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Modified resazurin microtiter assay for in vitro and in vivo assessment of sulfamonomethoxine activity against the fish pathogen *Nocardia seriolae*

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Abstract Resazurin microtiter assay (REMA) was carried out using four sulfonamides, three culture media, and four inoculum sizes as a first screening step to establish an easy-to-interpret sulfonamides susceptibility testing method for *Nocardia seriolae*. The in vitro activity of sulfamonomethoxine (SMM) against 190 clinical *N. seriolae* isolates was then examined, and in vivo experimental treatment was performed. When the culture medium and the inoculum size were considered in tandem, a 0.5× the original concentration of cation-adjusted Mueller–Hinton broth and an inoculum size of 10^2 CFU/well showed the clearest endpoint reading for all tested drugs, and the REMA-generated data were in excellent agreement with those generated by the reference Etest method. SMM activity showed minimum inhibitory concentration (MIC) values of 4–32 μg/ml against all tested *N. seriolae* isolates. Treatment of amberjack groups experimentally infected with *N. seriolae* isolates having SMM MICs of 4 and 32 μg/ml, resulted in survival rates of 100% and 87.5% in the two groups, respectively. In this study, we developed a simple visual method to test SMM activity against *N. seriolae*.

Keywords *Nocardia seriolae* · Resazurin microtiter assay · Sulfamonomethoxine · MIC · In vivo treatment

Introduction

Nocardia seriolae has been isolated from different marine fish species [1–9]. In recent years, *N. seriolae* infections have caused increasing damage to Japan's fish industry, especially yellowtail *Seriolae quinqueradiata* and amberjack *S. dumerili* farms, among others [2, 10]. In China and Korea, nocardiosis associated with mass mortality has been reported in farmed snakehead *Ophiocephalus argus* [6, 11]. Because outbreaks of nocardiosis often cause death of commercial-sized as well as small-sized intensively cultured fish species, studies investigating the treatment and prevention of nocardiosis in fish are necessary.

In Japan, sulfamonomethoxine (SMM) has been permitted as an effective drug for treatment of *N. seriolae* infections in fish farms. However, no recent data on the antimicrobial activity of SMM against *N. seriolae* have been published yet, and fish farms do not have updated guidelines for the monitoring of drug sensitivity or resistance of isolated strains. Broth microdilution is the recommended method for susceptibility testing of aerobic actinomycetes [12]. For sulfonamides, the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards

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(NCCLS), defined the minimum inhibitory concentration (MIC) as the concentration of drug that causes an approximately 80% inhibition of growth compared to the growth in the control with no drug [13]; this definition does not clarify an accurate endpoint estimation, and this may be a considerable limiting factor for studies in susceptibility testing.

Resazurin is a blue redox dye commonly used as an indicator for chemical cytotoxicity [14]. Resazurin turns pink when it is reduced by viable cells. Recently, a new method has been proposed that uses resazurin as an oxidation–reduction colorimetric indicator, and this method was found to be successful in determining the drug resistance and MICs of antimicrobial agents against *Mycobacterium tuberculosis* [15–17]. In order to establish a convenient and easy-to-interpret method for susceptibility testing, we used this resazurin indicator to investigate the influence of culture media type and concentration and inoculum size on the activity of sulfonamides against *N. seriolae* isolates. We then implemented the established method to point out the current condition of sensitivity or resistance of clinical isolates of *N. seriolae* to SMM treatment in fish farms.

Materials and methods

Bacterial strains

One hundred and ninety *N. seriolae* isolates were collected from four prefectures in Japan during 2008–2011. All isolates were from diseased yellowtail ($n = 136$), amberjack ($n = 44$), striped jack *Pseudocaranx dentex* ($n = 1$), and Japanese flounder *Paralichthys olivaceus* ($n = 9$). Primary isolation was performed using Ogawa medium (Nissui, Tokyo, Japan), brain heart infusion agar (BHIA; Difco, MI, USA), or Todd Hewitt agar (THA; Difco, MI, USA). After being subcultured for isolation of pure colonies, all the strains were cultured in Todd Hewitt broth, and were maintained at −80°C until use. Pure colonies obtained on the agar plates were subjected to Gram and Ziehl–Neelsen staining. All the strains were Gram-positive, acid-fast filamentous or branching bacilli as observed by light microscopy. They showed a positive reaction in the species-specific PCR targeting of the 16S rRNA gene, as previously described [2]. ChromID MRSA medium (bioMérieux, Marcy l'Etoile, France) was used to determine the α-glucosidase (α-glu) activity as previously described [18].

Resazurin microtiter assay (REMA)

A pilot study, with 10 representative α-glu-positive and 10 representative α-glu-negative *N. seriolae* isolates, was carried out using four sulfonamides, three culture media, and four inoculum sizes as a first screening step to establish an easy-to-interpret sulfonamides susceptibility testing method for *N. seriolae*. Log-phase cells were used for inoculum preparation. Before testing, the isolates were inoculated on THA plates and incubated at 25°C for 4 days to ensure suitable growth and purity. A loopful of the bacterial growth was then scraped from the medium and inoculated into 5 ml of cation-adjusted Mueller–Hinton broth II (CAMHB; Difco, MI, USA) containing approximately 2 g of sterile glass beads (2 mm in diameter) to assist cell dispersion. The cultures were incubated at 25°C until the optical density was approximately equivalent to that of the no. 1.0 McFarland standard. The incubation period required for adequate growth was about 3 days. During this period, tubes were shaken every day at 300 times/min for 10 min on a KM shaker (Iwaki Sangyo, Tokyo, Japan) to disperse the bacteria. In preparation for inoculation, suspensions equivalent to the no. 0.5 McFarland turbidity standard were adjusted by visual examination, and inocula of approximately 10^6, 10^5, 10^4, and 10^3 CFU/ml were prepared with sterile 0.85% NaCl.

The MICs of sulfamonomethoxine (SMM), sulfadimethoxine (SDM), sulfamethoxazole (SMX), and sulfisozole (SIZ) were determined. These drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) except for SIZ, which was obtained from Wako Pure Chemical Industries (Osaka, Japan), and were reconstituted in the recommended diluents to yield stock solutions according to the procedures proposed by the CLSI [13]. Serial two-fold dilutions, comprising original and double concentrations of CAMHB and a double concentration of yeast nitrogen base broth with glucose (YNB; Difco, MI, USA), were prepared immediately before use. The prepared dilutions were dispensed (100 µl/well) into sterile U-bottom 96-well microtiter plates (Becton–Dickinson Lab, NJ, USA). Next, 100 µl of the aforementioned inocula were added to each well, resulting in final concentrations of approximately 10^5, 10^4, 10^3, or 10^2 CFU/well.

Final drug concentrations were 0.50–512 µg/ml, and the final concentrations of media were 0.5× and 1× original concentration of CAMHB and 1× original concentration of YNB. The microplates were covered and incubated at 25°C under humidified conditions. The MICs were recorded after 5 days of incubation, at which time all isolates showed optimal growth. Each panel contained a control well without antibiotics or inoculum. The type strain *N. seriolae* ATCC 43993 was used as a control for validating the identification scheme. The MIC before adding the resazurin indicator was defined as the lowest concentration of the antimicrobial agent that inhibited visible growth.

The resazurin reagent was obtained as a resazurin sodium salt powder (Sigma-Aldrich, USA). A working

solution was prepared at a concentration of 0.01% (w/v) in distilled water and filtered through a 0.2-μm membrane (Millipore Corp., New Bedford, MA, USA); this working solution was stored at 4°C for up to 1 week.

After the microplates were incubated at 25°C for 5 days and the observed MIC was recorded, 30 μl of the resazurin working solution was added to each well, and the plates were incubated overnight for color development. A change from blue to pink indicates the reduction of resazurin and, therefore, bacterial growth. The MIC after adding the resazurin indicator was defined as the lowest drug concentration that prevented this color change [16].

Comparison between REMA and Etest

To evaluate the results of REMA, we performed Etest (bioMérieux, Marcy l'Étoile, France) to compare the activity of SMX against 50 *N. seriolae* isolates, concurrently with REMA (SMX was the only available tested sulfonamide for Etest strips). As previously described, REMA was performed in the aforementioned pilot study (using 0.5× the original concentration of CAMHB and an inoculum size of 10^2 CFU/well). The results obtained, using this combination of medium and inoculum size, were reproducible and easy to interpret. Etest was performed according to the manufacturer's instructions. CAMHB agar plates (90 mm in size) were inoculated by confluent swabbing on the surface with a no. 0.5 McFarland standard *N. seriolae* suspension, prepared as described above. An SMX Etest strip was applied to each plate, and the plates were incubated at 25°C in ambient air for 5 days. The Etest MIC was the lowest drug concentration at which the border of 80% growth inhibition within the elliptical zone intercepted the strip scale. The interpretive breakpoints used in this study were recommended for the *Nocardia* species by the CLSI [12]. Using Etest as the reference standard, we calculated essential agreement of REMA as the percentage of isolates having MICs within one double-fold dilution, and categorical agreement as the percentage of isolates having identical interpretations of sensitive or resistant [19].

In vitro activity of SMM

We screened the in vitro activity of SMM against all tested *N. seriolae* isolates ($n = 190$) by using the established REMA. The final concentration of SMM ranged from 0.50 to 512 μg/ml. The final medium concentration was 0.5× the original concentration of CAMHB, and the final inoculum size was 10^2 CFU/well. After the microplates were incubated at 25°C for 5 days, 30 μl of the resazurin working solution was added to each well, and the plates were incubated overnight for color development. The MIC was defined as the lowest drug concentration that prevented color change [16].

Experimental treatment

To date, no criteria have been established for interpreting the MIC results of SMM in *N. seriolae* isolates. Therefore, an in vivo trial was conducted to assess the potency of SMM in controlling nocardiosis in amberjack fish experimentally infected with *N. seriolae* isolates having SMM MICs of 4 and 32 μg/ml.

Amberjack ($n = 80$; average body weight 110 g) were used in this experiment. The fish were acclimated in a 200-l tank for 1 week. The tank was aerated and supplied with a continuous flow of seawater. The temperature and dissolved oxygen level were maintained at approximately 25.0°C and 6.0 mg/l, respectively. The fish were fed a 4.0-mm pellet commercial diet (Chubo Shiryo Co., Japan) once a day, and the daily feeding rate was 2% of body weight. After acclimation, the fish were randomly assigned to five groups (16 fish per group): two treated groups, two untreated groups, and one negative control group. Each group was transferred to its own 200-l tank. The tanks were aerated, and the temperature was maintained at approximately 25.0°C throughout the trial.

N. seriolae UT10 isolate (α-glu-negative and an SMM MIC of 4 μg/ml) and AM802 isolate (α-glu-positive and an SMM MIC of 32 μg/ml) were grown on BHIA plates at 25°C for 5 days. A homogenous bacterial suspension was prepared in sterile 0.85% NaCl to a final concentration of approximately 10^7 CFU/ml. Experimental infection was performed by intraperitoneal injection of 0.1 ml of the bacterial suspension of UT10 or AM802 in each fish (approximately 10^6 CFU/fish; two tanks per isolate; Table 1). The fish in the negative control group were injected intraperitoneally with 0.1 ml sterile 0.85% NaCl instead of the bacterial suspension. Diluted bacterial suspensions were inoculated on BHIA plates, and the resulting colonies were counted.

Medicated feed was prepared by adding sulfamonomethoxine sodium (SMM-Na; Daimeton®; Meiji Seika Pharma Co., Japan) to the commercial diet with the aid of a small amount of fish oil (1% w/w). SMM-Na was used at the manufacturer's recommended oral dose for fish nocardiosis (50 mg/kg body weight/day). The fish were fed once a day, and the daily feeding rate was 2% of body weight. The fish in the two treatment groups were hand-fed with the medicated pellets for five consecutive days starting from the day of infection (8 h after bacterial

Table 1 Experimental design of chemotherapeutic treatment by sulfamonomethoxine-Na (SMM-Na) against *Nocardia seriolae* infection in amberjack fish

	Group number				
	1	2	3	4	5
Number of fish/tank	16	16	16	16	16
Challenge type	UT10	UT10	AM802	AM802	0.85% NaCl
α-Glucosidase activity	−	−	+	+	
MIC (μg/ml) of the challenge isolate	4	4	32	32	
Oral dose of SMM-Na in medicated feed (mg/kg fish body weight/day)	50	No treatment	50	No treatment	No treatment
Onset of treatment after challenge	8 h	No treatment	8 h	No treatment	No treatment

Table 2 MIC results of four sulfonamides, two culture media, and four inoculum sizes of 20 representative *Nocardia seriolae* isolates determined by REMA

Drug	Culture medium	Most frequent MIC (μg/ml)							
		10^5 (CFU/well)		10^4 (CFU/well)		10^3 (CFU/well)		10^2 (CFU/well)	
		MIC	Range	MIC	Range	MIC	Range	MIC	Range
SMM	1× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32
	0.5× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32
SDM	1× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32
	0.5× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32
SMX	1× CAMHB	>512	>512	>512	>512	64	16–128	32	4–32
	0.5× CAMHB	>512	>512	>512	>512	64	16–128	32	4–32
SIZ	1× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32
	0.5× CAMHB	>512	>512	>512	>512	64	16–128	16	4–32

SMM sulfamonomethoxine, *SDM* sulfadimethoxine, *SMX* sulfamethoxazole, *SIZ* sulfisozole, *CAMHB* cation-adjusted Mueller–Hinton broth

inoculation). The same amount of untreated feed was given to the fish in the two untreated groups and in the negative control group at the same time. After the 5-day treatment, untreated feed was given (once a day at 2% of body weight per fish) to groups in which the fish maintained normal feeding behavior. The experiment was terminated 32 days after inoculation. The fish were continuously monitored for changes in feeding behavior, and mortality. Mortality was recorded every day, and an autopsy of the dead fish was performed to confirm the typical clinical signs of nocardiosis and re-isolate *N. seriolae*. All experimentally infected fish that survived to the end of the experiment were examined externally for clinical signs of the disease, and we attempted to re-isolate *N. seriolae* from the surviving fish by inoculating kidney and spleen samples onto BHIA plates for bacterial growth.

Statistical analysis was performed using SPSS for Windows (version 11.0; SPSS, Chicago, IL, USA). The chi-square (χ^2) test was used to compare the two treatment groups at the end of the experiment, and a p value of less than 0.05 was considered significant.

Results

Resazurin microtiter assay

The MIC results obtained by the pilot REMA are summarized in Table 2. The susceptibilities of 20 representative *N. seriolae* isolates to SMM, SDM, SMX, and SIZ were similar to each other for all three tested media and four inoculum sizes. Moreover, no differences in MICs were observed between α-glu-positive and α-glu-negative *N. seriolae* isolates. With regard to the three tested media, all *N. seriolae* isolates grew well in 1× and 0.5× original concentrations of CAMHB, whereas no growth was observed in YNB. The MICs of all tested sulfonamides determined in 0.5× concentration of CAMHB were similar to those obtained in 1× CAMHB.

Figure 1 shows the effects of inoculum size on the MICs of tested sulfonamides with the aid of the resazurin oxidation–reduction colorimetric indicator. Larger inoculum sizes, specifically 10^5 and 10^4 CFU/well, resulted in all tested *N. seriolae* isolates having high MICs (greater than 512 μg/ml for all tested sulfonamides). MICs for 10^3 CFU/

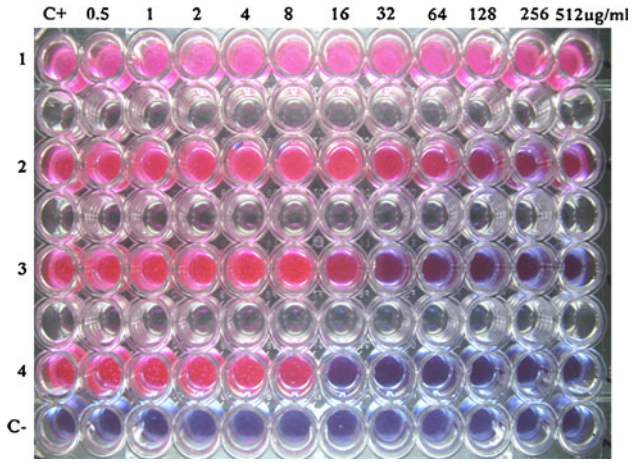

Fig. 1 REMA of the SMM activity against different inoculum sizes of the type strain *Nocardia seriolae* ATCC 43993 in 0.5× the original concentration of CAMHB: *row 1* an inoculum size of 10^5 CFU/well results in an MIC of greater than 512 μg/ml (the color of all the wells changed from *blue* to *pink*); *row 2* an inoculum size of 10^4 CFU/well results in an MIC of greater than 512 μg/ml; *row 3* an inoculum size of 10^3 CFU/well results in an MIC of 64 μg/ml, with the presence of the trailing effect; *row 4* an inoculum size of 10^2 CFU/well results in a clear MIC endpoint of 16 μg/ml. *C+* indicates a well containing no antibiotic and *C−* indicates a well containing no bacteria

well were two double-fold dilutions higher than those obtained using 10^2 CFU/well, and were generally associated with a trailing growth (i.e., progressive diminution of growth), which resulted in difficulty in determining the exact endpoint. This trailing growth was not observed when using an inoculum size of 10^2 CFU/well, and a clear endpoint was able to be determined at this size. In addition, the observed MICs of all strain–antibiotic combinations were not affected by the prolonged incubation period (up to 2 weeks).

In the evaluation of endpoint readings, all tested strains showed the same MIC results before and after the addition of resazurin. Because resazurin's color change was very clear, REMA was superior to the traditional reading method.

Comparison between REMA and Etest

Fifty *N. seriolae* isolates were simultaneously tested for susceptibility to SMX by using both REMA and Etest. The susceptibility breakpoint for SMX in *Nocardia* species is less than or equal to 32 μg/ml [12]. On the basis of this breakpoint, all the tested isolates were susceptible to SMX with MICs of 4–32 and 2–16 μg/ml for REMA and Etest, respectively. When Etest was used as the reference method, REMA showed a categorical agreement of 100.0%. The essential agreement between the two methods was 96.0%, as only two out of 50 tested isolates showed MICs of 2 μg/ml in Etest method and 8 μg/ml in REMA.

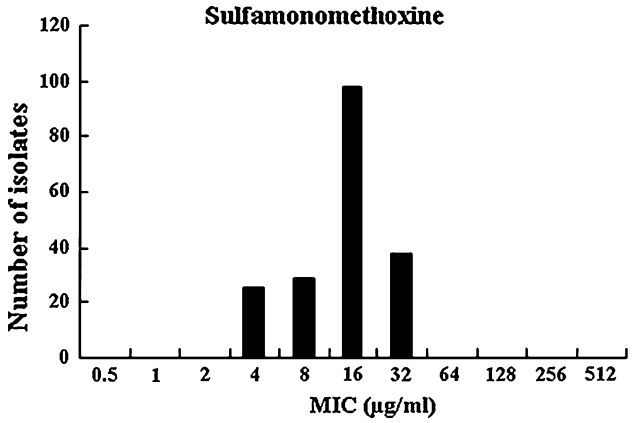

Fig. 2 Frequencies of SMM MICs (μg/ml) for *Nocardia seriolae* isolates ($n = 190$) determined by REMA

In vitro activity of SMM

Figure 2 shows the MICs of SMM against all the tested *N. seriolae* isolates. SMM was highly active toward all the tested *N. seriolae* isolates ($n = 190$), exhibiting low MICs ranging from 4 to 32 μg/ml.

Experimental treatment

The feeding behavior of the fish belonging to the two treated groups and one negative control group did not change throughout the experiment. In contrast, the fish in the two untreated groups exhibited diminished appetite 5 days after the infection, followed by complete anorexia 12 days after the infection. The survival rates of fish fed a medicated diet 8 h after the challenge were 100% (G1; MIC of 4 μg/ml) and 87.5% (G3; MIC of 32 μg/ml), whereas those of fish fed a non-medicated diet were 25% (G2; MIC of 4 μg/ml) and 0% (G4; MIC of 32 μg/ml) 32 days after the infection (Fig. 3). The fish in the negative control group (G5) were all alive throughout the experiment. All dead fish from G2, G3, and G4 showed typical internal signs of nocardiosis, and the percentage of re-isolation of *N. seriolae* was 100%. In the examination of the fish surviving at the end of the experiment, external necrotic lesions were observed in five individuals in group 1 (31.3%, $n = 16$), in all individuals in group 2 (100%, $n = 4$), and in two individuals in group 3 (14.3%, $n = 14$). The re-isolation rates of *N. seriolae* were 25, 100, and 14.3% in groups 1, 2, and 3, respectively. Statistical analysis revealed insignificant differences in the survival rate, rate of external signs, and rate of bacterial re-isolation between the two treated groups (G1 and G3; $p > 0.05$).

Discussion

Few in vitro studies investigating the sensitivities of *N. seriolae* against antimicrobials have been published

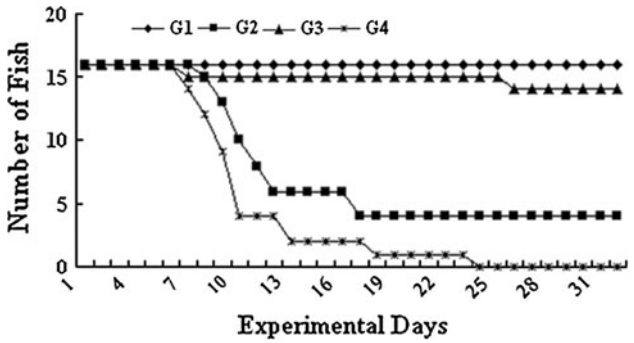

Fig. 3 Survival of experimentally infected amberjack fish: *G1* fish infected with the *Nocardia seriolae* UT10 isolate (MIC 4 μg/ml) and fed medicated pellets containing sulfamonomethoxine sodium (SMM-Na) (50 mg/kg body weight/day) for 5 consecutive days, *G2* fish infected with the *N. seriolae* UT10 isolate (MIC 4 μg/ml) and fed unmedicated pellets, *G3* fish infected with the *N. seriolae* AM802 isolate (MIC 32 μg/ml) and fed medicated pellets containing SMM-Na (50 mg/kg body weight/day) for 5 consecutive days, and *G4* fish infected with the *N. seriolae* AM802 isolate (MIC 32 μg/ml) and fed unmedicated pellets

[5, 18, 20, 21]. In all of these studies, antimicrobials other than SMM were used as the test drugs. In the present study, we established a modified REMA to examine the sensitivity of *N. seriolae* to sulfonamides, and we used the modified REMA to investigate the in vitro and in vivo activity of SMM against the clinical isolates of *N. seriolae.*

Sulfonamides act as competitive antagonists of *p*-aminobenzoic acid (PABA), which is an integral component of the folic acid structure. Medium ingredients are thought to play important roles in mediating antibacterial activity when sulfa drugs are used [22]. YNB is a synthetic medium that does not contain PABA or PABA-related compounds. As a test medium, YNB did not support the growth of *N. seriolae,* although it was considered to be a useful medium for measuring the MICs of sulfonamides against the clinical isolates of *Aspergillus* and *Cryptococcus* species [22]. In contrast, both 1× and 0.5× original concentrations of CAMHB greatly enhanced the growth of *N. seriolae*, and the different concentrations of CAMHB did not affect the observed MICs. These results were consistent with the CLSI recommendation for Mueller–Hinton broth as the medium for susceptibility testing of aerobic bacteria [13]. In addition, this result represented a new method for lowering the amount of antagonists in the culture medium that may otherwise allow some slight bacterial growth and hence hinder the development of a clear endpoint of sulfonamides [12]. In the present study, the inoculum size was also a crucial factor for the development of the clear endpoint. With large inoculum sizes (approximately 10^5 and 10^4 CFU/well, all tested sulfonamides (maximum concentration, 512 μg/ml) failed to inhibit all the tested representative *N. seriolae* isolates. However, when the smallest inoculum size (10^2 CFU/well) was used, all the tested isolates were inhibited with a maximum concentration of 32 μg/ml, and with easy-to-interpret clear endpoints. The results obtained in this study did not support using the NCCLS-recommended inoculum dose of 10^4 CFU/well [12]. On the other hand, our results do support previously reported results in which sulfonamides exhibited in vitro inhibitory activity only when *Nocardia* inocula of 10^2 CFU were employed [23, 24]. When the type and concentration of the medium and the inoculum size were considered in tandem, 0.5× the original concentration of CAMHB and the smallest inoculum size (10^2 CFU/well) could be used for the clearest endpoint reading of the microdilution test results, and all isolates were inhibited by all the tested sulfonamides at concentrations ranging from 4 to 32 μg/ml. In addition, we found an excellent agreement between the MIC endpoint readings before and after adding the resazurin indicator. Thus, the modified REMA is not particularly subject to observer's misinterpretation, and it therefore provides an entirely objective, simple, and visual means of determining the sulfonamide MIC endpoint in *N. seriolae* isolates that can be performed by either professional or non-specialized researchers. The validity of the modified REMA was verified at the categorical and essential agreement levels of 100.0 and 96.0%, respectively, by using the Etest reference method.

Although SMM-based therapy for the treatment of fish nocardiosis is generally effective, optimal treatment regimens must be guided by in vitro antimicrobial susceptibility testing of isolates in combination with in vivo therapeutic response experiments. In this study, the in vitro activity of SMM (with range of 4–32 μg/ml) against 190 clinical isolates of *N. seriolae* revealed that 80% of the isolates were inhibited by 16 μg/ml, and 100% were inhibited by 32 μg/ml. Despite the absence of the standard interpretation criteria for SMM against *N. seriolae*, the low MICs of SMM recorded in this study could suggest that all the tested isolates are sensitive to SMM in particular and the other tested sulfonamides in general as similar results for SMM, SDM, SMX, and SIZ were observed in the pilot REMA. This conclusion was supported by the results obtained in the experimental treatment, in which SMM treatment resulted in survival rates of 87.5 and 100.0% in the two treated groups. In this study, we could not find *N. seriolae* isolates with high MICs of SIZ, whereas six out of 60 isolates were previously reported to have high MICs of 100 μg/ml or more for SIZ [21]. Differences in the activity among sulfonamides may be caused by differences in the methodologies used for susceptibility testing.

N. seriolae is an intracellular pathogen that can survive and multiply within phagocytes [25]. In the present study, *N. seriolae* was re-isolated from surviving fish in the treated groups at the end of the experiment. This result

indicated that the bacteria were not completely eliminated by SMM, and that they may still be able to multiply when SMM treatment was stopped and SMM concentrations in the fish dropped to below the MIC. Thus, treatment of nocardiosis with SMM for five consecutive days can reduce mortality in fish populations; however, the disease may recur. Hence, repeated or long-term treatment may be required to maintain a low mortality rate in infected farms. These data were consistent with those in a previous report in which the treatment outcome for nocardiosis was improved after long-term medication for 3 weeks with trimethoprim and SMM in feed [1].

In conclusion, we have developed a simple and visual modified REMA for screening the in vitro activity of sulfonamides in *N. seriolae*. In addition, we have reported that clinical isolates of *N. seriolae* with MICs up to 32 μg/ml are sensitive to SMM treatment with a survival rate of 87.5–100%, and that an MIC 32 μg/ml may be taken as a preliminary guide in monitoring the sensitivity of *N. seriolae* to SMM in amberjack. However, continuous follow-up for the appearance of *N. seriolae* isolates with higher MICs and their response to SMM treatment should be applied in amberjack and other fish farms to establish a solid breakpoint for interpreting SMM MIC results in *N. seriolae*.

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