Crayfish Plague

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SUMMARY

Crayfish Plague is a contagious fungal disease of all freshwater crayfish of non-North American origin and is caused by Aphanomyces astaci*, an Oomycete fungus . The fungus affects the Noble crayfish (*Astacus astacus*), the stone crayfish (*Austropotamobius pallipes*) of south-west and west Europe, the related* Austropotamobius torentium *found in mountain streams of south-west Europe and the slender clawed crayfish (*Astacus leptodactylus*) of Eastern Europe and Asia Minor. Crayfish plague is found throughout North America and Europe. In the past 100 years, the disease has spread throughout Europe.*

Infected crayfish present a wide range of gross signs of infection, or none at all. Focal whitening of musculature beneath transparent areas of thin cuticle, often accompanied by focal areas of brown melanisation is the most consistent sign. Infection occurs in the connective tissue in the abdomen, limb joints, gills, the telson near the anus and occasionally the eyes. In terminal stages animals show a limited range of behavioural signs principally wandering into bright daylight where they appear disoriented and confused and may leave the water. The animals have a 'walking on stilts' appearance. There is loss of limb coordination, falling over, and the appearance of woolly tufts of fungal hyphae between the abdominal segments of infected individuals in terminal stages. There is total mortality (100%) in susceptible species and this massive mortality affecting only crayfish over large areas of waterway is often the first indication of infection and is diagnostic. American crayfish Orconectes limosus, Pacifastacus leniusculus and Procambarus clarkii are all resistant to the fungus and are considered to be partially or completely asymptomatic carriers

Identification of the agent: *Diagnosis requires isolation and characterisation of* A. astaci *using mycological media fortified with antibiotics to inhibit bacterial growth. Isolation is difficult unless within 12 hours of host death because of overgrowth of secondary invaders*

Serological tests: *Serological tests are not available for crayfish plague.*

Status of Australia and New Zealand: *Crayfish plague is exotic to both Australia and New Zealand.*

Introduction

Significance

The fungal disease caused by *Aphanomyces astaci* (crayfish plague) is a highly infectious disease of all freshwater crayfish of non-North American origin. The disease causes 100% mortality in susceptible crayfish and has devastated stocks in Europe. As a result it is probably the most intensively investigated and well documented disease of freshwater Crustacea. Crayfish plague affects crayfish only and naturally affects no other crustaceans. The disease is thought to have been introduced to Europe in the 19th century through imports from America and its subsequent spread with movements of infected animals is well documented[1].

Crayfish plague has had a serious impact on the ecology of European waterways. In countries such as Sweden, native crayfish populations have been eradicated within weeks or months after infestation[2]. This has changed the ecology of affected waterways, lakes have become weedy and turbid and the fishing industry has suffered[3].

Australia is considered to be free of this disease, though Australian *Cherax* species are highly susceptible[4].

Article 3.3.4.1 of the OIE *International Aquatic Animal Health Code* requires that Competent Authorities in countries or zones where crayfish plague has never been reported should prohibit the importation of live crayfish (other than for direct human consumption) from countries or zones where the disease has been reported or where its absence cannot be guaranteed.

In an export situation the receiving country will in most cases request that Health Certification for the absence of this disease be provided prior to export.

Case definition

The disease is caused by the fungus *Aphanomyces astaci* Schikora (a member of the Saprolegniales). Infection by other species of fungi in the absence of the above species does not constitute "crayfish plague".

Distribution (in Australia or Overseas)

Aphanomyces astaci is widespread in Europe and North America. It has not been reported in Japan (where the asymptomatic carrier *Procambarus clarkii* has been introduced). It does not occur in Australia, based on the lack of major mortalities, and targeted surveillance in Western Australia since 1989.

Limitation statement

There are many described species of *Aphanomyces* in the aquatic environment including *Aphanomyces invaderans* (the agent responsible for Epizootic Ulcerative Syndrome (EUS) in fish). EUS occurs widely in Australian waterways (see SDT for EUS). There are also many other genera of fungi that will grow in or on freshwater crayfish. This Diagnostic Technique refers only to *A. astaci*, which is considered exotic to Australia. Growth of fungi on moribund crayfish or on the recommended culture media is not uncommon. The SDT should, therefore, be carefully studied and suspect cases will need to be sent to Europe for final confirmation of Crayfish Plague. Live *A. astaci* is not held in Australia.

Clinical Signs

These are of limited diagnostic value. Infected crayfish present a wide range of gross signs of infection, or none at all. Focal whitening of musculature beneath transparent areas of thin cuticle, often accompanied by focal areas of brown melanisation is the most consistent sign. Infection occurs in the connective tissue in the abdomen, limb joints, gills, the telson near the anus and occasionally the eyes. In terminal stages animals show a limited range of behavioural signs principally wandering into bright daylight where they appear disoriented and confused and may leave the water[3]. The animals have a 'walking on stilts' appearance. There is loss of limb coordination, falling over, and the appearance of woolly tufts of fungal hyphae between the abdominal segments of infected individuals in terminal stages. There is total mortality (100%) in susceptible species and this massive mortality affecting only crayfish over large areas of waterway is often the first indication of infection and is diagnostic.

Pathology

The encysted fungal spores accumulate on and around superficial breaks in the epicuticle, possibly attracted by leakage of salts from the wound. Hyphae penetrate the cuticle through use of a combination of enzyme attack and pressure, preferentially moving along the cleavage planes in the chitin. Outside of the wound area the hyphae ramify, becoming more dense. There is a marked haemocyte response at the base of the wound and in

the vicinity of the epicuticle or epidermis within 48 hours[5]. Encapsulation of the hyphae by haemocytes results in melanisation and gives a knobbly appearance to the hyphae.

Diagnostic Tests

Range of tests available and appropriate applications

Surveillance

Wet Mounts: Examine muscle or soft cuticle of moribund crayfish for hyphae. Melanized cuticle of North American crayfish may indicate focal infections of hyphae.

Fungal isolation**:** Growth of fungus on IM agar.

Exclusion

Fungal Isolation: Growth of fungus on IM agar.

Diagnosis

Fungal Isolation**:** Growth of fungus on IM agar.
Production of spore clusters typical of *A. astaci*.
Challenge infection of susceptible uninfected animals.

Specimens required

The most suitable specimens are those freshly (<24h) dead from the infection[6]. Otherwise, live or moribund crayfish, preferably with melanised lesions suggestive of fungal infection are acceptable (Figure 1).

Tests available

Histopathology

Wet Mounts: Examine soft cuticle of moribund crayfish under low power compound microscope for hyphae. Melanized cuticle of crayfish may indicate focal infections.

Histological sections: Fungal hyphae stain with Haematoxylin and Eosin Stain or Grocott's modification of Gomori stain (personal communication, DJ Alderman).

Agent isolation

Aphanomyces astaci can be isolated from any infected tissue of freshwater crayfish, although most conveniently from soft parts of the cuticle such as the abdomen. Excise small pieces of tissue (uncalcified cuticle, connective tissue, muscle, nerve gut or eye are all suitable). Pieces of tissue 1-2mm^3 are inoculated onto IM agar for culture. Incubate plates at 16^o C. A 10 mm dia *A. astaci* colony should grow in 4-7 days at this temperature. The surface of a 90 mm dia. petri dish is colonised in about 15 days and remains viable for another 15 days but no resting stages are formed. Growth is skewed with an optimum at 22-24^o C.

Agent identification

This section is adapted from the 1997 OIE diagnostic manual:

Colonies are colourless. Vegetative hyphae are aseptate and 7-9 µm in dia., minimum observed dia. 5µm, max. 10 µm. Young, actively growing hyphae are densely packed with coarsely granular cytoplasm and refractile globules. Older hyphae are largely vacuolate with the cytoplasm restricted to the periphery leaving only thin strands of protoplasm bridging the large central vacuole. Oldest hyphae appear devoid of contents. Hyphae branch profusely, with vegetative branches often tending to be somewhat narrower than the main hyphae for the first 20-30 µm of growth.

When actively growing portions of thalli from agar culture are transferred to tap water, sporangia form readily in 20-30 hours at 16°C and 12-15 hours at 20°C. Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. Sporangial form is variable: terminal sporangia are simple, developing from new extramatrical hyphae, while intercalary sporangia can be quite complex in form. Intercalary sporangia develop by the growth of a new lateral extramatrical branch, which forms the discharge tube of the sporangium.

Formation of sexual structures cannot be induced by growth on suitable indicator media such as corn meal agar. Spore production should be observed after transfer of mycelia from IM media into tap water at 16° or 22°C.

molecular tests

DNA Probes: Arbitrary primers and DNA polymerase chain reaction (PCR) techniques have been used to identify four groups among *A. astaci* isolated from Sweden[7] and Germany[8]. These primers and probes are not commercially available.

Other techniques

Morphological identification of the fungus using the criteria based on Diéguez-Uribeondo et al.[9].

Exposing susceptible crayfish to zoospores produced by suspect isolates will result in characteristic rapid (2 to 8 days) mortality. Subsequent re-isolation of the fungus will give firm confirmation of crayfish plague[10].

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Appendices

APPENDIX 1 - Test details (comprehensive)

List of equipment for wet smears

Scalpel or a knife if it is a large animal
Scissors
Forceps
Slides, frosted at one end
Lead Pencil
Cutting board
Slide holder
Diff Quik stain
Plastic sealed container
Petri dish
Tap water

Major equipment
Compound microscope
Refrigerated Incubator
Steriliser

IM Media

IM medium: 12.0 g agar; 1.0 g yeast extract; 5.0 g glucose; 10 mg oxolinic acid; 1000 ml sterile tap water; and 1.0 g penicillin G added after autoclaving and cooling to 40°C. Do not use demineralised water, as calcium ions are required. Use either sterile river water (any natural river or lake water), or autoclaved tap water (chlorine is driven off in the sterlisation process) (Pers. com. Alderman 1998).

Quality control

No live *Aphanomyces astaci* is held in Australia. Media can only be tested in Australia for its ability to grow other fungi species.

Procedures

Euthanasia

The animals are kept refrigerated until slaughter. Slaughter is performed by holding the live animal on a cutting board and with a stout knife or scalpel, cutting the animal from a point just anterior to the heart on the dorsal carapace, through the mouth area in a downwards motion through the walking legs. This destroys the ventral nerve ganglia and exposes the internal organs. Examine ventral cephalothorax and tail for lesions, especially dark melanised patches. Take test tissue from these areas for preference. If no lesions are evident smear muscle and cuticle from at least three sites in each animal, especially around the base of the walking legs close to the body and inside the thorax, as outlined below.

Tissue for culture

A small portion (about $2mm^2$) of soft cuticle is cut out using sterile instruments and, using forceps, is rinsed in sterile R.O. water before placing on the surface of a pre-prepared sterile IM culture plate. Three or four pieces of cuticle from one animal can be placed on one 90mm IM plate. Use a new plate for each animal. Place plates in sealed plastic containers to reduce drying of the agar surface. The organism does not like dry surfaces, therefore do not dry plates prior to inoculation. Incubate plates between 15 to 24 °C and examine daily over 15 days for fungal growth. Slower growth occurs at lower temperatures.

Fungal colonies should appear after 4 to 7 days. Colonies are slow growing, flat and colourless, with growth within and on the surface of the agar but no aerial hyphae. Some superficial growth may occur at 7 °C. A Swedish isolate was noted to produce superficial ropey, slightly spiral growth in contrast to British isolates[11]. Hyphae are aseptate, 10 μm in width and when actively growing contain a granular cytoplasm. Branched hyphae are numerous and the first 20 – 30 μm of the branch is narrower than the main branch. Coiled hyphae may be seen. Reduced or absent granulation and vacuoles are seen in older hyphae.

Test for Sporulation:

Formation of clusters of encysted spores is diagnostic for *A. astaci*. Using a coverslip, cut a thin slice from the growing edge of the fungal colony. Place in a petri dish and add sufficient tap water to cover the slice. Leave overnight at 20 °C. After an optimum of 18 h incubation, examine the plug under an inverted microscope. Sporangia of *A. astaci*- line up in a single file at the end of the hyphae. Discharge occurs in less than five minutes and spores encyst at the sporangial orifice in grape-like clusters of 15 – 30 spores (Fig 3). These clusters remain encysted for 8 – 12 h. Refer to Alderman and Polgase[11] for excellent descriptions and photographs of this process.

Tissue for wet smear.

The tail muscle is cut longitudinally and a scraping of muscle tissue (about $2mm^3$) is smeared onto a slide. A scraping is also taken of soft cuticle from under the carapace over the top of the walking legs which is smeared on a separate slide. The slides are left to air dry and stained with Diff-Quik® or giemsa stain. Stained air dried slides are examined under the microscope for fungal hyphae.

Reagent

Diff-Quik® (LabAids Pty; available from Perth Scientific Pty)
Wrights-Giemsa is a substitute.

Interpretation (including limitations)

Wet Smears

A slide is positive if it has the following characteristics:-

(a) Frequently branching aseptate (except for septae delimiting zoosporangia) hyphae.
(b) The diameter of the hyphae is uniform, ca 8-10 um, and the hyphal tips are rounded.
(c) Fungal growth must be within the cuticle not only at the surface.
(d) No spore structures except, at late stages of infection, zoosporangia or primary cysts.

A wide range of fungi will grow on the media and may be isolated from freshwater crayfish. A 'positive' smear must be identified as a species of *Aphanomyces*, and the specific nature of the fungus determined by a reference laboratory before 'plague' is diagnosed.