CHAPTER 2.2.9.

INFECTION WITH MIKROCYTOS MACKINI

1. Scope

For the purpose of this chapter, infection with Mikrocytos mackini is considered to be infection with Mikrocytos mackini the causative agent of Denman Island Disease in oysters.

2. Disease information

2.1. Agent factors

Protozoa of unknown taxonomic affiliations. Phylogenetic analyses suggested that M. mackini may be a basal eukaryote, but almost certainly is not closely related to other known protistan taxa (Carnegie et al., 2003).

2.1.1. Aetiological agent, agent strains

No known strains (Carnegie et al., 2003; Farley et al., 1988) and total lack of genetic variation within Mikrocytos mackini across the complete ITS1-5.8S-ITS2 array in rDNA in over 70 samples collected throughout its range (Abbott et al., 2011).

2.1.2. Survival outside the host

Unknown.

2.1.3. Stability of the agent

Unknown. However, direct transmission between oysters occurs via the water column (Bower, 1988; Hervio et al., 1996; Quayle, 1982).

2.1.4. Life cycle

The life cycle is direct from host to host.

2.2. Host factors

Infectious to all species of oysters challenged artificially in the laboratory and naturally in the field.

2.2.1. Susceptible host species

Pacific oyster (Crassostrea gigas), eastern oyster (Crassostrea virginica), European flat oyster (Ostrea edulis) and Olympia oyster (Ostrea lurida) are susceptible to infection (Bower et al., 1997). At least two species of clams, the geoduck clam (Panope abrupta) and the Manila clam (Venerupis [=Tapes, =Ruditapes] philippinarum), are resistant to infection (Bower et al., 2005; and Meyer et al., 2008, respectively).

2.2.2. Susceptible stages of the host

All life stages of oysters after settlement are susceptible to infection (Bower et al., 2005). The susceptibility of larvae is not known.

2.2.3. Species or subpopulation predilection (probability of detection)

All susceptible species held at least 10°C for at least 3 months are vulnerable to the disease (Bower et al., 1997; Hervio et al., 1996). Crassostrea virginica, Ostrea edulis and Ostrea lurida appear to be more susceptible to infection and disease than Crassostrea gigas (Bower et al., 1997).

NB: Version adopted by the World Assembly of Delegates of the OIE in May 2013. This disease is no longer listed by the OIE.
2.2.4. Target organs and infected tissue

*Mikrocytos mackini* usually resides in the cytoplasm of the vesicular connective tissue cells of all organs, and adductor muscle fibres, but has also been observed in haemocytes and in the epithelium of the digestive gland (Hine et al., 2001; Meyer et al., 2005).

2.2.5. Persistent infection with lifelong carriers

Infection can be fatal depending on host and environmental conditions (Bower, 1988; Bower, 2001; Bower & Meyer, 1999). Subclinical infections occur but the persistence of infection over several years and occurrence of lifelong carriers is not known (Bower et al., 1994a).

2.2.6. Vectors

No vectors are required for the transmission of infection.

2.2.7. Known or suspected wild aquatic animal carriers

Not applicable.

2.3. Disease pattern

2.3.1. Transmission mechanisms

Transmission is direct from host to host (Hervio et al., 1996). Viable *M. mackini* released upon death of the host are probably acquired by the next host through feeding mechanisms. *Mikrocytos mackini* in haemocytes may also exit from its living host via diapedesis through the intestinal tract and gills.

2.3.2. Prevalence

During the 1960s, mortalities up to 40% during the spring (April and May) at low intertidal levels among old (3+ years) *C. gigas* grown on the beach substrate were attributed to *M. mackini*. More recently, *C. gigas* (2 years old) from a suspended culture site experienced about 10% mortality with heavy *M. mackini* infections during the spring. Up to 35% of the oysters from this infected population had symptomatic lesions (yellowy green pustules) and the affected crop was rejected by the market processor. This was an unusual occurrence as this disease does not usually have an impact on oysters that are under intensive culture and harvested within 3 years. Laboratory exposure experiments indicated that disease is exacerbated by cool temperatures suggesting that the impact of *M. mackini* may be more severe if it is inadvertently introduced into cooler locations. Also, in the laboratory, juvenile (seed) oysters are susceptible to infection and resulting high mortality (Bower et al., 2005). The impact of *M. mackini* on juveniles during commercial culture is not known but expected to be negligible if the seed are deployed after the end of the natural transmission period that occurs in the spring (Quayle, 1982).

2.3.3. Geographical distribution

On the west coast of Canada, *M. mackini* appears to be ubiquitous throughout the Strait of Georgia and confined to other specific localities around Vancouver Island. This parasite has also been detected in oysters from adjacent areas of the State of Washington, USA without evidence of associated mortalities (Abbott et al., 2011).

2.3.4. Mortality and morbidity

Infection can be lethal if environmental temperatures are conducive as indicated below. However, about half of the exposed oysters seem to be resistant to infection or the resulting disease.

2.3.5. Environmental factors

Cool temperatures of <10°C for 3–4 months seem to be a requirement for disease development (Bower & Meyer, 1999; Hervio et al., 1996). Experimentally exposed oysters held at 15°C for 3 months did not develop the disease until they were transferred and maintained at 10°C for an additional 4 months. The annual appearance of the disease only during the spring (March to June) could be explained by the cool temperature requirements for disease development.
2.4. Control and prevention

Management techniques can be employed by the aquaculture industry to circumvent the impact of *M. mackini* (Bower, 1988).

2.4.1. Vaccination
Not applicable.

2.4.2. Chemotherapy
Not applicable.

2.4.3. Immunostimulation
Not applicable.

2.4.4. Resistance breeding
Not applicable.

2.4.5. Restocking with resistant species
Not applicable.

2.4.6. Blocking agents
Not applicable.

2.4.7. Disinfection of eggs and larvae
Not applicable.

2.4.8. General husbandry practices
Harvest market-sized oysters within 3 years of planting and prior to February of the third year of grow-out. Oyster juveniles (seed) should not be deployed at lower tide levels or adjacent to infected stock in suspended culture before June (Bower, 1988; Quayle, 1982).

3. Sampling

3.1. Selection of individual specimens
Live or freshly dead oysters should be sampled.

During the spring, select old oysters (3+ years) from low intertidal levels. Focus sampling from locations experiencing recent mortalities. Extract oysters from the shell and select specimens that have small lesions (ulcerations, abscesses, pustules usually green in colour but can be yellow-brown or colourless and up to 5 mm in diameter) in the vesicular connective tissue of the body, mantle and labial palps, and/or in the adductor muscle (for images of the lesions see: http://www.dfo-mpo.gc.ca/science/aah-saa/diseases-maladies/mikmacoy-eng.html

3.2. Preservation of samples for submission
For histology the best preservative is Davidson’s solution, but 10% buffered formalin or other standard histology fixatives are also acceptable. For polymerase chain reaction (PCR) assays, samples must be preserved and stored in 95% undenatured ethanol until DNA extraction using a commercially available kit (e.g. DNeasy Kit; QIAGEN).

3.3. Pooling of samples
Pooling lesions from the same oyster is acceptable for PCR and histology.
3.4. Best organs or tissues

It is important that any lesions in the labial palps, mantle, body wall or adductor muscle are preserved for both histology and PCR. It is recommended that representative lesions are excised and bisected, with half of each fixed for histology and for PCR.

For histology, in the absence of lesions (or in addition to) a transverse section (approx. 3 mm thick) through the anterior portion of the visceral mass that includes labial palps, mantle, digestive gland and stomach is used. Vesicular connective tissue (VCT) is best for visualising *M. mackini* by histopathology.

For PCR, in the absence of lesions (or in addition to), a transverse section (slice approx. 3 mm thick) is cut through the middle of the visceral mass. From this section small piece(s) of tissue (approx. 25 mg total weight) are excised near the base of the gills that includes: VCT, gonad, digestive gland and gill.

3.5. Samples/tissues that are not suitable

*Mikrycytos mackini* associated with the digestive tract are not evident by routine histological examination on sections stained with haematoxylin and eosin stain. *In situ* hybridisation is usually required to see the parasite in these tissues. Also, it is near impossible to detect *M. mackini* by histopathology in tissues of naturally infected oysters that are not associated with lesions of haemocyte infiltration.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Small focal lesions (ulcerations, abscesses, pustules usually green in colour but can be yellow–brown or colourless) up to 5 mm in diameter observed in the soft tissues and often with brown scars on the shell, adjacent to abscesses on the mantle surface. Apart from the lesions, infected oysters are usually in good condition up to the time of death. These clinical signs are not specific to infection with *M. mackini*.

4.1.2. Behavioural changes

Moribund, gaping or weak oysters (slow to tightly close their valves) occur among affected stocks during the spring or when held in tanks maintained at <10°C. These behavioural changes are not specific to infection with *M. mackini*.

4.2. Clinical methods

4.2.1. Gross pathology

Small focal lesions of intense haemocyte infiltration (ulcerations, abscesses, pustules usually green in colour but can be yellow–brown or colourless) up to 5 mm in diameter occur within the body wall, adductor muscle or on the surfaces of the labial palps or mantle (Bower, 2005). These gross signs are not specific to infection with *M. mackini*.

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

Accumulation (infiltration) of numerous haemocytes in the vicinity of a focal infection.

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

Tissue imprints of lesions that have been stained with a Giemsa-type stain and examined using 1000x magnification light microscopy (oil immersion) may reveal *M. mackini* liberated from host cells.
4.2.6. Fixed sections

Foci of haemocyte infiltration in the mantle, labial palps and adductor muscle can indicate the location of *M. mackini* within the cytoplasm of adjacent vesicular connective tissue cells and myofibres. Tissue necrosis may occur at the centre of the lesion (Bower et al., 1994b). In high intensity infections induced in the laboratory, haemocyte infiltration may not be present.

4.2.7. Electron microscopy/cytopathology

The intimate relationship of *M. mackini* with the organelles of the host cell suggests that this parasite is able to obtain energy directly from the mitochondria of the host cell (Hine et al., 2001).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

The small size of *M. mackini* (2–3 µm in diameter) makes it impossible to see without the aid of high power (about 1000×) magnification.

4.3.1.1. Microscopic methods

With light microscopy, *M. mackini* appears morphologically similar to *Bonamia* sp. except for its occurrence in the cytoplasm of vesicular connective tissue cells and myocytes where *Bonamia* sp. does not occur. With Electron microscopy, morphological ultrastructure can be used to differentiate *M. mackini* from other known protozoa. However, very heavy infections are required for the use of this tool.

4.3.1.1.1. Wet mounts

Not applicable.

4.3.1.1.2. Smears

4.3.1.1.2.1. Samples to be taken

Live hosts with lesions.

4.3.1.1.2.2. Technical procedure

Excise lesion and cut in half with scalpel. Blot lesion side of tissue on absorbent paper to remove excess fluid. Touch tissue to several areas of a clean glass slide and air dry. Observations are made at ×1000 (oil emersion) after staining with Wright-Giemsa type stains or with a commercially available staining kit for blood cells (e.g. Hemacolor®; EMD Chemicals Inc.) in accordance with the manufacturer's instructions.

4.3.1.1.2.3. Positive controls

Tissue imprints of lesions from infected oysters are available from the OIE Reference Laboratory. However, it is important to note that *M. mackini* in tissue imprints is virtually impossible to differentiate from *Bonamia* sp. in tissue imprints.

4.3.1.1.2.4. Levels of validation

This assay has not been formally validated.

4.3.1.1.2.5. Specificity and sensitivity

Very low specificity because *M. mackini* resembles other microcells in tissue imprints; sensitivity may be better than routine histology but only when lesions are present (Carnegie et al., 2003).

4.3.1.1.2.6. Interpretation of results

Presence of small microcells (can be distorted to about 4 µm in diameter) that are usually observed outside of the host cells. The parasite, usually 2–3 µm in diameter has light blue (basophilic) cytoplasm and a small red (eosinophilic) nucleus (colours may vary with stain used). The technique is not species specific.
4.3.1.1.3. Fixed sections

4.3.1.1.3.1. Samples to be taken

Live or freshly dead oysters.

4.3.1.1.3.2. Technical procedure

Sections of tissue that include lesions should be fixed for approximately 24 hours in Davidson’s solution or 10% buffered formalin followed by normal processing for paraffin histology and staining with haematoxylin and eosin stain. Observations are made at increasing magnifications to ×1000.

4.3.1.1.3.3. Positive controls

Histological sections (stained and with cover-slips or unstained paraffin sections on a glass slide) are available from the OIE Reference Laboratory. Frequent reference to positive control slides while conducting a survey for *M. mackini* is recommended because of the cryptic nature and small size of this parasite.

4.3.1.1.3.4. Levels of validation

This assay has not been formally validated.

4.3.1.1.3.5. Specificity and sensitivity

Species specificity is very low for microcells observed in haemocytes but high when microcells are found within the cytoplasm of vesicular connective tissue cells; sensitivity is good for moderate to high intensity infections especially when the connective tissue immediately around lesions is examined, but low for tissues distant from lesions and for low intensity infections.

4.3.1.1.3.6. Gold standard

Currently, histopathology of lesions in oysters is considered to be the assay of choice for detecting and diagnosing infections with *M. mackini*.

4.3.1.1.3.7. Interpretation of results

A positive result is the occurrence of spherical microcells about 2‒3 µm in diameter within the cytoplasm of vesicular connective tissue cells and/or myocytes, usually intracellular in host cells immediately adjacent to focal intense haemocyte infiltration.

In susceptible host species within the known range of *M. mackini*, a positive result is evidence of *M. mackini* infection. Outside the known range, a positive result must be confirmed by DNA sequencing of the small-subunit ribosomal RNA gene (SSU rDNA) region and comparison of the sequence with the 1457 bp fragment from *M. mackini* published in GenBank. If the SSU rDNA sequence is suggestive of *M. mackini*, the recommendation is to sequence ITS1-5.8S-ITS2 rDNA using methods in Abbott et al., 2011 to confidently determine if it is genetically identical to *M. mackini* in the currently described range.

4.3.1.2. Agent isolation and identification

Pure isolates of *M. mackini* have not been produced. However, identification procedures in addition to light microscopy are under development.

4.3.1.2.1. Cell culture/artificial media

Not applicable.

4.3.1.2.2. Antibody-based antigen detection methods (IFAT, ELISA, etc.)

Not applicable. Monoclonal antibodies have been produced but have not been developed into an immunological diagnostic assay.

---

4.3.1.2.3. Molecular techniques (PCR, ISH, sequencing, etc.)

4.3.1.2.3.1. SSU rDNA region PCR assay

Primer pairs that target the SSU region have been developed for *M. mackini*. Primers are 5'-AGA-TGG-TTA-ATG-AGC-CTC-C-3' and 5'-GCG-AGG-TGC-AC-AAG-GC-3' and they amplify a 546 bp product (Carnegie et al., 2003). Samples should be diluted with either sterile ddH2O or sterile Tris/EDTA (TE) buffer (pH 7.0-7.2) to a concentration between 10 and 40 ng/µl before being assayed. A PCR reaction mixture contains the following ingredients at final concentrations: 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl2, 200 µM each dATP, dCTP, dGTP, and dTTP, 0.05 µM of each primer and 0.05 units/µl and 1.5 µl DNA template in a total volume of 15 µl. Cycling parameters begin with an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 1 minute, 60.5°C for 1 minute, and 72°C for 1 minute, and end with a final extension at 72°C for 10 minutes. Products can be electrophoresed on 1.5% agarose gels containing 2.0 µl 5000X SybrGreen or 0.1 µg/ml ethidium bromide and visualised by exposure to UV light.

4.3.1.2.3.1.1. Positive/negative controls

These are compulsory. Positive controls are genomic DNA from heavily infected hosts which can be obtained from the OIE Reference Laboratory. Negative controls are either genomic DNA from uninfected hosts or no template DNA reactions.

4.3.1.2.3.1.2. Levels of validation

This assay has not been formally validated.

4.3.1.2.3.1.3. Specificity and sensitivity

The SSU rDNA region assay detected three to four times more *M. mackini* infections in 1056 wild oysters from Denman Island, British Columbia than did standard histopathology (Carnegie et al., 2003). However, subsequently it has proven to be problematic because it does not distinguish between *M. mackini* and a genetically different *Mikrocytos* sp. that was detected in multiple disparate geographic locations (Abbott et al., 2011).

4.3.1.2.3.1.4. Interpretation of results

A positive result is a PCR amplicon of the appropriate size, with all negative controls negative and all positive controls positive.

4.3.1.2.3.2. TaqMan qPCR assay in ITS2-28S rDNA

Recently a new real-time qPCR diagnostic assay has been developed using primers and probe in ITS2-28S rDNA in *M. mackini* that does not cross-react with *Mikrocytos* sp. Bench validation of this assay has shown that it is highly specific and sensitive for detection of *M. mackini*. Formal diagnostic validation of the qPCR assay is currently in progress.

4.3.1.2.3.3. In situ hybridisation (ISH)

4.3.1.2.3.3.1. Samples to be taken

Follow the procedure for fixed sections (4.3.1.1.3) above, except that tissue sections must be placed on positively charged slides or slides coated with aminopropylsilane.

4.3.1.2.3.3.2. Technical procedure

Tissue sections are deparaffinised, rehydrated and then hybridised with labelled oligonucleotide probes. Probes labelled with 5’ Oregon Green hybridise strongly to *M. mackini* (Carnegie et al., 2003). However, host tissue orientation is difficult when using this fluorescent probe. Hybridisation with the probe MACKINI-1 (5’-AGC-CCA-CAG-CCT-TCA-C-3’) with a 3’ end digoxigenin label and a counter stain of 0.5% Bismark Brown Y in 30% ethanol (Meyer et al., 2005) depicts the parasite location within the host tissues.

4.3.1.2.3.3.3. Positive/negative controls

These are compulsory. Positive controls are tissue sections from an oyster infected with *M. mackini*. Negative controls are either no-probe assays or assays with uninfected oysters.
4.3.1.2.3.4. Levels of validation

This technique has not been formally validated.

4.3.1.2.3.5. Specificity and sensitivity

The MACKINI-1 probe hybridised strongly to *M. mackini*, but did not hybridise to oyster tissues or with the other shellfish parasites and a bacterium tested: *Bonamia ostreae* in *O. edulis*; *Perkinsus qugwadi* in *Patinopecten yessoensis*; *Trichodina* sp. in *C. gigas*; an amoeba-like protistan in *Protophaca staminea*; *Hematodinium* sp. in *Chionoecetes tanneri*; SPP (a protistan parasite of uncertain taxonomic affiliation) in *Pandalus platyceros*; and *Nocardia crassostreae* in *C. gigas* (Meyer et al., 2005). This probe with a digoxigenin label was considerably more sensitive for detecting infections when compared with standard histological sections stained with haematoxylin and eosin stain. Infecion could be detected at lower magnification (×100 in comparison with ×1000) and in basophilic-staining tissues such as the digestive gland, gut epithelium and gonad (Meyer et al., 2005).

4.3.1.2.3.6. Interpretation of results

A positive result is the presence of blue–black labelling of the parasite cells (appropriate size and tissue location) with all negative controls negative and all positive controls positive.

4.3.1.2.4. Agent purification

Isolates of *M. mackini* free of contamination host cell nuclei have not been obtained. However, a filtration technique that concentrates this parasite has been described (Joly et al., 2001).

4.3.2. Serological methods

Monoclonal antibodies were developed and hybridomas cryopreserved about 15 years ago. The specificity of the monoclonal antibodies has not been fully validated and they have not been developed as a diagnostic test.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of *Mikrocytos mackini* are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

### Table 5.1. Methods for targeted surveillance and diagnosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance</th>
<th>Presumptive diagnosis</th>
<th>Confirmatory diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Juveniles</td>
<td>Adults</td>
<td></td>
</tr>
<tr>
<td>Gross signs</td>
<td>d</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>Smears (tissue imprints)(^1)</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>Histopathology</td>
<td>c</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td>In situ DNA probes</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>PCR</td>
<td>b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Sequence</td>
<td>d</td>
<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

1. The technique is not species specific, but can be used in susceptible hosts and areas and during the spring of the year when disease caused by *M. mackini* is evident.
2. Only used in conjunction with a positive result by PCR.

EM = electron microscopy; PCR = polymerase chain reaction.
6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Mikrocytos mackini*

Histopathology and PCR assays should be used for targeted surveillance to declare freedom from infection with *M. mackini*. Oysters that are 2+ years old from low intertidal levels or suspended culture should be collected during the spring of the year and the presence of any lesions should be targeted.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

In known susceptible species within the known geographical range of *M. mackini* during the spring of the year, a suspect case of infection with *M. mackini* is gross signs of the disease in combination with a positive result by tissue imprint of lesions.

In other host species, or outside the known range of *M. mackini*, a suspect case is positive result by histology or PCR.

7.2. Definition of confirmed case

In known susceptible species within the known geographical range of *M. mackini* during the spring of the year, a confirmed case is a positive result by one of the following methods: histology, PCR or ISH.

In other host species or outside the known range of *M. mackini* a confirmed case is a positive result by histology or PCR combined with a positive result by *in situ* hybridisation, electron microscopy or homology with the published rDNA sequences (Abbott et al., 2011; Carnegie et al., 2003). Sequencing of the SSU and/or ITS1-5.8S-ITS2 regions is recommended as a final step for a confirmatory diagnosis of *M. mackini*.

8. References


* * *

**NB:** There is an OIE Reference Laboratory for Infection with *Mikrocytos mackini* (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: [http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/](http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/)). Please contact the OIE Reference Laboratories for any further information on Infection with *Mikrocytos mackini*.