Crayfish Plague

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Summary

Crayfish plague is a contagious fungal disease affecting freshwater crayfish (Decapoda: Astacidae, Cambaridae) of non-North American origin. Susceptible species include the Noble crayfish (*Astacus astacus*) of north-west Europe, the white clawed or stone crayfish (*Austropotamobius pallipes*) of south-west and west Europe including *Austropotamobius torrentium*, which is found in mountain streams of south-west Europe, and the slender clawed or Turkish crayfish (*Astacus leptodactylus*) from eastern- Europe and Asia Minor. Crayfish of North American origin, the mud or spiny cheeked crayfish (*Orconectes limosus*), the signal crayfish (*Pacifastacus leniusculus*) and the red swamp crayfish (*Procambarus clarkii*) are considered to be either carriers or resistant. The disease is exotic to Australia and New Zealand but Australian freshwater crayfish are susceptible as proven under experimental conditions.

The causative agent *Aphanomyces astaci*, an Oomycete fungus, is extremely virulent causing up to 100% mortality in infected animals.

Often the first sign of infection is massive stock losses. Clinical signs in infected animals may be non-existent or difficult to detect, but are more easily discernible in the terminal stages of the infection. These include gross appearance such as whitening of the musculature beneath the soft cuticle of the abdomen, and areas of brownish-red melanisation where haemocytes have encapsulated the hyphae. Areas of infection are the connective tissue of the abdomen, limb joints, gills, the telson near the anus and occasionally the eyes. Animals in the terminal stages of infection show signs of neurotoxicity such as being seen in daylight hours, loss of limb coordination, a ‘walking on stilts’ phenomenon, and lack of tail flip response. Loss of limbs or eye stalks can occur. Crayfish may even have tufts of hyphae growing out of the soft areas of the cuticle.

Crayfish plague is diagnosed by culture and identification of *A astaci*. This may be difficult to achieve in contaminated samples as the fungus does not compete well against contaminating microflora. Crayfish that are still alive (if any are remaining), moribund and recently dead (within 12 hours) should be collected from the infected area and transported to the laboratory in containers that maintain the temperature between 7-25°C. The fungus does not survive temperatures outside this range.

No serological tests are available for diagnosis. A PCR with limited validation is available.

Crayfish plague is exotic to Australia and New Zealand.

Guidelines for emergency response to crayfish plague are available in AQUAVETPLAN. The OIE manual provides details on crayfish plague including diagnostic tools and techniques.

Part 1. Diagnostic Overview

Aetiology

The causative agent of crayfish plague is the aquatic fungus, *Aphanomyces astaci* Schikora.[1] The *Aphanomyces* genus comprises water moulds (Oomycetes) belonging to the family Saprolegniaceae [2] many of which are pathogens or saprophytes on fish, plants and animals. The Oomycetes are members of the Oomycotina and are not directly related to the true fungi, the Eumycota, but are more closely related to brown algae and diatoms.

The fungus is highly virulent and causes up to 100% mortality following a disease outbreak.[3,4]

A astaci is a non-septate, branching fungus with hyphae approximately 7-10 micron in thickness. Biflagellate zoospores are produced by asexual reproduction: a process typical of many of the Saprolegniaceae.

Four genetic groups have been identified by random amplification of polymorphic DNA (RAPD).[5,6] Group A comprises *A astaci* strains isolated from the European crayfish species *Astacus astacus* and *A leptodactylus*; Group B comprises strains isolated from crayfish species that originated in California, *Pacifastacus leniusculus*; Group C consists of strains from *P leniusculus* of Canadian origin, and Group D comprises strains isolated from *Procambarus clarkii*. Group A-C strains are from crayfish of cold-water origin (4-21°C) and Group D are strains that originated from subtropical regions of the south east of the United States of America. The warmer climate strains are more adapted to growing at temperatures of 20-26°C. Virulence at 10°C, morphology and a number of physiological characteristics of the four groups are similar. At temperatures above 20°C strains in Group D are able to grow faster, and release zoospores, which means that it is virulent at higher temperatures compared with the cold water strains comprising groups A-C.[6]

Clinical Signs

The first sign of an infection is often massive stock losses of up to 100% in susceptible species and is indicative of crayfish plague. Death in only freshwater crayfish distinguishes the disease from other causes that result in the death of other aquatic species at the same location. Clinical signs may not be seen or can be non-specific, and include behavioural anomalies indicating neurotoxicity where animals fail to respond to external stimuli. Crayfish may be seen in daylight hours, can be caught easily, may have missing limbs, lack the tail flip response, or have an appearance of ‘walking on stilts' due to loss of limb coordination. Often these signs are seen only at the terminal stages of infection. Gross appearance can include whitening of the musculature beneath the thin areas of the cuticle particularly of the ventral abdomen and in the limb (periopod) joints (Figure 1).

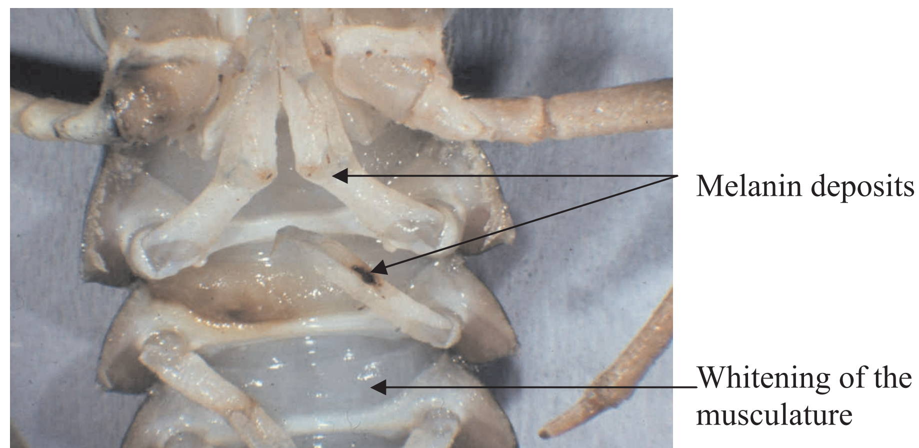

Figure 1. Infected white-claw crayfish showing areas of whitening of the musculature and melanin deposits

These areas may also show a brown melanisation, a result of the host's defence system against the hyphae (Figure 2). Melanisation is non-specific and reflects the crayfish immune response to infection from disease agents.

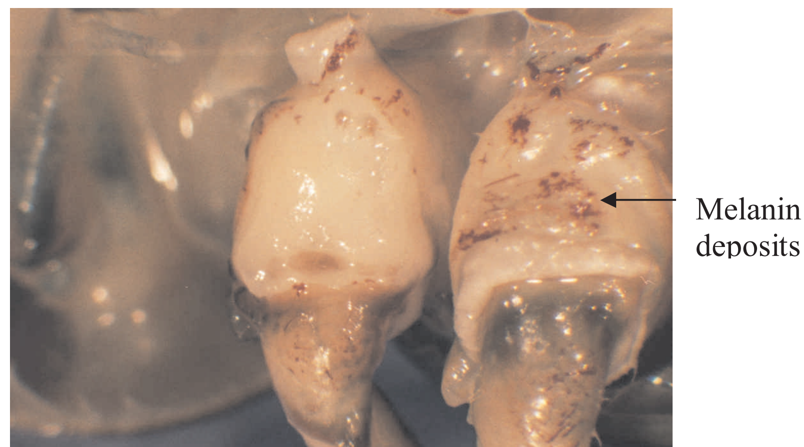

Figure 2. Melanin deposits on claws of white-clawed crayfish

Paralysis of the entire abdomen frequently occurs one to two days before death.[7-9] The gross appearance of a crayfish infected with porcelain disease or Thelohaniasis may appear to be similar to crayfish plague. The causative agent, *Thelohania contejeani* Henneguy, a microsporidian, invades the muscle fibres producing a 'porcelain whiteness' that can be seen through the exoskeleton.

Epidemiology

Freshwater crayfish from Europe, Britain, New Guinea, Japan and Australia are susceptible to the disease. Species include the Noble crayfish (*Astacus astacus*, Linnaeus), red swamp crayfish (*Procambarus clarkii* Girard), the stone crayfish or white-clawed crayfish (*Austropotamobius pallipes*, Lereboullet), *Austropotamobius torrentium*, and the slender clawed or Turkish crayfish (*Astacus leptodactylus*).[10] *Cambaroides japonicus* from Japan is susceptible to the disease [11] as is the *Cherax papuanus* from New Guinea.[12] Crayfish plague is exotic to Australia, but *Cherax*

species are highly susceptible under experimental conditions.[12] The Chinese crab (*Eriocheir sinensis* Milne Edwards), a pest in Europe, is mildly susceptible to artificially induced infection with *A astaci*.[13]

North American crayfish (*Pacifastacus leniusculus* Dana – signal crayfish; *Orconectes limosus* Rafinesque – mud crayfish or spinycheek crayfish; *Procambarus clarkii* – Louisiana swamp crayfish) can be asymptomatic carriers or resistant to the disease [14,16], but may die if their immune system is stressed.[17] These crayfish can act as vectors for the disease and are thought to be the source of a European outbreak when the crayfish were introduced into Europe via Italy around 1860 [18] and ten years later in France. The disease has spread to the Black Sea, Russia, Balkans, Germany, Finland, and in 1907 to Sweden. Outbreaks occurred in 1960s in Spain and then in the 1980s in Turkey, Greece, Norway and Britain.[10,19]

Molecular typing by random amplification of polymorphic DNA (RAPD) of 15 isolates of *A astaci* from Sweden, Turkey, USA and Canada indicated that the introductions of the North American crayfish into Europe were the likely sources of the European disease outbreaks.[5] Prior to Swedish outbreaks, rivers and waterways had been stocked with signal crayfish from North America. Four genetic groups have been identified. Group A represents isolates introduced into Europe in the 19th century. Group B isolates are from the more recent introductions in the 1960s with links to importation to Sweden of crayfish from Lake Tahoe in the USA. Group C contains a strain originating from Canada and imported into Sweden, and Group D contains isolates from Spain.[5, 6, 20]

Infection causes massive loss of stocks in rivers and waterways with up to 100% mortality.

Incubation period

The crayfish plague fungus can spread rapidly in a body of water and can eradicate a population of susceptible crayfish in a large lake within a month, or less, for smaller lakes and riverways.[20] The mean time of death for an infected animal is six days [14] but may be up to 15 days or longer depending on the infective dose.

Strategies used by the fungus for survival and maintenance

The fungus survives by producing zoospores that can swim and attach to another host. In the later stages of the infection, fungal hyphae protrude through the cuticle at the original entry site. Due to the lack of nutrients in the water body outside the host, the fungus is stimulated to produce spores, which line up within the hyphal tips. The spores cluster externally at the tip and encyst within the cluster. Each spore produces a flagellum and once motile the zoospores leave the host in search of another crayfish. The swimming spores will survive about three to seven days at 10°C or only a few minutes at 28-30°C before they must find a new host.[21,22] The swimming zoospores are attracted to scratches or damaged areas of the soft cuticle and will attach and encyst in this area. During encystment the flagellum is shed, a cell wall is formed and the fungus infects the new host. If encystment happens on non-crayfish substrate, germination does not occur (due to lack of appropriate nutrients such as $CaCl_2$) and the zoospores have the ability to re-emerge from the cysts and swim away to find a new host. This is termed zoospore re-emergence and is typical of *A astaci* and some other Saprolegniaceae.[23] A maximum of three generations of zoospores is possible. Zoospore production and mobility to find and attach to (chemotaxis) and thereby infect a new host by triggering germination determine virulence of the fungus.[24]

Chitinases and serine proteases (subtilisin and trypsin) are produced, which facilitate penetration of the fungus into the host. [25,30]

Methods of transmission
During a disease outbreak the zoospores move more quickly downstream with the currents, whereas movement upstream is limited. No known intermediate or secondary hosts have been detected for the fungus.[31] The disease is primarily transmitted by carrier crayfish, but damp contaminated equipment, such as boots, and fishing nets, can transport the disease. Zoospores can remain viable for a short time in the mucus of freshly caught fish.[10,32,33] Drying contaminated materials is effective, as *A astaci* is sensitive to desiccation. Sodium hypochlorite and iodophores are effective for disinfection of contaminated equipment.[34] Zoospores are non-viable after drying for 48 hours, freezing for two hours, or incubation at 30°C for 30 hours.[31]

Other Aphanomyces *strains present on freshwater crayfish*
A new strain, tentatively named *Aphanomyces repetans*, was isolated from crayfish of North American origin (*Procambarus clarkii*, *Pacifastacus leniusculus*) in Italy and Spain.[35] This strain did not cause disease in European species of crayfish normally susceptible to infection with *A astaci*. *A repetans* is similar to *A astaci* in possessing repeated zoospore emergence, lack of sexual reproduction, germination in response to nutrients, but unlike *A astaci* did not express chitinase during growth or sporulation. *A repetans* had a high similarity to *A laevis* based on internally transcribed spacer (ITS) regions, but differed to this organism, which does not undergo repeated zoospore emergence.[35] *A laevis* is a saprophyte but can be an opportunist in wound infections of crayfish.[36]

Occurrence and Distribution

Crayfish plague is exotic to Australia and New Zealand. A targeted surveillance program has existed in Western Australia since 1989 to meet requirements of countries importing *Cherax destructor*.

Gross Pathology

Very few signs are seen early in the disease process, and may depend on the infective dose. A whitening of the musculature beneath the thin areas of cuticle may occur, but this is more commonly seen in advanced infection or at death. Fungal hyphae are seen where penetration has occurred, which is usually at sites of injury. The non-chitinous proteolipid epicuticular membrane is the first line of defence for the crayfish as seen by the melanisation reaction along the fungal hyphae in this region. Penetration occurs in and on the exoskeleton in the soft areas of the joints and the segments of the abdomen. Involvement of the cuticle is limited and may be difficult to detect. The fungus grows along the ventral nerve cord and the brain ganglion from the area of penetration, and numerous blood cells and melanin can be seen around the hyphae. It may be seen in the eye, but rarely in other organs. Limited fungal growth occurs in the musculature due to the rapid melanisation of the hyphae.[8] In advanced infection, hyphae will grow out of the exoskeleton at the penetration points to form zoosporangia and produce zoospores ready to infect another host.
Invertebrate animals lack antibodies and therefore an immune system, but deal with infections using an innate immune response based on the prophenoloxidase-activating system.[37, 38]

Diagnostic Tests (General)

The diagnosis of crayfish plague based on clinical findings, is confirmed by the presence of non-septate hyphae *within* the cuticle of freshwater crayfish, typical morphology on culture, and the sporulation test.
Culture and identification of the fungus is the primary means of diagnosing crayfish plague. However, the finding of almost 100% mortality of infected freshwater crayfish is considered diagnostic. Examination for fungal hyphae in wet mounts prepared from the soft cuticle, and microscopic examination of formalin-fixed, paraffin-embedded sections using appropriate stains are useful. PCR tests have been published but have yet to undergo sufficient validation.
See flowchart of the methods for the diagnosis of crayfish plague, and sensitivity and specificity of methods in table 2 at the end of Part 2. Test methods.
OIE recommended tests and culture techniques are described in the Appendices and in the OIE Manual of Diagnostic Tests for Aquatic Animals 2006.

Transport of samples to the laboratory

The fungus is killed by exposure to drying and to temperatures below 7°C; therefore consideration needs to be given for appropriate packaging and transportation of animals and samples to the laboratory. Ideally, samples (whole animals) should be collected within 12 hours of death and transported to the laboratory at temperatures between 7-25°C. An insulated container can be used to transport samples. A cool brick with wads of paper to maintain separation from the samples can assist in maintaining the correct temperature range.

Culture and identification

Small pieces of material should be collected from areas where the infection is likely – the soft cuticle of the abdomen, connective tissue, the limb joints, gills, nerve cord, gut, the eye, the telson near the anus, and particularly areas that show melanisation. The samples should be washed three times in sterile distilled water to wash off as much contaminating material as possible, as *A astaci* does not compete well against contaminating microflora.
Small pieces (>2 mm^3) of sample are embedded into the moist surface of Isolation Medium (also referred to as RGY agar) and incubated at 15-24°C for seven days.[39] If there is gross bacterial contamination, hyphae are subcultured to a fresh plate to improve likelihood of uncontaminated culture. Colonies of *A astaci* are colourless and grow within the top millimetres of the agar surface. A colony will grow to 10 mm in diameter in 4-7 days.[39] No aerial hyphae are produced. Placing the tissue sample within a glass cylinder (0.5 cm in height, and 1 cm diameter) that has been embedded a few millimetres into the agar [40] or a sterile metal washer, may assist in allowing the fungus to grow through the agar and away from the bacterial contamination (Figure 3).

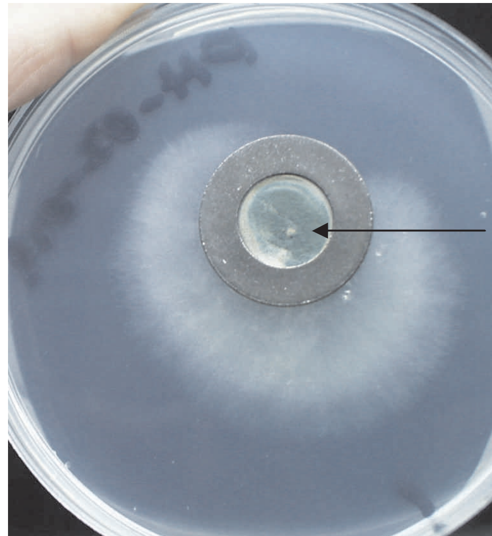

Figure 3. Growth of *Aphanomyces* (*A invadans*) demonstrating the use of a sterile washer to ensure growth of contaminating *Aspergillus* species is contained within the centre of the washer

Wet preparation

Small samples of tissues from appropriate collection sites are placed into a few drops of water on a glass slide and flattened under a coverslip. Examine for the presence of non-septate branching fungal hyphae approximately 7-10 micron in width (Figure 4).

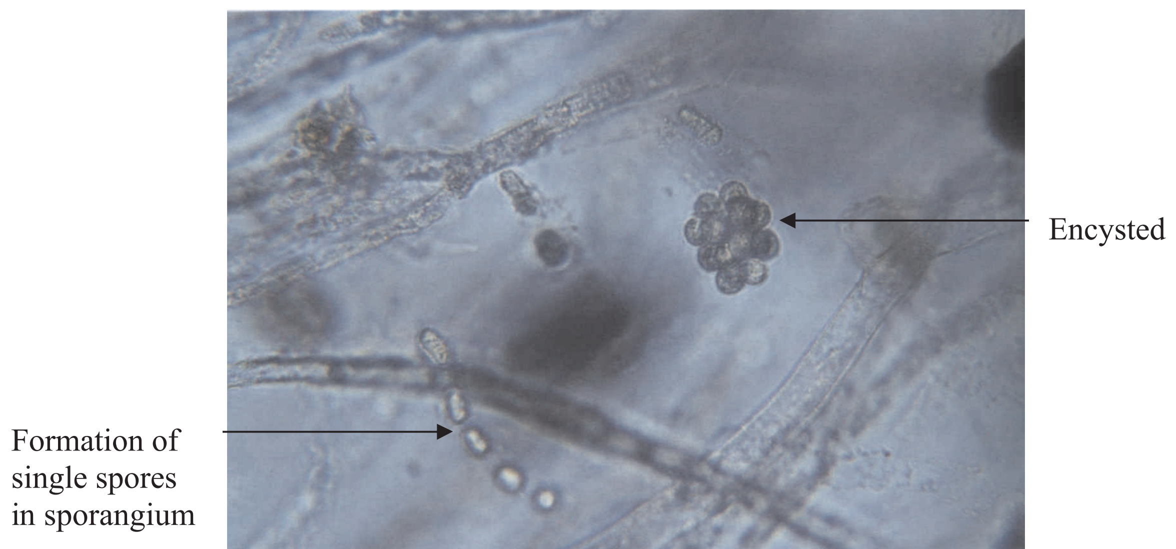

Figure 4. Fungal hyphae, formation of single spores in sporangium, and encysted spores in a wet preparation

The hyphae may be densely packed with coarse granular cytoplasm with highly refractive globules (young active hyphae), or vacuolate with cytoplasm restricted to the periphery (older hyphae) or entirely devoid of contents (very old hyphae).[39] Suspending soft cuticle in 10% KOH and leaving for 15 minutes may assist in 'clearing' some of the cuticle so that the hyphae are more easily seen within the cuticle.

Histopathology

The same types of samples collected for culture should also be collected for histopathology. Because the hyphae of *A astaci* are thin-walled they are rarely detected using the standard haematoxylin and eosin stain. A combined Grocott silver stain with haematoxylin and eosin used as a counter stain can improve the visualisation of the hyphae in the crayfish tissue (Figures 5 & 6).

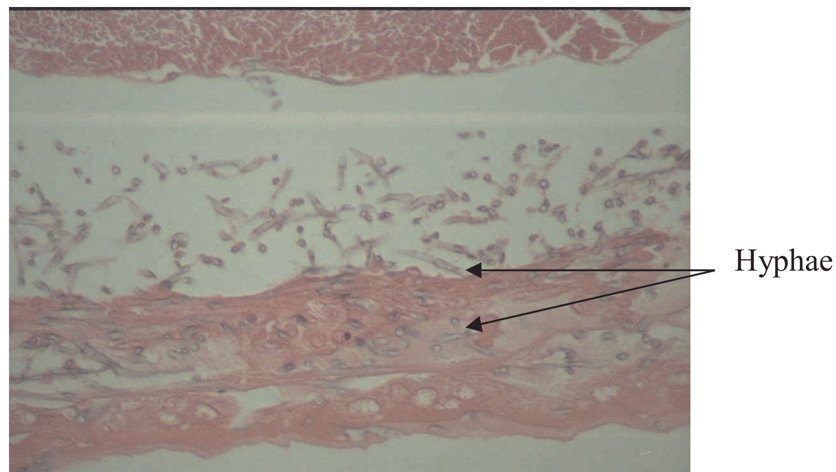

Figure 5. Grocott silver stain combined with a haematoxylin and eosin counter stain showing fungal hyphae in connective tissue

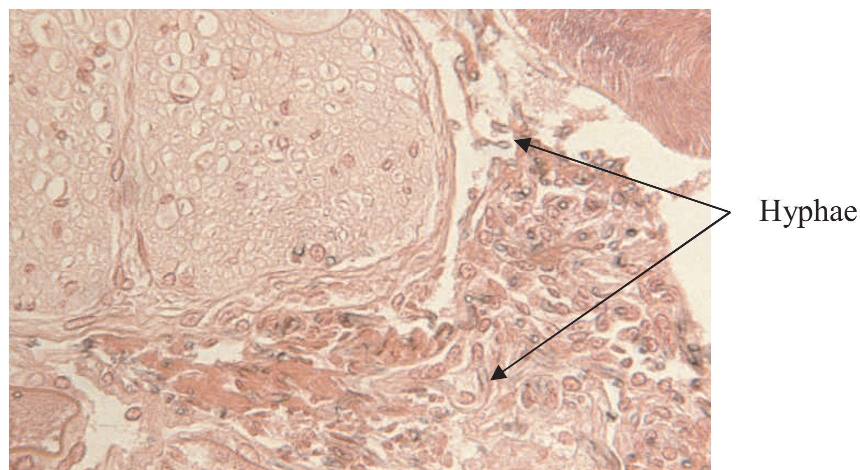

Figure 6. Grocott silver stain combined with a haematoxylin and eosin counter stain showing fungal hyphae in connective tissue around nerve cord

Grocott's modification of Gomori's hexamine silver method can also be used.[41, 42] Haemocytes surround and encapsulate the hyphae, which become melanised giving a knobbly appearance to the hyphae.

Serology
No serological tests exist for diagnosis of crayfish plague.

Nucleic acid detection
PCRs have been published for the detection of *A astaci* from culture material [43,44] but *A astaci* cross-reacted with *A invadans* and *A frigidophilus*. A second primer (42f) was designed which was specific for *A astaci* [45] and amplifies DNA from the internal

transcribed spacer region (ITS) and the 5.8 rRNA gene. The detection limit was 100 fg for DNA prepared from culture material, and amplicon was obtained from one spore. The PCR method was partially validated in the paper[45] and in Fisheries Research and Development Corporation (FRDC) project 2004/091, but further work needs to be done on diagnostic sensitivity and specificity. The advantage of the PCR published in 2004 using primers 525 and 640 was that a short amplicon of 115 bp was produced [43]. This was found to be very sensitive when used to detect *A astaci* in paraffin-embedded sections (FRDC 2004/091) but has the disadvantage of cross-reacting with *A invadans* and *A frigidophilus*, as previously stated. The amended PCR reported in 2006 was not tested on paraffin-embedded sections.[45] Generally, to obtain successful amplification from formalin-fixed, paraffin-embedded material a primer combination that generates a short amplicon is required. The recent PCR is described in the appendix to provide laboratories with a molecular detection method with the provision that it is used with caution. For a specific PCR for use on fresh tissue or cultured hyphae use the 42f and 640r primer combination, which generates an amplicon of 569 bp. This primer combination does not amplify *A astaci* DNA from paraffin-embedded material due to the large size of the amplicon. However, although cross-reaction with *A invadans* (weak amplification) and *A frigidophilus* occurs, DNA from formalin-fixed paraffin-embedded material can be amplified using the primer combination 525 and 640, which generates a short amplicon of 115 bp.

Part 2. Test Methods

Sporulation for the identification of A astaci

Principle of the test
The identification of Oomycetes to genus level is based on sporangial morphology, and to species level on the morphology of the sexual reproductive stages (oogonia and antheridia). The latter cannot be done in *Aphanomyces* as they reproduce asexually. Instead the process of sporulation, where spores are produced to discharge from the hyphal tip and encyst before producing motile spores (zoospores) that swim away, is used to identify *A astaci*.

Reagent and materials

Culture plate (IM medium) of actively growing fungus
Sterile scalpel blade or cover slip
Sterile Petri dish 9 mm
Distilled water
Incubator set to 20°C
Light microscope or stereomicroscope

Test procedure
Using a sterile scalpel blade (or sterile coverslip) cut a thin slice (2 cm x 2 cm x 2 mm) from the edge of an actively growing culture and place into sterile distilled water in a Petri dish. Some methods suggest using tap water, but the presence of ions may not be consistent between different geographical locations, therefore tap water can be used if it comes from the same geographical area where the suspect crayfish plague animals were collected. Sterile lake water that doesn't contain too high a salt content (optimal 150 mOsm/kg) [46] can be used or 1 mM $CaCl_2$.[40] Optimal pH is 5-7, and temperature 16-20°C. For strains from Spain and warmer climates, a temperature of up to 24°C may be required. Sporulation begins after 4-8 hours and is completed in 10-25 hours, and up to 30 hours at 16°C.
The process begins with the transformation of a long piece of hyphae into a sporangium, which is delineated by a highly refractive septum. Primary spores (cytoplasmic units) are formed from the sporangium's contents (Figure 7) and are released (within 5 minutes) from the sporangial discharge tube and accumulate at this point.

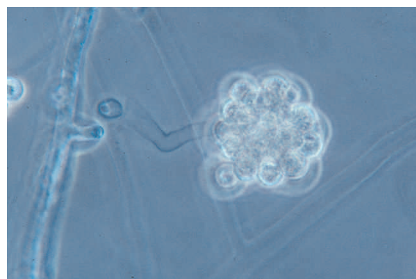

Figure 7. Primary spores encysted at sporangial tip (wet preparation)

The second stage is encystment where the spores become round and a cyst wall develops (Figures 4 & 7). Most spores remain as a cluster (15-30 spores) at the sporangial tip, but some encyst away from the sporangial tip. The number of spores in a cluster is generally less than that of other *Aphanomyces* species. The clusters are adherent and fairly resistant to physical disturbance, and spores will remain encysted for 8-12 hours.
The third stage is the formation of a flagellum to become a reniform shaped zoospore (motile spore) of 8 x 12 micron. Motility takes 5-20 minutes to develop and the zoospores emerge from the cysts and swim away leaving the empty capsules of the encysted spores.[13, 39, 47] A stereomicroscope, or light microscope that has a stage that can accommodate a Petri dish, is used to view the sporulation process.

Quality control aspects
A astaci is exotic to Australia; therefore, a positive control cannot be used. All three stages must be observed.

Interpretation of results
Subtle differences in sporangial types and release patterns are observed between the genera and species of the Saprolegniaceae, but may only be recognised by an experienced mycologist. Genera include *Saprolegnia*, *Leptolegnia*, *Achlya*, *Thraustotheca*, and *Aphanomyces*. However, the results of the sporulation test combined with typical colourless, flat growth on IM medium and huge stock losses should be sufficient to diagnose *A astaci*.

Flowchart for diagnosis of crayfish plague

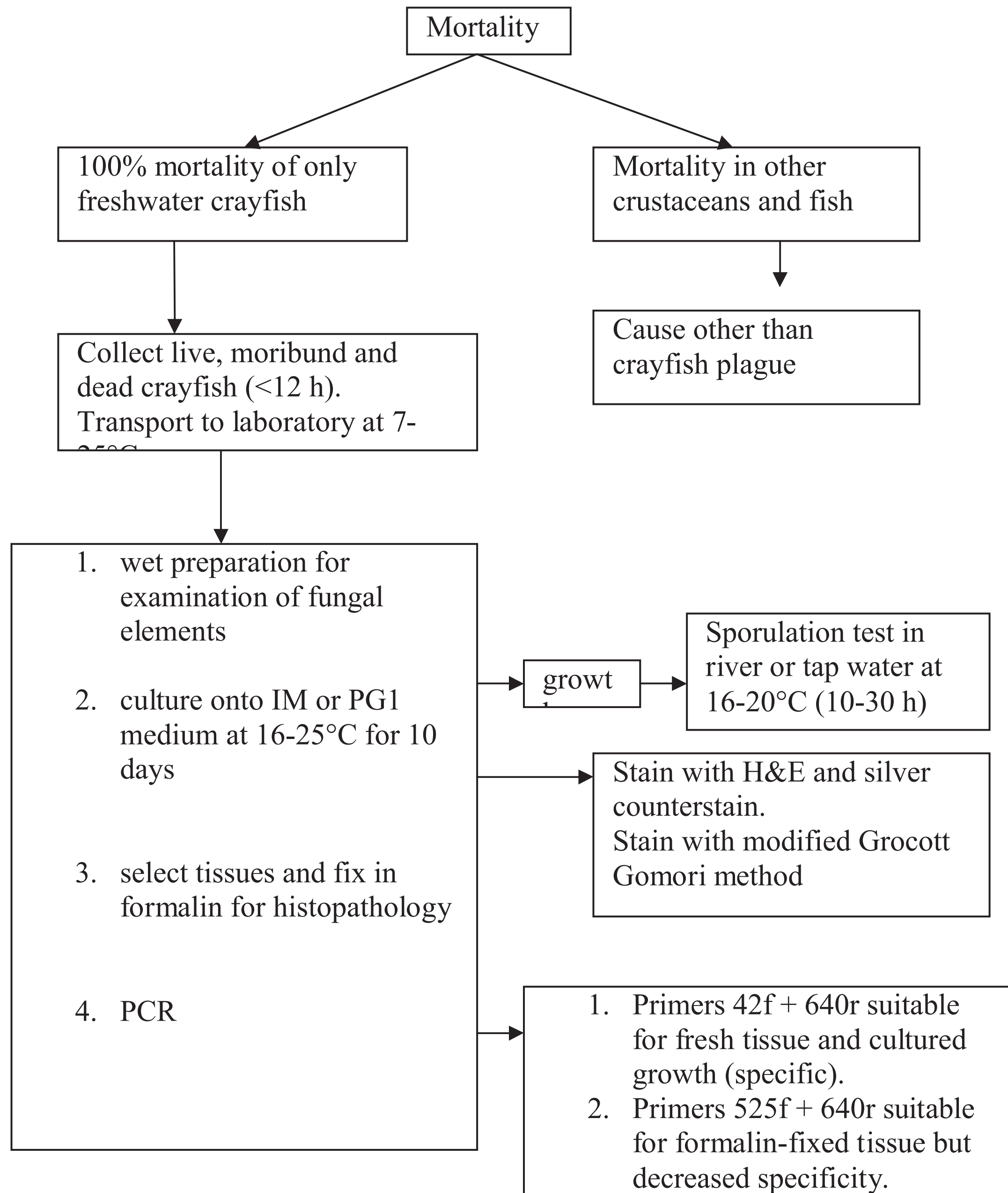

Table2. Sensitivity and specificity of diagnostic test methods

Test Method	**Sensitivity**	**Specificity**
Wet preparation	M	L
Culture	M	L
Sporulation	H	H
Histopathology	M	L
PCR using Primers 42f + 640r on fresh tissue and cultured growth. Not suitable for paraffin-embedded sections	H	H
Primers 525f + 640r on formalin-fixed tissue.	H	M (weak cross-reaction against *A invadans* and *A frigidophilus*)

H = High; L = low; M = medium

Appendix

Aphanomyces astaci Isolation Medium (IM)

Reagents

Oxoid Agar No1	7.2 g
Difco Yeast Extract	0.6 g
Glucose	3.0 g
Sterile (autoclaved) Tap Water	600 mL
Oxolinic Acid	6.0 mg
Penicillin G	600 mg

Suspend agar, yeast extract and glucose in water in a 1 L Schott bottle with a magnetic stir-bar. Autoclave at 121°C for 15 min then cool to 50°C in a waterbath. Add oxolinic acid and penicillin to 10 mL of distilled water and filter through a sterile 0.22 μm filter into a sterile McCartney container. Add filtered antibiotics to the cooled medium while stirring. Pour media into plates to an approximate depth of 3 mm, and then gently swirl plate to obtain complete coverage. Label “IM” and store at 4°C.
Note: **Media can be prepared in sterile (autoclaved) tap water, sterile river water, or sterile distilled water. Any chlorine in the tap water is driven off in the autoclave.**

Quality Control

A astaci is exotic to Australia, but quality control for fungal growth can be tested using *Aphanomyces invadans*. Other quality control, checks such as plate sterility and pH, should be tested according to NATA requirements.

Aphanomyces astaci PG1 medium (Basal medium #2) [48]

Reagents

$MgCl_2.6H_2O$	0.165 g
$CaCl_2.2H_2O$	0.147 g
KCl	0.373 g
$FeCl_3.6H_2O$	0.020 g
0.2 M EDTA	1.5 mL
(ethylene diamino tetraacetic acid (EDTA neutralised with solid NaOH))	
peptone	0.3%
glucose	0.6%
phosphate buffer	1/75 mol
trace element stock solution	1 mL
pH	6.0-7.0

Trace element stock solution

$CuSO_4.5H_2O$	0.25 g
H_3BO_3	0.286 g
$MnCl_2.4H_2O$	0.181 g
$ZnSO_4.7H_2O$	0.022 g
$H_2MoO_4.4H_2O$	0.009 g
Water	1 L

Phosphate buffer $NaH_2PO_4.H_2O + Na_2HPO_4.2H_2O$.

The phosphate buffer (5/3 x final concentration) is autoclaved in the flasks and the rest of the basal medium (10 x conc) is autoclaved separately and added aseptically to the flasks.

If using PG1 medium grow cultures at 20-25°C with shaking.

Storage of cultures

Aphanomyces cannot be freeze-dried or placed at –80°C for long-term storage as this treatment kills the fungus. Cultures are maintained on IM medium prepared as sloped agar in 20 mL sterile plastic McCartney containers (or "universal" containers). A small plug of an actively growing culture is placed into the centre of the slope and incubated at 16-22 °C until the growth almost reaches the sides of the container. The container is then filled with sterile paraffin or mineral oil and the culture stored in the dark at 7°C. Cultures must be subcultured every six months, or every three to four months if stored at 15-18°C (D Alderman, personal communication, 2003). Mineral oil appears to lower the metabolic activity of the organism.[48]

Nucleic acid detection of A astaci [45]

Principle of the test

Primers (42f and 640r) were developed that specifically amplify *A astaci* DNA extracted from culture material and from soft cuticle infected with the fungus.[45] A 569 bp product was obtained that was shown to be specific when tested against a total of 31 isolates representing strains of *Aphanomyces*, *Saprolegnia*, other Oomycetes, crayfish parasites, bacterial species and other fungal species. These primers generate a product that is too large for amplification from formalin-fixed, paraffin-embedded tissue. Primers (525f and 640r) previously designed [43], but shown to produce weak product from *A invadans* and *A frigidophilus*, produced a short amplicon of 115 bp that successfully amplified DNA from paraffin-embedded sections (FRDC 2004/091).

Reagents and materials

Liquid nitrogen
Filter paper
Cold mortar and pestle
DNeasy tissue kit (Qiagen)
Primers 42f & 640r
Reddy Mix PCR Mastermix (Abgene AB-0575)
Microfuge tubes
Microfuge
Thermocycler
Electrophoresis equipment
Ethidium bromide solution
UV transilluminator and gel documentation system

Test procedure

DNA extraction from culture mycelium

The fungus is grown in a broth culture using PG1 medium incubated at 20-25°C with shaking. After three days the mycelium is removed and drained onto filter paper. Once the moisture is removed the mycelium is placed into a cold mortar containing liquid nitrogen and ground to a powder using a cold pestle. The powder is transferred to a 50 mL centrifuge tube and resuspended in an equal weight/volume ratio of lysis buffer (9 mM Tris-HCl, 0.9 mM EDTA, pH 8.0; 2.0% SDS). To 10 mL of the suspension is added 250 µL of Proteinase K (18 mg mL^{-1}, Boehringer Mannheim) and incubated at 56°C for 2 hours. DNA is extracted with phenol, followed by phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1). One volume of isopropanol is added to the aqueous supernatant for DNA precipitation. The DNA is redissolved in 10 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA).[43]
DNAzol reagent (Invitrogen, Life Technologies), and DNeasy plant mini kit (Qiagen) successfully extracted DNA from *Aphanomyces invadans* mycelium grown in broth culture [49] (FRDC project 2001/621) and could be used instead of the phenol/chloroform method. The DNeasy tissue kit (Qiagen) was used for the extraction of *A astaci* DNA from crayfish cuticle[45] therefore it is highly likely it would be successful for mycelium alone. Many of the commonly used kit methods to extract DNA from True fungi are incapable of extracting DNA from the Oomycetes (FRDC 2001/621). Oomycetes contain cellulose in their cell walls as opposed to chitin found in True fungi.

DNA extraction from crayfish cuticle

The cuticle is cleaned of as much contaminating material as possible using cotton swabs moistened with sterile distilled water. Cuticle (30-50 g) is excised using flame-

sterilised scissors and forceps. Any attached muscle or connective tissue is removed before the cuticle is ground in liquid nitrogen. DNA is extracted from the ground material using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. An additional centrifugation is done after digestion and before the DNA loading onto the column. Only fluid supernatant is transferred to the extraction column as any undigested cuticle can cause blockage of the column.[45]

Primers

Table 1. Primers used for amplification of *A astaci*

Primer name	Sequence 5'-3'
42f	GCT TGT GCT GAG GAT GTT CT
640r	CTA TCC GAC TCC GCA TTC TG

f = forward primer. r = reverse primer

Primer 525f = 5' – AAG AAG GCT AAA TTG CGG TA – 3'. This primer combined with 640r produces a short amplicon of 115 bp that successfully amplifies DNA from formalin-fixed, paraffin-embedded sections. However, is not as specific as the primer combination of 42f and 640r.

Thermocycling protocol

A 50 µL reaction volume was used, which contained Reddy Mix PCR Mastermix (Abgene AB-0575 with 1.5 mM $MgCl_2$) and primers 42f and 640r at a final concentration of 0.5 µM each.

DNA was denatured at 96°C for 5 min, followed by 50 cycles of 96°C for 1 min, 59°C for 1 min, 72°C for 1 min, with a final cycle containing the extension step at 72°C for 7 min.

Gel electrophoresis

Six microlitres of amplified product plus 6 µL of 2 x loading buffer is placed into the wells of a 1.3% agarose gel. A 100 bp molecular weight marker is included in the first and final wells. Electrophoresis is carried out at 100 volts for 45 minutes. The gel is immersed in a solution of ethidium bromide (50 µL per 1 litre) for 20-40 min. Amplified DNA is visualised using a UV transilluminator. The expected product size is 569 bp.

Quality control aspects

A positive control of *A astaci* DNA, and a DNA-free control should be used for each PCR test. *A astaci* is exotic to Australia, but DNA is available in a limited supply from Animal Health Laboratories, Department of Agriculture Western Australia.

Interpretation of results

Primers 42f & 640r give a product of 569 bp. All four RAPD groups of *A astaci* have been tested and all produce the same product size. If final confirmation is needed the amplified product can be sequenced.

Primers 525f and 640r give a product of 115 bp, which is suitable for amplification of DNA from formalin-fixed, paraffin-embedded tissue, but weak reactions may occur with *A invadans* and *A frigidophilus*. If no other material is available, then these primers could be used, but the interpretation meeds to be treated with caution. *A invadans* has never been reported from freshwater crayfish, but *A frigidophilus* has

recently been isolated from dead freshwater crayfish, possibly as a secondary invader.[50]

Sensitivity
For the analytical sensitivity the lowest level of detection was 100 fg of DNA. Tests done with spores showed that the PCR could detect a single spore.
The samples that gave the greatest number of positive reactions in infected crayfish were those collected from the soft abdominal areas or the telson, compared with samples taken from proximal walking leg, dorsal abdomen, cephalothorax and eyestalk. Differences occurred between samples taken from different species of crayfish tested. Soft abdominal cuticle gave a greater number of positives for *Orconectes limosus*, whereas the telson produced a greater number of positives in *Pacifastacus leniusculus*. For the diagnostic sensitivity 139 animals were tested and 75 of these were diagnosed as being infected. All animals diagnosed as being infected tested positive by PCR when samples from both the soft abdominal cuticle and the telson were tested. If only one sample was taken from one site the number of positives decreased to 48 and 55 for abdominal and telson, respectively. It is recommended that both telson and soft abdominal cuticle should be collected from each animal.[45]

Specificity
For primers 42f and 640r, no cross-reactions occurred in the testing of 31 species from fungi, bacteria and parasites. Oomycetes that were tested included, *Aphanomyces brassicae, A cladogamus, A cochloides*, *A euteiches*, *A frigidophilus*, *helicoides*, *A invadans* (seven strains), *A irregulare*, *A iridis*, *A laevis*, *A stellatus*, *Saprolegnia declina*, *S ferax*, *S furkata*, *S litoralis*, *S* parasitica, *S terrestris*, *Leptolegnia*, *Pythium flevoense*, *Isoachlya turoloides*, and *I eccentrica*. Bacterial species likely to be contaminants on crayfish tissue (*Aeromonas hydrophila*, *Citrobacter freundii*, *Hafnia alvei*) were all negative. Crayfish parasites *Psorospermium haeckeli* and *Thelohania contejeani* were negative. Also negative were some true fungi that can be contaminants on crayfish tissue or laboratory contaminants: *Fusarium solani*, *Mucor* species, *Aspergillus* species, *Trichosporan beigelii* and the yeast *Candida albicans*. No reaction with the primers occurred for crayfish muscle tissue from *Orconectes limosus*, *Pacifastacus leniusculus* and *Procambarus clarkii*.
Primers 525f and 640r produce weak amplicon from *A invadans* and *A frigidophilus*.

Acknowledgment

Photographs 1, 2, 3, 4, 6, and 7 courtesy Dr David Alderman, CEFAS, UK.

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