CHAPTER 3.1.2.

MSX DISEASE
(*Haplosporidium nelsoni*)

GENERAL INFORMATION

MSX disease is caused by the protistan Haplosporidium nelsoni (= Minchinia nelsoni) *of the phylum Haplosporidia (14).* Haplosporidium nelsoni *is commonly known as MSX (multinucleate sphere X). The oysters* Crassostrea virginica *and* Crassostrea gigas *are infected by* H. nelsoni*; however, the prevalence and virulence of this pathogen are much higher in* C. virginica *than in* C. gigas *(1, 5, 10, 11).*

The geographical distribution of H. nelsoni *in* C. virginica *is the east coast of North America from Florida, USA, to Nova Scotia, Canada (9, 12). Enzootic areas are Delaware Bay and Chesapeake Bay, with occasional epizootics in North Carolina estuaries, Long Island Sound, Cape Cod and Nova Scotia (1, 8, 22).* Haplosporidium nelsoni *has been reported from* Crassostrea gigas *in California, USA (10, 11), and in Korea, Japan, and France (5, 10, 15, 16, 18). Another* Haplosporidium *sp.also occurs in* C. gigas *in France (6).*

The plasmodium stage of H. nelsoni *occurs intercellularly in connective tissue and epithelia. Spores of* H. nelsoni *occur exclusively in the epithelium of the digestive tubules. Sporulation of* H. nelsoni *is rare in infected adult oysters, but is frequently observed in infected juvenile oysters (2, 3). Infection by* H. nelsoni *takes place between mid-May and the end of October. Mortalities from new infections occur throughout the summer and peak in July/August. Mortalities may occur in the spring from over-wintering infections. MSX disease is restricted to salinities over 15 ppt (parts per thousand); rapid and high mortalities can occur at 20 ppt (1, 8, 13). Holding infected oysters for 2 weeks in water of 10 ppt salinity or less at 20°C kills* H. nelsoni, *but not* C. virginica *(7).*

Mortality of highly susceptible oysters from H. nelsoni *infections is rapid and occurs without loss of condition. More tolerant oysters survive longer and show reduction in condition index correlated with infection intensity (9). At death they are grossly emaciated and the storage cells appear shrunken and disrupted. Sporulation causes disruption of digestive tubule epithelium.*

It has not been possible to transmit H. nelsoni *experimentally in the laboratory. No life cycle has been elucidated for any member of the phylum Haplosporidia, but an intermediate host is suspected (12).*

For diagnosis, the recommended guidelines for sampling are those stated in Chapter I.2. of this Manual.

EXAMINATION PROCEDURES

1. Screening Methods

1.1. Histological examination

General histological procedures are detailed in Chapter I.2. of this *Manual.* Cut a transverse section through the visceral mass that includes mantle, gill and digestive gland, and place the sample in a fixative, such as Davidson's or Carson's fluid (the latter enables the samples to be re-used for electron microscopy, if necessary). The ratio must be no more than one volume of tissue to ten volumes of fixative. The sections are subsequently treated by conventional histological

procedures (4). Haplosporidia are revealed by many nonspecific stains, such as haematoxylin & eosin (H&E).

Multinucleate plasmodia of *Haplosporidium nelsoni* (usually 5–15 μm in diameter, but can be up to 25 μm) occur throughout the connective tissue and often in epithelia of the gill and gut. Plasmodia are detectable throughout the year. Sporocysts containing spores are restricted to the epithelium of the digestive tubules. Sporulation of *H. nelsoni* is rare in infected adult oysters, but is frequently observed in infected juvenile oysters. Mature spores measure about 6 ´ 8 μm. Sporocysts can disrupt the digestive epithelium releasing developing and mature spores into the lumen of the digestive tubules. Spores can be found from June through December in *C. virginica*.

Infection intensity has been rated (4) as follows: localised (LO) = any infection where plasmodia are localised in one small area in one tissue type, usually the epithelium of gills or gut; rare (R) = systemic infections with less than ten plasmodia in the entire section; light (L) = systemic infections with less than two plasmodia per field at ´400 magnification, but more than ten plasmodia in the entire section; moderate (M) = systemic infections with 2–5 plasmodia per field at ´400 magnification; heavy (H) = more than five plasmodia per field at ´400 magnification; sporulation (S) = any infection where spores are present.

2. Presumptive Diagnostic Methods

2.1. Histology

See Section 1.1. above for the technical procedure. When spores are present, *H. nelsoni* can be presumptively diagnosed in oysters if the spores are the correct size and occur exclusively in the epithelium of the digestive tubules. In areas along the east coast of the USA where salinity is consistently less than 25 ppt., haplosporidian plasmodia in oysters can be presumed to be *H. nelsoni*. In areas where salinity is consistently greater than 25 ppt., *H. nelsoni* and *H. costale* (the causative agent of SSO disease) co-occur and plasmodia of the two species cannot be distinguished by histology (see Chapter 3.2.1. SSO disease).

2.2. Polymerase chain reaction of DNA from oyster tissue

A positive polymerase chain reaction (PCR) amplification is only a presumptive diagnosis because it detects DNA and not necessarily a viable pathogen. Other techniques, preferably *in-situ* hybridisation, must be used to visualise the pathogen.

The PCR primers developed for *H. nelsoni* detection, MSX-A' (5'-CGA-CTT-TGG-CAT-TAG-GT-TTC-AGA-CC-3') and MSX-B (5'-ATG-TGT-TGG-TGA-CGC-TAA-CCG-3'), target small subunit ribosomal DNA and have been shown to be sensitive and specific for this pathogen (18, 21). A multiplex PCR (17) for *H. nelsoni* and *H. costale* (SSO) has been developed using these primers, but it has not been validated. PCR reaction mixtures contain reaction buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 10 μg/ml gelatin), 400 μg/ml bovine serum albumin, 25 pmoles each of MSX-A' and MSX-B, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.6 units Ampli*Taq* DNA polymerase (Applied Biosystems), and template DNA, in a total volume of 25 μl. The reaction mixtures are cycled in a thermal cycler. The programme for the GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) is: initial denaturation at 94°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1.5 minutes, and a final extension at 72°C for 5 minutes. An aliquot (10% of reaction volume) of each PCR reaction is checked for the 573 base pairs (bp) amplification product by agarose gel electrophoresis and ethidium bromide staining.

3. Confirmatory Identification of the Pathogen

3.1. *In-situ* hybridisation examination of *Haplosporidium nelsoni*

In-situ hybridisation is the method of choice for confirming identification because it allows visualisation of a specific probe hybridised to the target organism. DNA probes must be thoroughly tested for specificity and validated in comparative studies before they can be used for confirmatory identification.

In-situ hybridisation has recently been developed to differentiate plasmodia of *H. nelsoni* from those of *H. costale* in tissue sections (19, 20). Species-specific, labelled oligonucleotide probes hybridise with the small subunit ribosomal RNA of the parasites. This hybridisation is detected by an antibody conjugate that recognises the labelled probes. Substrate for the antibody conjugate is added, causing a colorimetric reaction that enables visualisation of probe–parasite RNA hybridisations.

The procedure for *in-situ* hybridisation is conducted as follows. Positive and negative controls must be included in the procedure.

i) A transverse section is cut through the visceral mass and placed in Davidson's AFA fixative (glycerin [10%], formalin [20%], 95% ethanol [30%], dH_2O [30%], glacial acetic acid [10%]) for 24–48 hours, and then transferred to 70% ethanol until processed by histological procedures (step ii). The ratio must be no more than one volume of tissue to ten volumes of fixative.

ii) The samples are subsequently embedded in paraffin by conventional histological procedures. Sections are cut at 5–6 µm and placed on positively-charged slides or 3-aminopropyl-triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C.

iii) The sections are deparaffinised by immersion in xylene or another less toxic clearing agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The sections are then washed twice for 5 minutes in phosphate buffered saline (PBS).

iv) The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 15 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2 ´ SSC (standard saline citrate) for 10 minutes.

v) The sections are prehybridised for 1 hour at 42°C in prehybridisation solution (4 ´ SSC, 50% formamide, 5 ´ Denhardt's solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-denaturated herring sperm DNA).

vi) The prehybridisation solution is then replaced with prehybridisation buffer containing 2 ng/µl of the digoxigenin-labelled oligonucleotide probe. The sequence of the probe designated MSX1347 (19) is 5'-ATG-TGT-TGG-TGA-CGC-TAA-CCG-3'. The sections are covered with *in-situ* hybridisation plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridisation overnight at 42°C in a humid chamber.

vii) The sections are washed twice for 5 minutes each in 2 ´ SSC at room temperature, twice for 5 minutes each in 1 ´ SSC at room temperature, and twice for 10 minutes each in 0.5 ´ SSC at 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

viii) The sections are placed in Buffer 1 (see step vii) supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer's recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue

sections. The sections are covered with *in-situ* hybridisation cover-slips and incubated for 3 hours at room temperature in the humid chamber.

ix) The slides are washed twice in Buffer 1 for 5 minutes each (see step vii) and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 5 minutes each. The slides are then placed in colour development solution (337.5 µg/ml nitroblue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2 hours in the dark. The colour reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

x) The slides are then rinsed in dH_2O. The sections are counterstained with Bismarck Brown Y, rinsed in dH_2O, and cover-slips are applied using an aqueous mounting medium. The presence of *H. nelsoni* is demonstrated by the purple-black labelling of the parasitic cells.

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