Fluorescent Antibody Technique Compared to Standard Media Culture for Detection of Pathogenic Bacteria for Yellowtail and Amberjack

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During a one year period, 355 net pen cultured yellowtail (*Seriola quinqueradiata*) and amberjack (*Seriola dumerili*) mortalities were examined for bacterial infections. A comparison of detection of pathogenic bacteria by direct fluorescent antibody technique (FAT) and media culture was performed. Fluorescein labeled rabbit immunoglobulin G (IgG: FI) was prepared against *Streptococcus* sp., *Pasteurella piscicida*, *Nocardia kampachi* and *Vibrio anguillarum*. Heat fixed smears of kidney from each mortality were stained with each one of the IgG: FI preparations and examined using fluorescent microscopy. In addition, kidney samples were inoculated on brain heart infusion agar and Ogawa media. From the 355 mortalities, FAT detected 485 infections as compared to 347 by culture. The respective numbers of fish that bacteria were detected by method were (bacteria, by FAT, by culture): *Streptococcus* sp., 89, 55; *P. piscicida*, 293, 269; *N. kampachi*, 91, 17; and *V. anguillarum*, 12, 7. The FAT diagnosed 127 mixed infections as compared to 20 by culture. Three mortalities were not infected by FAT (confirmed by culture) as compared to 28 mortalities where no infection was apparent by culture. The FAT proved to be a rapid (1 h), effective and powerful method for diagnosis of bacterial infection, especially of mixed infection which the culture method frequently failed to detect.

Yellowtail (*Seriola quinqueradiata*) and amberjack (*Seriola dumerili*) are intensively cultured in Japan. Bacterial infections are often a limiting factor in the production of these food fishes. Infections by *Streptococcus* sp., *Pasteurella piscicida*, *Nocardia kampachi* and *Vibrio anguillarum* are known to occur (respectively: KUSUDA, *et al.*, 1976; SNIESZKO, *et al.*, 1964; KUSUDA, 1975; KUSUDA *et al.*, 1979). The standard method used to diagnose these pathogens is culture on bacteriological media. However, this procedure is time consuming and does not always detect the infecting pathogen.

The fluorescent antibody technique (FAT) for detection of bacterial infections was developed by COONS, *et al.* in 1942. The technique offers the advantage of being rapid where a presumptive diagnosis can often be made in less than one hour as compared to days using culture media. In addition, the method is highly sensitive where low numbers of infecting pathogens can be detected.

This report describes a diagnostic system based on FAT for detection of bacterial disease in cul-

tured yellowtail and amberjack. Fluorescein labeled, purified, rabbit immunoglobulin G (IgG: FI) was prepared against the four bacterial pathogens of these fish. Over a one year period, 355 mortalities were examined comparing FAT to standard culture method.

Materials and Methods

Sample Collection

Yellowtail and amberjack mortalities which suspected to be infectious disease were brought to Kochi University Fish Disease Laboratory on ice from the culture areas shown in Fig. 1. Kind, number and weight of fish and culture area are shown in Table 1.

Rabbit Vaccination

Streptococcus sp. (strain YT-3), *P. piscicida* (strain 2337), *N. kampachi* (strain N-1) and *V. anguillarum* (serotype J-0-3, strain 1109) cells were killed with 0.5% formalin for 2 d at 4°C. The cell suspensions were washed three times with

Fig. 1. Map showing Kochi Prefecture yellowtail and amberjack culture areas and the Kochi University Fish Disease Laboratory.

Table 1. Source of yellowtail and amberjack mortalities

Date	Fish	Number of fish	Weight (g)	Culture area	Date	Fish	Number of fish	Weight (g)	Culture area
83-12-7	Yellowtail	2	ND ^{a)}	Sukumo	84-7-9	Yellowtail	12	46	Uranouchi
12-13	Yellowtail	2	ND	Sukumo	7-9	Amberjack	5	51	Nomi
84-2-20	Amberjack	1	1009	Nomi	7-14	Yellowtail	4	121	Uranouchi
3-19	Amberjack	3	842	Nomi	7-15	Yellowtail	29	65	Nomi
4-14	Amberjack	3	375	Nomi	7-18	Yellowtail	21	ND	Uranouchi
6-26	Yellowtail	3	18	Nomi	7-19	Amberjack	5	167	Nomi
6-28	Yellowtail	7	122	Nomi	7-26	Yellowtail	12	128	Sukumo
6-29	Yellowtail	20	23	Nomi	8-31	Yellowtail	2	279	Uranouchi
6-30	Yellowtail	6	11	Sukumo	9-8	Yellowtail	4	368	Sukumo
7-2	Yellowtail	4	13	Uranouchi	9-20	Yellowtail	21	328	Sukumo
7-2	Yellowtail	3	100	Nomi	9-22	Yellowtail	9	336	Sukumo
7-3	Yellowtail	7	132	Uranouchi	10-2	Yellowtail	16	565	Sukumo
7-4	Yellowtail	5	29	Nomi	10-4	Amberjack	8	756	Nomi
7-4	Yellowtail	79	43	Uranouchi	10-6	Yellowtail	3	1413	Sukumo
7-6	Yellowtail	15	27	Sukumo	10-24	Yellowtail	9	290	Sukumo
7-7	Yellowtail	30	23	Saga	11-10	Yellowtail	5	573	Sukumo

a) ND=not determined.

phosphate buffered saline (PBS, pH 7.2) by centrifugation ($12,000 \times g$, 10 m) and concentrated to an optical density (OD) of 2.0 at 525 nm. Lack of viability was confirmed by inoculating the preparations on agar media. A 1:1 emulsion with

Freunds complete adjuvant (Difco, Detroit, MI) was prepared and each preparation was injected into rabbits: intradermally into each hind foot pad (0.4 ml) and intradermally between the scapula (1.2 ml). Booster were prepared and injected in

the same manner at ten week intervals. Rabbits were bleed weekly, sera obtained and stored at -20°C .

Purification and Fluorescein Labeling of Rabbit IgG

The purification and labeling of rabbit IgG was performed using a modification of the method of BANNER *et al.* (1982). In brief, IgG was partially purified from sera using DEAE Affi-Gel Blue chromatography (Bio-Rad Laboratories, Richmond, CA). The IgG fraction was concentrated to 20 mg/ml (1.0 OD at 280 nm approximately equals 0.69 mg/ml) in a dialysis bag coated with polyethylene glycol (PEG 20,000, Wako Chemical Ltd., Osaka). The concentrated protein was dialyzed against fresh 0.025 M sodium carbonate-bicarbonate buffer (pH 9.0, two changes in 18 h), then fluorescein labeled with celite fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/mg protein with constant stirring at 40°C for 15 m. The preparation was centrifuged ($12,000 \times g$, 5 m) and further purified using G-75 column chromatography (Pharmacia, Tokyo) into PBS with 0.01 % sodium azide. The mole fluorescein to mole IgG (M Fl: M IgG) was determined by the method of HUDSON and HAY (1976): $\text{M Fl: M IgG} = 2.87 \times (\text{OD at } 495 \text{ nm}) / (\text{OD at } 280 \text{ nm}) - [0.35 \times (\text{OD at } 495 \text{ nm})]$. Anti-*Streptococcus* sp. M Fl: M IgG was 5.1; anti-*P. piscicida* M Fl: M IgG was 1.1; anti-*N. kampachi* M Fl: M IgG was 3.5; and anti-*V. anguillarum* M Fl: M IgG was 4.4. Each preparation was stored in the dark at -20°C .

Fluorescence Titer and Specific Activity

The IgG: Fl preparations were examined for staining titer by applying two-fold dilutions to microscope slides coated with heat fixed bacterial fish pathogens. Each slide was then washed with PBS and examined by fluorescence microscopy. Anti-*Streptococcus* sp. IgG: Fl titer was <1 : 10,000. Anti-*P. piscicida* IgG: Fl was 1: 1,000. Anti-*N. kampachi* IgG: Fl was 1: 100. And anti-*V. anguillarum* IgG: Fl was 1: 1,000. The preparations were not diluted this much for use for detection of pathogens, because too high of dilution reduces the fluorechrome concentration (NARIN, 1969). Anti-*Streptococcus* sp. IgG: Fl was diluted 1: 100 for use. Anti-*P. piscicida* IgG: Fl was diluted 1: 500. Anti-*N. kampachi*

IgG: Fl was diluted 1: 10. And anti-*V. anguillarum* IgG: Fl was diluted 1: 100.

To determine if cross reaction occurred, fish pathogen bacterial suspensions were heat fixed to slides and stained with the dilute IgG: Fl preparations. Cross reactivity was assessed using fluorescent microscopy. The bacteria were: *Streptococcus* sp. (strain YT-3), *S. iniae* (strain KK-3), *P. piscicida* (strain P-2337), *N. kampachi* (strain N-1), *V. anguillarum* (serotype J-O-1, strain V-36), *V. anguillarum* (serotype J-O-2, strain V-1155), *V. anguillarum* (serotype J-O-3, strain V-1109), *Pseudomonas fluorescens* (ATCC 13525), *Ps. putida* (ATCC 12633), *Edwardsiella tarda* (strain EF-1) and *Aeromonas salmonicida* (ATCC 14174). Anti-*Streptococcus* sp. IgG: Fl and anti-*P. piscicida* IgG: Fl did not cross react with any of the bacteria tested. Anti-*N. kampachi* IgG: Fl had slight cross reactions (very faint fluorescence) with *V. anguillarum* (serotype J-O-2, strain V-1155) and *Ps. putida*. Anti-*V. anguillarum* (serotype J-O-3, strain V-1109) IgG: Fl had a slight cross reaction with *E. tarda*.

Diagnosis using Fluorescent Antibody

Five microscope slides were smeared with kidney samples from each morbid fish and heat fixed. Each slide was stained with one of the four diluted fluorescent antibody preparations for 30 m and then washed with PBS for 5 m. An additional smear was not stained and used as a control. A cover glass was affixed with elvanol mounting medium (RODRIGUEZ and DEINHARDT, 1960). The stained slides were examined using fluorescence microscopy.

Diagnosis using Culture Media

Kidney samples were inoculated using platinum loop on brain heart infusion agar (BHI, Difco, Detroit, MI) and Ogawa media (Eiken Chemical Co., Ltd., Tokyo). The cultures were incubated for two weeks and examined daily for the presence of bacterial growth. Selected colonies were gram stained and the identities of the isolated bacteria were confirmed using the rapid slide agglutination technique.

Microscopy

A Nikon FLUOPHOT Microphot V Series microscope (Nippon Kogaku K. K., Tokyo) was used to examine the preparations. The fluorescence filters were excitation filters (type IF 420-

490 and auxilliary 460), a dichroic mirror (DM 505) and eyepiece-side absorption filter (515W).

Results

During a one year period, 355 yellowtail and amberjack mortalities were examined for bacterial pathogens using FAT and media culture (Table 2). A comparison of FAT and culture diagnosis

revealed 485 bacterial infections detected by FAT and 347 infections by culture, a 29% difference at $P < 0.01$ (Table 3). No fish had more pathogens detected by culture than by FAT. The reason for the difference was examined by analyzing the detection of each pathogen separately.

Culture detected 55 *Streptococcus* sp. infections whereas FAT detected 89, a 38% difference at $P < 0.01$ (Tables 2 and 3). The majority of the

Table 2. Detection of pathogenic bacteria from yellowtail and amberjack using the fluorescent antibody technique and culture on media

Date	Fish	Number of fish	FAT ^{a)}					Culture ^{b)}				
			S	P	N	V	ND	S	P	N	V	ND
83-12- 7	Yellowtail	2	2	0	1	0	0	2	0	1	0	0
12-13	Yellowtail	2	2	0	0	0	0	2	0	0	0	0
84- 2-20	Amberjack	1	1	0	1	0	0	1	0	1	0	0
3-19	Amberjack	3	0	0	3	3	0	0	0	0	3	0
4-14	Amberjack	3	3	0	1	2	0	3	0	0	1	0
6-26	Yellowtail	3	0	2	0	1	0	0	2	0	0	1
6-28	Yellowtail	7	0	7	0	0	0	0	7	0	0	0
6-29	Yellowtail	20	0	20	0	1	0	0	19	0	0	1
6-30	Yellowtail	6	6	1	0	5	0	2	0	0	3	3
7- 2	Yellowtail	4	0	2	0	0	2	0	2	0	0	2
7- 2	Yellowtail	3	0	3	0	0	0	0	2	0	0	1
7- 3	Yellowtail	7	0	7	0	0	0	0	6	0	0	1
7- 4	Yellowtail	5	0	5	0	0	0	0	5	0	0	0
7- 4	Yellowtail	79	0	79	55	0	0	0	75	0	0	4
7- 6	Yellowtail	15	1	15	0	0	0	0	8	0	0	7
7- 7	Yellowtail	30	1	30	0	0	0	0	29	0	0	1
7- 9	Yellowtail	12	0	12	0	0	0	0	12	0	0	0
7- 9	Amberjack	5	3	5	0	0	0	0	5	0	0	0
7-14	Yellowtail	4	0	4	0	0	0	0	3	0	0	1
7-15	Yellowtail	29	4	29	2	0	0	0	29	0	0	0
7-18	Yellowtail	21	2	21	1	0	0	0	20	0	0	1
7-19	Amberjack	5	0	5	1	0	0	0	5	0	0	0
7-26	Yellowtail	12	2	12	0	0	0	0	8	0	0	4
8-31	Yellowtail	2	2	0	2	0	0	2	0	2	0	0
9- 8	Yellowtail	4	4	0	1	0	0	4	0	1	0	0
9-20	Yellowtail	21	18	10	8	0	0	12	9	2	0	0
9-22	Yellowtail	9	8	4	6	0	0	6	4	4	0	0
10- 2	Yellowtail	16	9	11	0	0	0	5	10	0	0	1
10- 4	Amberjack	8	5	8	0	0	0	0	8	0	0	0
10- 6	Yellowtail	3	2	0	1	0	1	2	0	1	0	1
10-24	Yellowtail	9	9	1	6	0	0	9	0	5	0	0
11-10	Yellowtail	5	5	0	2	0	0	5	0	0	0	0
Total		355	89	293	91	12	3	55	268	17	7	29

a) Abbreviations used: S=*Streptococcus* sp.; P=*Pasteurella piscicida*; N=*Nocardia kampfchi*; V=*Vibrio anguillarum*; ND=no detection.

b) Culture on Ogawa media and brain heart infusion agar. Selected colonies were gram stained and tested by the rapid slide agglutination technique.

Table 3. Comparison of fluorescent antibody technique (FAT) and media culture method for detection of bacterial pathogens of yellowtail and amberjack

Pathogen Detected	Detected by		Percent	
	FAT	Culture	Difference ^{a)}	Statistic ^{b)}
<i>Streptococcus</i> sp.	89	55	38	P<0.01
<i>Pasteurella piscicida</i>	293	268	9	P<0.05
<i>Nocardia kampfachi</i>	91	17	81	P<0.001
<i>Vibrio anguillarum</i>	12	7	NS ^{c)}	NS
Total	485	347	29	P<0.01

a) Percent Difference = (number detected by FAT) - (number detected by culture) / (number detected by FAT) × 100.

b) Chi square was performed comparing the difference between the number of diagnosis detected by FAT to detections by the culture method.

c) NS = not significant at P=0.05.

Streptococcus sp. infections were detected from Sukumo Bay yellowtail mortalities: 68/89 by FAT and 49/55 by culture. When mixed infections with *P. piscicida* and *Streptococcus* sp. occurred, there were 27 fewer isolations of *Streptococcus* sp. by culture. This may have indicated either *P. piscicida* numbers in the culture inoculum were greater than *Streptococcus* sp. or during culture, there was inhibition of *Streptococcus* sp. by the *P. piscicida*. When *Streptococcus* sp. occurred alone as detected by FAT, the same isolation frequency occurred using culture.

Culture detected 268 *P. piscicida* infections whereas FAT detected 293, an 8% difference at $P<0.05$ (Tables 2 and 3). The majority of the difference was found where no detection of a pathogen was present by media culture but *P. piscicida* was detected by FAT. No infection was detected in three fish by FAT (confirmed by culture); but, there were 29/355 fish where no infection was apparent by culture. However, 21 of these fish were infected with *P. piscicida* as detected by FAT. The reason *P. piscicida* could not be cultured may be due to the low numbers of infecting microorganisms.

Culture detected 17 *N. kampfachi* infections whereas FAT detected 91, an 81% difference at $P<0.001$ (Tables 2 and 3). The majority of this difference occurred in 55/79 fish on 7/4/84, a mixed infection of *N. kampfachi* and *P. piscicida*. The difference was always found where *N. kampfachi* was not isolated by culture in mixed infected fish but detected by FAT (Table 4). Ogawa media, the culture media for *N. kampfachi*, contains

Table 4. Detection of mixed infections in yellowtail and amberjack using the fluorescent antibody technique or culture on media

Mixed Infections	Detected by	
	FAT	Culture
<i>Streptococcus</i> sp. and <i>P. piscicida</i>	30	0
<i>Streptococcus</i> sp. and <i>N. kampfachi</i>	22	15
<i>Streptococcus</i> sp. and <i>V. anguillarum</i>	6	3
<i>P. piscicida</i> and <i>N. kampfachi</i>	59	1
<i>P. piscicida</i> and <i>V. anguillarum</i>	1	0
<i>N. kampfachi</i> and <i>V. anguillarum</i>	3	0
<i>Streptococcus</i> sp., <i>P. piscicida</i> and <i>N. kampfachi</i>	5	1
<i>Streptococcus</i> sp., <i>P. piscicida</i> and <i>V. anguillarum</i>	1	0
Total	127	20

malachite green to selectively inhibit other bacteria. However, *V. anguillarum* and *P. piscicida* were not inhibited and grew on Ogawa media. This indicated *N. kampfachi*, a slow growing microorganism, did not compete well on this medium.

Culture detected seven *V. anguillarum* infections whereas FAT detected 12 (Tables 2 and 3). This was not statistically different. Throughout the one year period, *V. anguillarum* infections were infrequent and only occurred in mixed infected fish as detected by FAT.

The largest difference between diagnosis by FAT and culture occurred in the detection of mixed infections (Table 4). Culture detected 20 mixed infections whereas FAT detected 127, an 81% difference at $P<0.001$. The *Streptococcus* sp.

with *P. piscicida* mixed infection was detected 30 times by FAT and not detected by culture. And *P. piscicida* with *N. kampachi* mixed infection was detected 59 times by FAT and only one time by culture. These differences were possibly due to *P. piscicida* over growing the cultures. The detection frequency of the other mixed infections was not statistically different. However, FAT always detected a larger number than did culture.

Discussion

The fluorescent antibody technique proved to be a rapid and efficient method to diagnose bacterial infections in yellowtail and amberjack. The power of the technique was proven where in no case out of 355 fish diagnosed did the FAT fail to detect an infection detected by culture on media. The converse was not true because 138 fewer infections were detected by culture than by FAT.

Although FAT has been performed for single infections (KITAO and KIMURA, 1974; MORI *et al.*, 1976), the culture method has been used extensively to diagnose bacterial disease in cultured yellowtail and amberjack. The results of this study showed culture method did not detect all infections and infrequently identified mixed infections. The media culture method as compared to FAT diagnosed totally only 33 % of the single and mixed infections in the 355 mortalities. The largest source of error in culture diagnosis was not identifying mixed infections. The reasons why culture method frequently failed to detect mixed infection that FAT could detect are 1) FAT has high specificity that enabled to detect small amount of less dominant pathogens which exist together with large amount of dominant pathogens; 2) the less dominant pathogens were suppressed to grow on media by the dominant pathogens; or 3) FAT could also detect pathogens which showed weak and slow growth or were in a static state by the effect of previously administered therapeutic drugs.

These results suspect that diagnostician can not detect less dominant pathogen and lead him to prescribe drug therapies which would have little value in controlling the mixed infection. This is particularly true for mixed infections with *N. kampachi* where the present antibiotics employed do not control *N. kampachi* infections. Because of the prevalence of antibiotic resistant bacterial

fish pathogens (AOKI *et al.*, 1981), the culture method should be performed to determine the minimum inhibitory concentration (MIC) of antibiotics used to control the infection. However, FAT should be used with culture because a presumptive diagnosis can be obtained within one hour. This would allow drug therapy to begin immediately. After MIC determination has been performed, a reassessment of the prescribed therapy could be made. This would allow for rapid diagnosis and effective therapy.

This report is one of the first to describe mixed infections in fishes. Mixed infections detected by FAT occurred 127/255 times in the yell owtail and amberjack mortalities. The water temperature for culture of these animals is high and therefore may have lead to a higher incidence of mixed infections in which an observant examination should be accompanied on a role of the less dominant pathogen. Furthermore, because the aquatic environment allows a contagion to rapidly spread, studies to determine the incidence of mixed infections in other fishes should be made. Based on the culture results of this study, FAT should be employed in these studies.

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