

Simultaneous Detection of *Edwardsiella ictaluri* and *Flavobacterium columnare* by Dual Immunofluorescence Test

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Abstract

A rapid immunofluorescence test with fluorescent conjugated-antibodies having different spectral properties was compared with standard bacteriological culture for simultaneous detection of bacterial fish pathogens *Edwardsiella ictaluri* (*Ei*) and *Flavobacterium columnare* (*Fc*). Three groups of experimentally infected channel catfish (*Ictalurus punctatus* Rafinesque) and a fourth group that acquired an aquarium-infection with *F. columnare* were tested. Kidney, brain and nares tissues from 101 fish were concurrently examined by both tests. Fish in experimentally infected groups yielded bacteria with which they were infected, while the fish that acquired an aquarium-infection tested positive for *Fc* by culture and IFA test. The IFA test compared favorably in sensitivity (for *Ei* = 80.7%, and for *Fc* 87.2%) and specificity (for *Ei* = 83.9% and for *Fc* = 88.9%) with the standard bacteriological culture. Thus, the dual immunofluorescence test will serve as an efficient tool for rapid simultaneous detection of *E. ictaluri* and *F. columnare* in infected fish.

Introduction

Enteric septicemia of catfish (ESC) caused by *E. ictaluri*, reported by 67% of operations within one year and columnaris disease caused by *F. columnare*, reported by 50% of operations within one year, are the two most important bacterial diseases affecting the catfish aquaculture industry in the USA (1). Since both species of bacteria (*Ei* and *Fc*) are ubiquitous in the aquatic environment and fish are reared in extensive pond acreages with high stocking densities (8,000-12,000 fish per acre), the occurrence of co infections with both bacteria in the same host (fish) cannot be overlooked (2). Traditional methods of diagnosing bacterial infections using culture techniques require several days to arrive at a definitive diagnosis, resulting in increasing the potential of spreading of disease and delaying implementation of important disease control measures. The dual immunofluorescence test developed in this study will serve as an efficient tool for rapid simultaneous detection of *E. ictaluri* and *F. columnare* in outbreaks of disease.

Materials and Methods

Infection of fish and sample collection. Channel catfish were divided into groups/batches and infected with *E. ictaluri* and/or *F. columnare* as shown in Table 1. Except for the control fish in group 1, batch 5, which were euthanized prior to sampling, all other groups were sampled after death from bacterial infection. Three hundred and three samples (derived from kidney, brain and nares) from 101 fish were concurrently examined by bacteriological culture (accepted standard) and IFA.

Bacteriological culture. Initial culture was done on selective media (Shieh medium for isolation of *F. columnare* and Shotts medium for isolation of *E. ictaluri*), and randomly selected isolates biochemically characterized using API 20E test strips and compared with standard patterns determined for each bacterium. Additionally, fatty acid methyl ester (FAME) composition of the bacteria was determined using an Agilent 6850 gas chromatographic system.

Immunofluorescence staining and microscopy. The procedure used for immuno-staining impression smears made from kidney, brain and nares of infected fish is schematically presented in Fig.1. Mouse MAb AA224 against *E. ictaluri* diluted 1:100, and polyclonal goat anti-*F. columnare* antibody diluted 1:10 were used as primary antibodies. A mixture of fluorochrome-conjugated secondary antibodies (Alexa Fluor 488-conjugate rabbit anti-mouse IgG, emitting green fluorescence and Alexa Fluor 594-conjugated rabbit anti-goat IgG, emitting red fluorescence) was used for visualization of specific antigen-antibody reactions by epifluorescence microscopy.

Results

Bacteriological culture and IFA test results are presented in Table 1. Isolates were readily differentiated by culture as belonging to *Ei* or *Fc* on the basis of their morphologic appearance, distinct biochemical characteristics and FAME profiles. Fish in group I, batches 1-4 that died ($n = 35$) of *E. ictaluri* infection, yielded *E. ictaluri* from at least 1 of 3 tissues sampled. No *F. columnare* were isolated from this group by either method. The control fish in group I batch 5, were negative for both organisms by both tests. In group II, batches 1 and 2, all infected fish tested positive for *Ei* and *Fc* in at least one of the three sampled tissues (Table 1). Group III fish were positive for *Fc* only as were fish in group IV which acquired spontaneous *F. columnare* infection.

The relative efficiency of the IFA test compared to bacteriological culture (accepted standard) for *E. ictaluri* and *F. columnare* is summarized in Tables 2 and 3. Since *Ei* was the only organism detected in group I, batches 1-4, and *Ei* and *Fc* were both detected in group II, batches 1 and 2, the efficiency of the IFA test for detection of *Ei* was analyzed independently from that of *Fc* (Table 2) and the efficiency of the IFA test to detect *Fc* among the 3 groups, II (batches 1 and 2); III and IV, were analyzed separately (Table 3).

The IFA test compared favorably in sensitivity (*Ei* = 80.7%; *Fc* = 87.2%) and specificity (*Ei* = 83.9%; *Fc* = 88.9%) with the standard bacteriological culture. The positive predictive value (*Ei* = 96.2% group I, 90.8% group II, 93.7% groups I and II combined; *Fc* = 95.2% group II, 95.3% groups II, III, and IV combined) was high, while the negative predictive value (*Ei* = 66.7% group I, 31.3% group II, 59.5% groups I and II combined; *Fc* = 73.7% group II, 72.7% groups II, III, IV combined) was relatively low.

Estimate of IFA test efficiency compared with bacteriological culture (BC) for *Edwardsiella ictaluri*

Group	IFA + BC				SENS (%)	SPEC (%)	PPV (%)	NPV (%)	Agreement (%)
	TP ^a	FP ^b	FN ^c	TN ^d					
I: (a)	11	0	3	1	78.6	100.0	100.0	25.0	80.0
(b)	14	1	3	0	82.4	0.0	93.3	0.0	77.8
(c)	20	1	8	1	71.4	50.0	95.2	11.1	70.0
(d)	30	1	7	4	81.1	80.0	96.8	36.4	81.0
(e)	0	0	0	36	-	100.0	-	100.0	100.0
I: (a)-(e)	75	3	21	42	78.1	93.3	96.2	66.7	83.0
COMBINED	35	0	6	4	85.4	100.0	100.0	40.0	86.7
II: (a)	24	6	5	1	82.8	14.3	80.0	16.7	69.4
II: (b)	59	6	11	5	84.3	45.5	90.8	31.3	79.0
II: (a) & (b)	134	9	32	47	80.7	83.9	93.7	59.5	81.5

^a True positive (TP) = IFA positive (+) and BC positive (+).
^b False positive (FP) = IFA positive (+) and BC negative (-).
^c False negative (FN) = IFA negative (-) and BC positive (+).
^d True negative (TN) = IFA negative (-) and BC negative (-).
^e SENS = Sensitivity - The conditional probability that a unit of analysis has a positive test result, given the disease is present.
^f SPEC = Specificity - The conditional probability that a unit of analysis has a negative test result, given the disease is not present.
^g PPV = Positive predictive value. NPV = Negative predictive value.
^h Agreement = overall proportion correctly classified. [(TP+TN)/N]. (Grainer & Gardner 2000)

Table 1. Bacteriological culture and IFA test results on sampled fish in respective groups

Group	Number of fish	Bacteriological culture (BC)						IFA test (IFAT)					
		Kidney		Brain		Nares		Kidney		Brain		Nares	
		Ei ^a	Fc ^b	Ei	Fc	Ei	Fc	Ei	Fc	Ei	Fc	Ei	Fc
I ^c	5	5 ^d	0	5	0	4	0	5	0	3	0	3	0
I: (a)	6	6	0	6	0	5	0	6	0	4	0	5	0
(b)	10	10	0	10	0	8	0	9	0	5	0	7	0
(c)	14	14	0	14	0	9	0	13	0	10	0	8	0
(d)	12	0	0	0	0	0	0	0	0	0	0	0	0
(e)	15	15	15	13	6	13	14	15	15	8	5	12	13
II ^e (a)	12	11	12	10	6	8	11	12	10	9	7	9	12
(b)	12	0	9	0	9	0	5	0	10	0	6	0	5
III ^f	15	0	15	0	6	0	9	0	13	0	3	0	8
IV ^g	15	0	15	0	6	0	9	0	13	0	3	0	8

^a Ei = *Edwardsiella ictaluri*
^b Fc = *Flavobacterium columnare*
^c Number positive
^d Group I: (a) - (d) immersion immunized and challenged with *Edwardsiella ictaluri*. (e) unimmunized control batch.
^e Group II: (a) & (b) IP injected with *E. ictaluri* and *Flavobacterium columnare*.
^f Group III: IP injected with *F. columnare*.
^g Group IV: spontaneously acquired infection with *F. columnare*

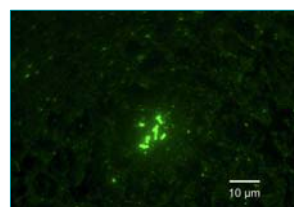


Fig. 1 (a). Impression smear of brain tissue, from a fish infected with *Edwardsiella ictaluri*, emitting apple green fluorescence of Alexa Fluor 488, by epifluorescence microscopy.

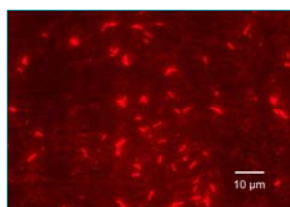


Fig. 1 (b). Impression smear of kidney tissue, from a fish infected with *Flavobacterium columnare*, emitting reddish fluorescence of Alexa Fluor 594, by epifluorescence microscopy.

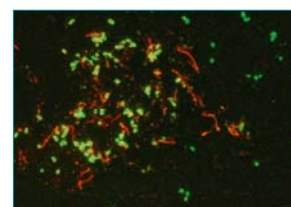


Fig. 1 (c). Impression smear of kidney tissue, from a fish simultaneously infected with *Edwardsiella ictaluri*, emitting green fluorescence of Alexa Fluor 488, and *Flavobacterium columnare*, emitting reddish fluorescence of Alexa Fluor 594, by epifluorescence microscopy.

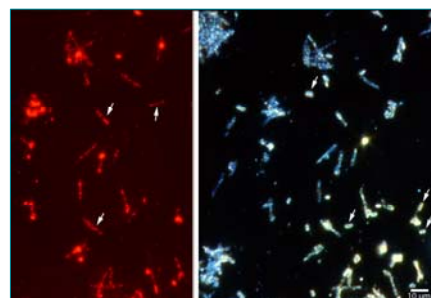


Fig. 2. Smear made from pure cultures of *Edwardsiella ictaluri* and *Escherichia coli* mixed and treated with MAb AA224 and Alexa Fluor 488-conjugated second antibody. 2(a) Shows the *Edwardsiella ictaluri* emitting apple green fluorescence when viewed with the FITC/Texas Red, blue/green band-pass filters ON. 2(b) Shows the presence of *Escherichia coli* in the same field when viewed under white-light, with the band-pass filters OFF.

Estimate of IFA test efficiency compared with bacteriological culture (BC) for *Flavobacterium columnare*

Group	IFA + BC				SENS (%)	SPEC (%)	PPV (%)	NPV (%)	Agreement (%)
	TP	FP	FN	TN					
II: (a)	32	1	3	9	91.4	90.0	97.0	75.0	91.1
II: (b)	27	2	2	5	93.1	71.4	93.1	71.4	88.9
II: (a) & (b)	59	3	5	14	92.2	82.4	95.2	73.7	90.1
III	19	2	4	11	82.6	84.6	90.5	73.3	83.3
IV	24	0	6	15	80.0	100.0	100.0	71.4	86.7
II, III & IV COMBINED	102	5	15	40	87.2	88.9	95.3	72.7	87.7

^a Ei = *Edwardsiella ictaluri*
^b Fc = *Flavobacterium columnare*
^c Number positive
^d Group I: (a) - (d) immersion immunized and challenged with *Edwardsiella ictaluri*. (e) unimmunized control batch.
^e Group II: (a) & (b) IP injected with *E. ictaluri* and *Flavobacterium columnare*.
^f Group III: IP injected with *F. columnare*.
^g Group IV: spontaneously acquired infection with *F. columnare*

SCHEMATIC OF FLUORESCENT ANTIBODY TEST

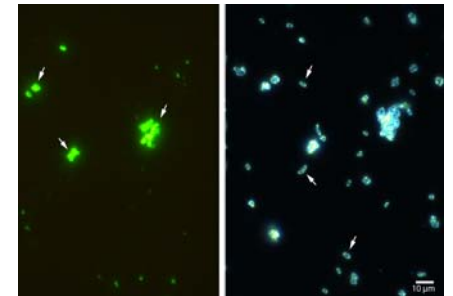
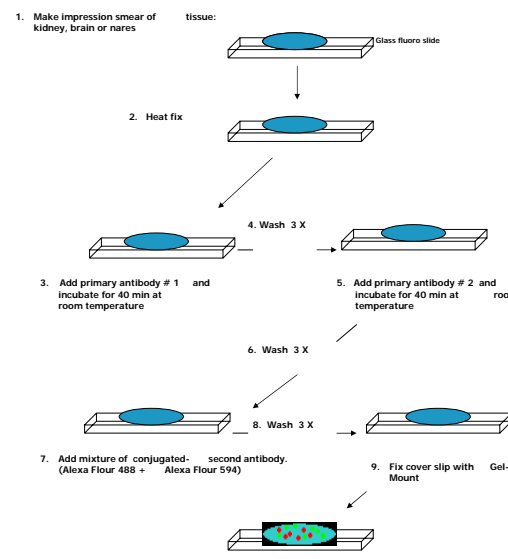


Fig. 3. Smear made from pure cultures of *Flavobacterium columnare* and *Yersinia ruckeri* mixed and treated with polyclonal goat anti-*Flavobacterium columnare* antibodies and Alexa Fluor 594-conjugated second antibody. 3(a) Shows the *Flavobacterium columnare* emitting red fluorescence when viewed with the FITC/Texas Red, blue/green band-pass filters ON. 3(b) Shows the presence of *Yersinia ruckeri* in the same field when viewed under white-light, with the band-pass filters OFF.

Discussion

Because *E. ictaluri* and *F. columnare* are ubiquitous and could cohabit the same aquatic environment, co-infections or dual infections in the same host by both organisms are not unusual (2, 3). The IFA test using a combination of antibody-conjugated fluorochromes (Alexa Fluor 488, emitting green fluorescence and Alexa Fluor 594, emitting red fluorescence) with distinct spectral properties was able to simultaneously detect *E. ictaluri* and *F. columnare* in the same host specimen. The IFA test compared favorably in sensitivity (*Ei* = 80.7%, *Fc* = 87.2%) and specificity (*Ei* = 83.9%, *Fc* = 88.9%) with the standard bacteriological culture. Bacteriological culture techniques usually take a long time (2 to 3 days) to attain a definitive diagnosis. Thus, a test such as the IFA with the potential to rapidly detect multiple bacterial pathogens simultaneously would have a distinct advantage over culture techniques, enabling fish-farmers to make management decisions promptly to avert the spread of infection from pond to pond or to neighboring farms. Recent innovative developments such as specifically tailored filter sets for simultaneous detection of multiple fluorochromes, efficient means for retarding fading, and the potency and specificity of antibody conjugates makes the IFA test an efficient tool for rapid diagnosis of ESC and columnaris disease in aquaculture farms.

Conclusions

- With specific primary antibodies and fluorescein-conjugated secondary antibodies with distinctive spectral properties, IFA test enabled simultaneous detection of specific bacterial fish pathogens *E. ictaluri* and *F. columnare* in the same host.
- In terms of sensitivity and specificity, the IFA test results were within the range considered as efficient for diagnosis of bacterial disease.
- The IFA test is rapid compared to standard bacteriological culture techniques.
- The IFA test is easy to perform and requires minimum equipment.

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