Video Article

Use of Fluorescent Immuno-Chemistry for the detection of *Edwardsiella ictaluri* in channel catfish (*I. punctatus*) samples

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Abstract

While *Edwardsiella ictaluri* is a major pathogen of channel catfish *Ictalurus punctatus* and has been discovered nearly three decades ago [1,2], so far, to the best of these authors' knowledge, no method has been developed to allow for the *in situ* visualization of the bacteria in histological sections.

While bacterial localization has been determined in vivo in previous studies using plate counts [3], radiometric labeled [4], or bioluminescent bacteria [5], most of these studies have only been performed at the gross organ level, with one exception [6]. This limitation is of particular concern because *E. ictaluri* has a complex infection cycle [1,7], and it has a variety of virulence factors [8,9]. The complex interaction of *E. ictaluri* with its host is similar in many respects to Salmonella typhi [10], which is in the same taxonomic family.

Here we describe a technique allowing for the detection of bacteria using indirect immuno-histochemistry using the monoclonal Ed9 antibody described by Ainsworth *et al.*[11].

Briefly, a blocking serum is applied to paraffin embedded histological sections to prevent non-specific biding. Then, the sections are incubated with the primary antibody: *E. ictaluri* specific monoclonal antibody Ed9. Excess antibodies are rinsed away and the FitC labeled secondary antibodies are added. After rinsing, the sections are mounted with a fluorescent specific mounting medium.

This allowed for the detection of *E. ictaluri* in situ in histological sections of channel catfish tissues.

Video Link

The video component of this article can be found at http://www.jove.com/video/2687/

Protocol

1. Bacterial challenge

1. Grow bacteria overnight at 30°C in shaking Brain Heart Infusion broth.
2. Stop water circulation in the fish tanks and add broth in concentration of 1 in 100 (10 ml per liter, for example).
3. After one hour incubation, restart water circulation in the tanks to flush out the remaining bacteria.

2. Sampling

1. Sampling times and organs depends of the objective of the study but in our study, we used the following sampling points: 1, 6, 16, 24, 36, 48, 60, 72, 96, 110, 125 and 175 hours post-infection.
2. At each time points, euthanize six fish by immersion in water containing MS222 and sample the following organs (selection of the organs sampled was based on what is known of the infection process): gills, posterior muscles along the lateral line, intestine, spleen, liver, stomach, heart, head kidney, and trunk kidney.
3. Upon sampling, immediately load the organs in histological cassettes and immerse them for two hours in 1% formalin.
4. After two hours fixation, transfer the cassettes from formalin to 50% ethanol where they remain until embedding.
5. Paraffin embed organs overnight using a tissue-Tek VIP 3000 tissue processor (Sakura Tek, Torrance, California) and section at 5µm using a Leica RM2255 microtone (Leica Microsystems, Bannockburn, Illinois).

3. Dewaxing sections

1. Immerse the sections in a staining trough filled with clear-rite for approximately 5 minutes to allow for all the wax to dissolve. Lift the rack and drain off excess wax solvent.
2. Immerse the sections in a second container of clear-rite for 5 minutes. After a few uses, replace the solvent in the first container and alternate between the first and second containers.
3. Immerse the sections in 100% alcohol to remove the wax solvent.
4. Immerse the sections in a second trough of 100% alcohol.
5. Immerse the sections in a trough of 70% alcohol.
6. Immerse the sections in running tap water to remove all traces of alcohol.

4. Buffer preparation

1. Prepare phosphate buffered saline by dissolving 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 into 1 liter of distilled water and pH to 7.4.
2. Dilute PBS to 0.9X by adding 900 ml of PBS to 100 ml of distilled water.
3. Prepare blocking serum by adding 2 ml mouse serum; 200 µl Triton-X 100 and 500 µl BSA to 50 ml of 0.9X PBS.
4. Prepare 50 mM Bicarbonate Buffered Saline is prepared by dissolving 2.1 g $NaHCO_3$ and 4 g NaCl in 500 ml distilled H_2O and titrated to pH 8.2.

5. Immunhistochemistry

1. Rehydrate sections by immersion in 0.9X PBS.
2. Incubate the slides for 30 minutes in blocking serum.
3. Incubate sections in Ed9 (diluted 1 in 100 in the blocking serum) for two hours in an opaque moist chamber at room temperature.
4. Rinse sections 4 times in PBS for 10 minutes each.
5. Incubate sections for two hours in 50 µl of the secondary antibody (goat anti mouse FitC labeled antibodies; diluted 500 times in blocking serum) in an opaque moist chamber at room temperature.
6. Rinse sections 3 times in Bicarbonate buffered saline.
7. Rinse sections briefly in deionized water.

6. Mounting

1. Leave the slides to dry in darkness for a few minutes.
2. Mounting is performed using permafluor.
3. Leave the mounting medium to dry, preferentially overnight, in a dark cool environment.

7. Microscope

1. Histological sections are ready to observe under a fluorescent microscope. In our experiment, we used an Olympus BX51 microscope equipped with a triple (FitC; MCA Texas-Red) filter. The Picture Frame software (MicroFire, Goleta, California) was used.

8. Representative Results:

Because the auto-fluorescence of fish tissues is so high, these tissues will appear orange under the Tri-filter, conveniently providing a background for the bacteria.

Against this background, the individual FitC stained bacteria will appear as bright green dots and, at 200X their rod shape will be recognizable (Fig 1).

Bad sections can constitute false positive (due to a problem in the rinsing phase), in which case a large number of bacteria will appear to be present uniformly on the slide.

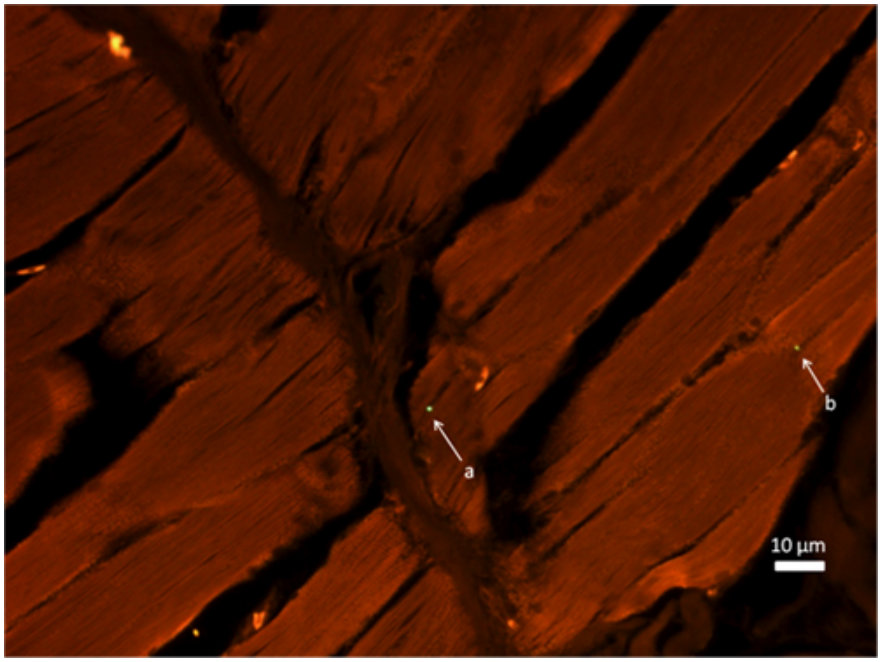

Figure 1. Micrograph of a muscle sections showing two *E. ictaluri* (arrows a and b) (200x).

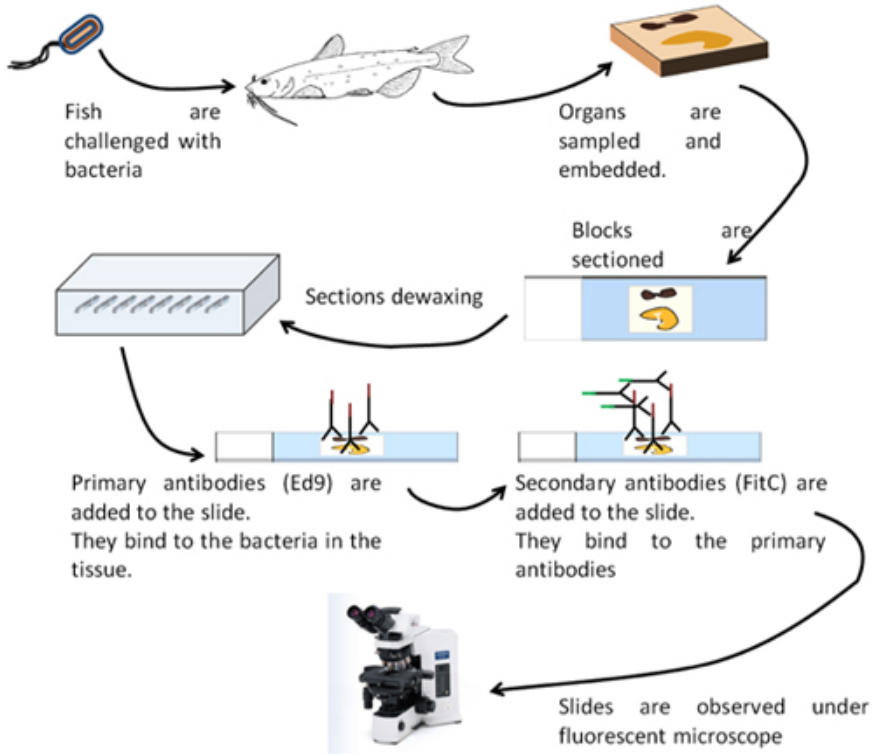

Figure 2. Overall scheme of the experiment

Discussion

This protocol details a technique for the *in situ* visualization of the catfish pathogen *Edwardsiella ictaluri* in histological sections. To the best of our knowledge, this is the first such protocol described.

The most critical step, in our experience, is the rinsing of the antibodies as described in the step 4.4 of the present protocol as insufficient washing may result in false positive.

The main problem with interpreting the results of this technique is the auto-fluorescence of the tissue. However, it was found that using the Tri-filter mostly solved that problem as the vast majority of the non-specific fluorescence occur outside of the green fluorescent spectrum. Also, while this technique is quite sensitive, it can fail to detect low bacterial loads.

Disclosures

No conflicts of interest declared.

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