The use of melanin bleaching for the immunohistochemical detection of *Renibacterium salmoninarum*.

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**Abstract**
Differentiating immunohistochemically stained bacteria on tissue sections, that possess high levels of melanomacrophages and free melanin can be difficult. This can be compounded due to the routine use of the chromogen diaminobenzidine for immunohistochemical procedures. Here we describe a technique for melanin bleaching prior to immunostaining to remove the subjectivity from results when studying bacterial pathogens of fish.

**Introduction**
Evaluation of immunohistochemically stained sections such as kidney tissue for bacterial pathogens is routinely performed across many laboratories. However, the interpretation of such results may be hampered by the presence of melanomacrophages and melanin granules in the tissues studied. This can lead to problems in identifying low levels of bacteria that are not forming obvious plaques or pathologies and for identifying intracellular pathogens. The use of diaminobenzidine as the chromogen for immunohistochemical staining can further confuse interpretation as it produces a brown precipitate (Nadji 1986, Haines and Chelack 1991). Although other chromogens can be used that produce different colour precipitates, the use of diaminobenzidine confers advantages in that it produces intense, insoluble, stable, deposits that remain indefinitely. Furthermore, bacteria intensely stained using alternative chromogens can still present problems for identification from melanin (Jansson, Hongslo et al. 1991).

In clinical immunohistochemical procedures melanin bleaching using Potassium permanganate/ Oxalic acid is used to remove the subjectivity from heavily pigmented tissues. However, to our knowledge, this technique has not been reported for the detection of bacterial pathogens in fish when used in conjunction with immunohistochemistry. Previous reports for melanin bleaching prior to immunohistochemical staining in the aquatic sciences have focused on the use of an extended 16-24 hour exposure of sections to Hydrogen peroxide (Jansson, Hongslo et al. 1991). However, some authors have reported loss of antigenicity when using this protocol (Gomez, Navarro et al. 1996). We therefore decided to examine the effectiveness of Potassium permanganate/ Oxalic acid bleaching for the immunohistochemical staining of the bacterial pathogen *Renibacterium salmoninarum* in kidney tissues of rainbow trout *Oncorhynchus mykiss*. 
Materials and Methods

Tissue sections of infected kidney were sectioned, serially, at 5µm onto 3’ aminoalkysilane coated slides and dewaxed through a xylene, ethanol series to distilled water. After dewaxing they were bleached by immersing into a Potassium permanganate bath 3.0g/L for 20 minutes. They were then washed in distilled water followed by immersion into 1% Oxalic acid for 90 seconds. The sections were then washed in running tap water and standard immunohistochemistry applied (adapted from Adams and Mateo 1994). Briefly, after encircling the sections with a PAP pen, endogenous peroxidase activity was blocked on the sections by incubating for 10 minutes in 10% hydrogen peroxide in methanol followed by washing in Tris buffered saline (TBS: 0.05M Tris base, 0.15 M Sodium Chloride, pH 7.6). They were then blocked by incubating for 10 minutes in 10% donkey serum in TBS. The slides were tapped dry before incubating with the primary antibody for 2 hours (rabbit polyclonal antiserum raised to *Renibacterium salmoninarum* diluted 1/100 in TBS). After washing in TBS the slides were incubated for 1 hour with anti-rabbit HRP (Diagnostics Scotland) diluted 1/50 in TBS. After washing in TBS the polyclonal antibody was detected using 0.15mg/ml diaminobenzidine in TBS, with 0.02% Hydrogen peroxide, for 10 minutes. The sections were immersed into tap water before counterstaining with Meyers haemotoxylin. After differentiating in tap water they were dehydrated through an alcohol series, cleared in xylene and coverslipping using Pertex. Immunohistochemical staining was evaluated microscopically. Control sections included in this study included slides that were not
bleached prior to staining, and bleached slides that were incubated with TBS instead of rabbit anti-sera.

**Results and Discussion**

The bleaching procedure removed all traces of melanin from melanomacrophages and melanin granules from the treated tissue sections (Figures 1 and 2). The procedure also did not appear to affect the intensity of the immunohistochemical staining of the polyclonal antibody (Figure 3). This made the full extent of the bacterial infection in the kidney tissue easy to assess and although there appeared to be a slight loss of cellular morphology there was no observable loss of overall tissue structure.

These results illustrate the successful use of melanin bleaching for the immunohistochemical detection of *R. salmoninarum*. Previously Bruno (1987) reported the usefulness of melanin bleaching using Permanganate/Oxalic acid when examining sections infected with *R. salmoninarum* stained with Haemotoxylin and eosin. However, subsequent immunohistochemical procedures on this pathogen have not incorporated this technique, thus leading to difficulties in detecting low numbers of bacteria (Hoffmann, Bell et al 1989). The combination of techniques outlined in this communication make diagnosis of this pathogen simpler. Furthermore, as the technique increases the standard immunohistochemical protocol by only 30 minutes it is considerably more rapid than using Hydrogen peroxide for bleaching tissues. This technique could be usefully incorporated into immunostaining protocols of a wide range bacterial and viral pathogens of fish in tissues where melanin is relatively abundant and causes problems in pathogen detection. However, it is recognised that the procedure, like Hydrogen peroxide bleaching, can adversely affect some antigens (Foss, Alexander et al. 1995). Therefore, appropriate controls should always be included into these procedures to assess possible adverse effects on the intensity of the resulting immunostaining.

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**References**


