered susceptible to a drug if their MICs were below or equal to the critical concentration (Heifets, 1991a). The results were evaluated until the bacteria grew in the control well or for 2 months. Then, the minimal bactericidal concentration (MBC) was evaluated. When growth did not appear in the wells, the broth was inoculated onto an agar plate and survival was examined. MBCs were the lowest concentration that exhibited no growth or the growth of less than five colonies on agar. MBC/MIC ratios were evaluated for bacteriostatic or bacteriocidal activities for each drug as described by Ho *et al.* (1997) and Kone *et al.* (2004). If MBC/MIC ratios were not greater than four as described by Kone *et al.* (2004), they were bactericidal.

Drug sensitivity test by the proportion method

Preparation of bacterial suspension

The aim of this test was to examine the drug sensitivity of *M. marinum* NJB 0419. The proportion method was performed according to Heifets (1991c). The bacterium previously adjusted with 0.5 McFarland was suspended in sterilized distilled water.

Drugs

SM and KM were selected from the results of the broth microdilution method. Sulfamonomethoxine (SMMX) was used instead of SMX because their mode of action is similar and SMMX is allowed to be used for control of nocardiosis in yellowtail. The original drug solution was diluted with sterilized distilled water and appropriate drug solutions were added to Middlebrook 7H10 agar with OADC. The final concentration of drugs incorporated into the medium referred to the MIC critical concentrations or the breakpoints for sensitivity testing with other *Mycobacterium* spp. (Cynamon and Klemens, 1991; Heifets, 1991c). However, data on *M. marinum* has not been reported for the proportion method (Heifets, 1991c). The critical or main concentrations of SM, KM and SMMX for *M. marinum* were 4.0, 5.0 and 32 µg/mL, respectively, with additional lower and greater concentrations (SM; 2–16 µg/mL; KM; 2.5–20 µg/mL; SMMX; 16–128 µg/mL).

Sensitivity testing

Ten microliters of two concentrations of bacterial suspension (2.5×10^4 and 2.5×10^6 CFU/mL) were inoculated onto each agar plate containing different concentrations of the drugs. Each plate was divided into four channels and was inoculated at three positions by 10 µL of a high and low concentration of bacteria in duplicate. Each plate was sealed and incubated at 25°C for 1–2 mo until bacteria without drugs grew on the medium. A proportion of resistant colonies was also observed.

Treatment of *Mycobacterium marinum* infection

Fish

Yellowtail (n = 120; average body weight, 236 g; average total length, 24.7 cm) were used for experimental treatment and were obtained from a commercial fish farm in Kagoshima Prefecture. The fish were first treated with tap water to remove external parasites and fish were acclimatized in the holding tanks at Kagoshima Prefecture Fisheries Technology and Development Center. The water temperature was between 24.0°C and 30.5°C during the experiment. Fish were fed pellets everyday and 20 fish were kept in each of six aquaria.

Bacteria

M. marinum NJB 0419 was used in this experiment. The number of CFU/ mL was counted by inoculating diluted bacterial suspension onto Middlebrook 7H10 agar with OADC and adjusting to 1.4×10^5 CFU/ mL. Intramuscular injection of 0.4 mL of the bacterial suspension was performed per fish. Fish used as negative controls were injected with 0.85% NaCl instead of the bacterial suspension.

Drugs

SM (Sigma) was mixed with commercial food using gluten. The fish were orally administered, based on a feeding ratio of 2% of their body weight per day. Commercial food without the drug was also fed daily to positive and negative control fish at the same ratio.

Treatment

All fish were fed with medicated diet or non-medicated diet (control) from the fourth hour after injection. The medicated diet of the two treatment groups were administered SM orally from the fourth hour after injection at a dosage of 25 and 50 mg/kg body weight for seven days. The other two treatment groups were also administered streptomycin at 25 and 50 mg/kg body weight from the 10th day after injection for seven days (Table 2). The fish were observed for 90 days and mortalities were recorded every five days and the fish were autopsied to confirm the typical clinical signs of the infection, but re-isolation of the bacterium from the moribund fish was not attempted.

Table 2. Experimental design of chemotherapeutic treatment by streptomycin against *Mycobacterium marinum* infection in yellowtail

Tank number	Treatment group				Control group	
	T1	T2	T3	T4	T5	T6
Bacterial challenge*	○	○	○	○		○
0.85% NaCl					○	
Oral administration of streptomycin (mg/kg BW)	25	25	50	50	0	0
Start of treatment after challenge	4 h	10 days	4 h	10 days	No treatment	No treatment

* Yellowtail were experimentally injected with *M. marinum* NJB 0419

Table 3. The MICs ($\mu\text{g/mL}$), MBCs ($\mu\text{g/mL}$) and MBC/MIC ratio of 19 drugs against *Mycobacterium marinum* isolated from yellowtail ($\mu\text{g/mL}$)

Drug	NJB 0419			MY 0401		
	MIC	MBC	Ratio* ²	MIC	MBC	Ratio* ²
Isoniazid	1.56	>200	—	3.12	3.12	1
Rifampin	0.09* ¹	0.39* ¹	4	0.09* ¹	0.39* ¹	4
Streptomycin	<0.04* ¹	0.39* ¹	—	1.56* ¹	100	64
Ethanbutol	1.56* ¹	>50	—	1.56* ¹	1.56* ¹	1
Ethionamide	6.25	>50	—	6.25	6.25	1
Kanamycin	0.09* ¹	>200	—	3.12* ¹	3.12* ¹	1
Cycloserine	25* ¹	>200	—	12.5* ¹	12.5* ¹	1
Amikacin	0.09* ¹	0.78* ¹	8	0.39* ¹	0.39* ¹	1
Ciprofloxacin	0.78* ¹	6.25* ¹	8	0.19* ¹	0.19* ¹	1
Ofloxacin	0.78* ¹	12.5	16	12.5	12.5	1
Doxycyclin	25	50	2	25	50	2
Erythromycin	50	50	1	3.12* ¹	200	64
Sulfamethoxazole	25* ¹	>200	—	12.5* ¹	100	8

*¹ The MIC and MBC were lower than the critical concentration (Cynamon and Klemens, 1991; Heifets, 1991c).

*² MBC/MIC ratios were evaluated. If the ratios are not more than four, it is bactericidal (Kone et al., 2004).

Results

Drug sensitivity test by the broth microdilution method

M. marinum were sensitive to RFP, SM, EMB, KM, CS, AMK, CPFX, EM and SMX.. Most drugs also had high bactericidal activity against *M. marinum*, where the MIC/MBC ratios were also lower than 4 and MBCs were substantially lower than the critical concentration (Table 3).

Testing of drug sensitivity using the proportion method

The efficacies of the drugs against *M. marinum* NJB 0419 by the proportion method showed that the low concentration of bacteria could not grow on agar with SM and SMMX, but were resistant to the critical concentration of KM. The high concentrations of bacteria were resistant to agar containing SMMX (Table 4). In addition, the results showed that the resistance of bacteria depended on the number of bacteria. SM only inhibited bacterial growth at both bacterial concentrations. The number of bacteria resistant to KM also showed a high percentage, whereas SM was 0% (Table 5).

Treatment of *Mycobacterium marinum* infection

The cumulative mortalities of treatments were dependent on the dosage and onset of the medicated diet. The cumulative mortalities of the fish fed with medicated diet four h after the challenge were 45% (group T1: 25 mg SM) and 20% (group T3: 50 mg SM), in contrast to 70% mortality in non-medicated fish (group T6) at 90 days post injection (Fig. 1). The negative control fish (T5: saline-injected) were alive throughout the experiment, whereas the first mortality in the medicated group started at day 50, with the exception of group T3, which started at day 58. On the other hand, the cumulative mortalities of the medicated fish 10 days after the

challenge were 75% (group T2: 25 mg SM) and 60% (group T4: 50 mg SM) at 90 days post injection (Fig. 2). The data showed that SM was not effective against *M. marinum* infections if it was administered ten days after injection.

The affected fish showed cutaneous nodules, ulcerative lesions and abdominal swelling. The necropsy findings showed lesions in the injection site and white nodules on internal organs.

Table 4. Drug sensitivities of *Mycobacterium marinum* NJB 0419 by proportion method on Middlebrook 7H10 agar with OADC

Drug concentration ($\mu\text{g/mL}$)	Bacterial concentration (CFU/mL)	
	Low (2.5×10^4)	High (2.5×10^4)
Streptomycin	2.0	—
	4.0*	—
	8.0	—
	16	—
Kanamycin	2.5	+
	5.0*	+
	10	+/-
	20	+/-
Sulfamonomethoxine	16	+
	32*	+
	64	+/-
	128	+/-

* The main concentrations, which were MIC critical concentrations or the breakpoints for sensitivity testing of the other *Mycobacterium* spp. (Cynamon and Klemens, 1991; Heifets, 1991c).

—: non-resistant or bacteria can not grow. +: resistant or bacteria can grow. +/-: thin film of bacteria was found and/or they did not grow on both two replication.

Table 5. Drug sensitivities and resistance measured by proportion method of *Mycobacterium marinum* NJB 0419 on Middlebrook 7H10 agar with OADC®

Drug concentration ($\mu\text{g/mL}$)	Number of colony (CFU)	Resistance*2 (%)
Control	0	252
Streptomycin	2.0	0
	4.0	0
	8.0	0
	16	0
Kanamycin	2.5	273
	5.0	142
	10	0
	20	0
Sulfamonomethoxine	16	124
	32	0
	64	0
	128	0

*1 The main concentrations, which were MIC critical concentrations or the breakpoints for sensitivity testing of the other *Mycobacterium* spp. (Cynamon and Klemens, 1991; Heifets, 1991c).

*2: Number of colonies on drug containing media/number of colonies on the control media

Fig. 1. The cumulative mortality of yellowtail artificially infected with *Mycobacterium marinum* NJB 0419. Streptomycin was orally administered to fish at 25 (◆, T1 in the text) and 50 (▲, T3) mg/kg BW/day for seven days after four h following the injection. ■: saline-injected group (T5), ●: *M. marinum*-injected group without medication (T6).

Fig. 2. The cumulative mortality of yellowtail artificially infected with *Mycobacterium marinum* NJB 0419. Streptomycin was orally administered to fish at 25 (□, T2 in the text) and 50 (△, T4) mg/kg BW/day for seven days after ten days following the injection. ■: saline-injected group (T5), ●: *M. marinum*-injected group without medication (T6).

Discussion

The definition used for "drug susceptible" is usually MIC (Ellner and Neu, 1981). However, the MBC/MIC ratio is usual standard for the expression of bactericidal potency (Heifets, 1991a). This ratio can be used to estimate the probability of a bactericidal effect upon strains, if the degree of susceptibility has been expressed as the MIC. For most infections, the relevance of bactericidal activity is not known, although there are some exceptions (Washington, 1981; Schonknecht *et al.*, 1985; NCCLS, 1987). However, the study showed that MBC/MIC ratios could not be accepted if a higher concentration required to inhibit or kill bacteria than the critical concentration, when compared with previous studies using *M. tuberculosis* (Heifets, 1991b).

The *in vitro* activity of the drugs against *M. marinum* was almost the same as that for *M. tuberculosis* (Table 3). *M. marinum* strains were uniform in their degree of susceptibility to the drugs, which were similar to their activity against *M. tuberculosis* (Heifets, 1991b). *M. marinum* showed a lower MIC than the critical concentration against *M. tuberculosis* and the rapidly growing *Mycobacterium* spp. (Wallace *et al.*, 1986).

Susceptibility data on *M. marinum* are scarce and rely upon a small number of strains and antibiotics tested. Consequently, intrinsic antibiotic susceptibilities of *M. marinum* are not well defined, and methods for their routine determination have not been evaluated (Aubry *et al.*, 2000). *M. marinum* was inhibited at the lowest concentration when compared with the critical concentration for *M. tuberculosis*. As mentioned above, it was thought that the efficacy of the drugs for *M. marinum* should be comparable with *M. tuberculosis* (Heifets, 1991b).

In our experiments, SM assessed using the proportion method with *M. marinum* NJB 0419 could inhibit bacterial growth at both low and high concentrations, while the bacteria showed resistance to SMX and KM. The MIC and MBC of SM were only lower than the critical concentration from the broth microdilution and proportion methods.

SM was effective against infection and the chemotherapeutic efficacy was dependent on the dosage and the onset of the medication. The treatments of T3 and T1, which were orally administered at 4 h after injection, and the high dosage showed greater efficacies than the other treatments. It showed that the bacterial growth was inhibited in the fish. However, bacteria were still alive in the fish, and infection in some fish developed slowly during 90 days of the experimental period.

The success of the treatment might also depend on the period of the treatment, because it takes time to kill all the bacteria in a fish. Treatment of *Mycobacterium* infection in humans requires a period of at least 4–8 wk (Johnston and Izumi, 1987; Edelstein, 1994; Harth *et al.*, 1994; Barton *et al.*, 1997; Shih *et al.*, 1997; Saadamand *et al.*, 1999; Van Seymortier *et al.*, 2004; Lam *et al.*, 2006). In addition, SM would be more effective when the fish were naturally infected with *M. marinum*, which are less number of bacteria than that in the artificial challenge test and developed the disease chronically. Colorni *et al.* (1998) also examined effectiveness of SM against sea bass experimentally infected with *M. marinum*. The method was that 1% solution of SM was intraperitoneally injected at week 9 post-inoculum, but the infection could not controlled. It showed that the treatment was impossible if granulomas were produced in fish.

As mentioned above, *M. marinum* was the most susceptible to SM *in vitro*. Therefore, SM was used for the treatment by oral administration against *M. marinum* experimental infection in yellowtail. However, the timing for initiating oral medication with SM requires further study.

References

- Aubry, A., V. Jarlier, S. Escolano, C. Truffot-Pernot and E. Cambau (2000): Antibiotic susceptibility pattern of *Mycobacterium* spp.

- bacterium marinum*. *Antimicrob. Agents Chemother.*, **44**, 3133–3136.
- Barton, A., R. M. Bernstein and J. K. Struthers (1997): *Mycobacterium marinum* infection causing septic arthritis and osteomyelitis. *Br. J. Rheumatol.*, **36**, 1207–1209.
- Coban, A. Y., A. Birinci, B. Ekinici and B. Durupinar (2004): Drug susceptibility testing of *Mycobacterium tuberculosis* by the broth microdilution method with 7H9 broth. *Mem. Inst. Oswaldo Cruz.*, **99**, 111–113.
- Colorni, A., R. Avtalion, W. Knibb, E. Berger, B. Colorni and B. Timan (1998): Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with *Mycobacterium marinum* and treated with streptomycin and garlic (*Allium sativum*) extract. *Aquaculture*, **160**, 1–17.
- Cynamon, M. H and S. P. Klemans (1991): Drug susceptibility test for *Mycobacterium fortuitum* and *Mycobacterium chelonae*. In "Drug susceptibility in the chemotherapy of mycobacterial infections" (ed. by L. G. Heifets). CRC Press. London. pp. 147–159.
- Decostere, A., K. Hermans and F. haesebrouch (2004): Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. *Vet. Microbiol.*, **99**, 159–166.
- Edelstein, H. (1994): *Mycobacterium marinum* skin infections: Report of 31 cases and review of the literature. *Arch. Intern. Med.*, **154**, 1359–1364.
- Ellner, P. D. and H. C. Neu (1981): The inhibitory quotient. A method for interpreting minimum inhibitory concentration Data. *JAMA*, **248**, 1575
- Harth, M., E. D. Ralph and R. Faraawi (1994): Septic arthritis due to *Mycobacterium marinum*. *J. Rheumatol.*, **21**, 957–960.
- Heifets, L. G (1991a): Drug susceptibility tests and the clinical outcome of chemotherapy. In "Drug susceptibility in the chemotherapy of mycobacterial infections" (ed. by L. G. Heifets). CRC Press. London. pp. 1–11.
- Heifets, L. G (1991b): Antituberculosis drugs: antimicrobial activity in vitro. In "Drug susceptibility in the chemotherapy of mycobacterial infections" (ed. by L. G. Heifets). CRC Press. London. pp. 13–57.
- Heifets, L. G (1991c): Drug susceptibility test in the management of chemotherapy of tuberculosis, In "Drug susceptibility in the chemotherapy of mycobacterial infections" (ed. by L. G. Heifets). CRC Press. London. pp. 89–121.
- Ho, Y. I. I., C. Y. Chan and A. F. B. Cheng (1997): *In vitro* activities of aminoglycoside-aminocyclitols against mycobacteria. *J. Antimicrob. Chemother.*, **40**, 27–32.
- Johnston, J. M. and A. K. Izumi (1987): Cutaneous *Mycobacterium marinum* infection ("swimming pool granuloma"). *Clin. Dermatol.*, **5**, 68–75.
- Kone, W. M., K. Atindehou, C. Terreaux, K. Hostettmann, D. Traore and M. Dosso (2004): Traditional medicine in North Cote-d'Ivoire: screening of 50 medicinal plants for antibacterial activity. *J. Ethnopharmacol.*, **93**, 43–49.
- Lam, A., W. Toma and N. Schlesinger (2006): *Mycobacterium marinum* arthritis mimicking rheumatoid arthritis. *J. Rheumatol.*, **33**, 817–819.
- Leite, C. Q. F., A. L. R. Z. Beretta, I. S. Anno and M. A. S. Telles (2000): Standardization of broth microdilution method for *Mycobacterium tuberculosis*. *Mem Inst Oswaldo Cruz.*, **95**, 127–129.
- National Committee for Clinical Laboratory Standards (1987): Methods for determining bactericidal activity of antimicrobial agents; proposed guidelines, NCCLS document M26-P. Villanova.
- Saadmand, B., J. K. Poulton and C. L. Kauffman (1999): *Mycobacterium marinum* with associated bursitis. *J. Cutan. Med. Surg.*, **3**, 218–220.
- Schonknecht, F. D., L. D. Sabath and C. Thonsberry (1985): Susceptibilities tests, In "Manual of clinical Microbiology, 4th ed." (eds. by E. H. Lennette, A. Balows, W. J. Jr. Hausler and H. J. Shadomy). American Society for Microbiology. Washington, DC. pp. 1000.
- Shih, J. Y., P. R. Hsueh and Y. L. Chang (1997): Osteomyelitis and tenosynovitis due to *Mycobacterium marinum* in a fish dealer. *J. Formos. Med. Assoc.*, **96**, 913–916.
- Van Seymourtier, P., K. Verellen and I. De Jonge (2004): *Mycobacterium marinum* causing tenosynovitis. 'Fish tank finger'. *Acta. Orthop. Belg.*, **70**, 279–282.
- Wallace, R. J. Jr, D. R. Nash, L. C. Steele and V. Steingrube (1986): Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 Broth. *J. Clin. Microbiol.*, **24**, 976–981.
- Washington, J. A., II (1981): Bactericidal test, In "Laboratory procedure in clinical microbiology" (ed. by J. A. Washington). Springer-Verlag. New York. pp. 715.
- Weerakhun, S., N. Aoki, O. Kurata, K. Hatai, H. Nibe and T. Hirae (2007): *Mycobacterium marinum* infection in cultured yellowtail *Seriola quinqueradiata* in Japan. *Fish Pathol.*, **42**, 79–84.