CHAPTER 2.2.2.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci* of the Family Leptolegniaceae, Phylum Oomycota (water moulds). The disease is commonly known as crayfish plague.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Aphanomyces astaci is a member of a group of organisms commonly known as the water moulds. Although long regarded to be fungi, this group, the Oomycetida or oomycota, are now considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista. Chromista are a eukaryotic supergroup, probably polyphyletic, which may be treated as a separate kingdom or included among the Protista.

Four groups (A–D) of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) (Huang et al., 1994; Diéguez Uribeondo et al., 1995): Group A (the so called *Astacus* strains) comprises a number of strains that were isolated from *Astacus astacus* and *Astacus leptodactylus*; these strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus* strains I) includes isolates from both *A. astacus* in Sweden and *Pacifastacus leniusculus* from Lake Tahoe, USA. Imported to Europe, *P. leniusculus* have probably introduced *A. astaci* and infected the native *A. astacus* in Europe. Group C (*Pacifastacus* strains II) consists of a strain isolated from *P. leniusculus* from Pitt Lake, Canada. Another strain (Pc), isolated from *Procambarus clarkii* in Spain, sits in group D (*Procambarus* strain). This strain shows temperature/growth curves with higher optimum temperatures compared with isolates from northern Europe (Diéguez Uribeondo et al., 1995). *Aphanomyces astaci* strains that have been present in Europe for many years (group A strains) appear to be less pathogenic than strains introduced more recently with crayfish imports from North America since the 1960s.

2.1.2. Survival outside the host

Aphanomyces astaci zoospores remain motile for up to 3 days and cysts survive for 2 weeks in distilled water (Svensson & Unestam, 1975; Unestam, 1966). As *A. astaci* can go through three cycles of zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension kept at 2°C for 2 months (Unestam, 1966).

2.1.3. Stability of the agent (effective inactivation methods)

Aphanomyces astaci, both in culture and in infected crayfish, is killed by a short exposure to temperatures of 60°C or to temperatures of –20°C (or below) for 48 hours (or more) (Alderman, 2000; Oidtmann et al., 2002). Sodium hypochlorite and iodophors are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection, since organic matter was found to decrease the effectiveness of iodophors (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation.

2.1.4. Life cycle

The life cycle of *A. astaci* is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores. These initially encyst, but then release a biflagellate zoospore (secondary zoospore). Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. Free-swimming zoospores appear to be chemotactically attracted to crayfish cuticle (Cerenius &

Söderhäll, 1984a) and often settle on the cuticle near a wound (Nyhlén & Unestam, 1980). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infective viability (Cerenius & Söderhäll, 1984b). Growth and sporulation capacity is strain-and temperature-dependent (Diéguez Uribeondo et al., 1995).

2.2. Host factors

2.2.1. Susceptible host species

To date, all species of freshwater crayfish have to be considered as susceptible to infection with *A. astaci*. The outcome of an infection varies depending on species. All stages of European crayfish species, including the Noble crayfish (*Astacus astacus*) of north-west Europe, the white clawed crayfish (*Austropotamobius pallipes*) of south-west and west Europe, the related *Austropotamobius torrentium* (mountain streams of south-west Europe) and the slender clawed or Turkish crayfish (*Astacus leptodactylus*) of eastern Europe and Asia Minor are highly susceptible (Alderman, 1996; Alderman et al., 1984; Rahe & Soylu, 1989; Unestam, 1969b; Unestam, 1976; Unestam & Weiss, 1970). Laboratory challenges have demonstrated that Australian species of crayfish are also highly susceptible (Unestam, 1976). North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Orconectes* spp. are infected by *A. astaci*, but under normal conditions the infection does not cause clinical disease or death. All North American crayfish species investigated to date have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (Oidtmann et al., 2006; Unestam, 1969b; Unestam & Weiss, 1970) and it is therefore currently assumed that this is the case for any other North American species.

The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) (Benisch, 1940).

2.2.2. Susceptible stages of the host

All live stages need to be considered as susceptible to infection.

2.2.3. Species or subpopulation predilection (probability of detection)

The host species susceptible to infection with *A. astaci* fall largely into two categories: those highly susceptible to infection with development of clinical disease and mortalities, and those which are infected without associated clinical disease or mortalities.

Highly susceptible species

*C*linical disease outbreaks caused by infection with *A. astaci* are generally known as ‘crayfish plague’ outbreaks. In such outbreaks, moribund and dead crayfish of a range of sizes (and therefore ages) can be found.

North American crayfish species

The prevalence of infection tends to be lower in animals that have gone through a recent moult (B. Oidtmann, unpublished data). However, through studies have not been undertaken to corroborate these observations. Juvenile crayfish go through several moults per year, whereas adult crayfish usually moult at least once per year in temperate climates. Therefore, animals in which the last moult was some time ago may show higher prevalence compared with animals that have recently moulted.

2.2.4. Target organs and infected tissue

The tissue that becomes initially infected is the exoskeleton cuticle. Soft cuticle, as is found on the ventral abdomen and around joints, is preferentially affected. In the highly susceptible European crayfish species, the pathogen often manages to penetrate the basal lamina located underneath the epidermis cell layer. From there, *A. astaci* spreads throughout the body primarily by invading connective tissue and haemal sinuses; however, all tissues may be affected.

In North American crayfish species, infection is usually restricted to the cuticle. Based on PCR results, the tailfan (consisting of uropds and telson) and soft abdominal cuticle appear to be frequently infected (Oidtmann et al., 2006; Vrålstad et al., 2011).

2.2.5. Persistent infection

A number of North American crayfish species have been shown to be infected with *A. astaci* (Oidtmann et al., 2006; Unestam, 1969a; Unestam & Söderhäll, 1977). Infected naturalised or aquaculture-reared North American crayfish populations usually do not develop clinical disease or increased mortalities (Oidtmann et al., 2006; Strand et al., 2011; Strand et al., 2012).

Based on the observations made in North American crayfish species, it seems reasonable to assume that all crayfish species native to the North American continent can be infected with *A. astaci* without development of clinical disease and they may therefore act as lifelong carriers of the pathogen.

A recent report from Finland also suggests that noble crayfish populations in cold water environments may be chronically infected at low prevalence (Viljamaa-Dirks et al., 2011).

2.2.6. Vectors

Finfish movement may facilitate the spread of *A. astaci* in a number of ways, such as through the presence of spores in the transport water, co-transport of infected crayfish specimens, or a combination of both (Alderman et al., 1987; Oidtmann et al., 2002). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.) (Alderman et al., 1987).

2.3. Disease pattern

2.3.1. Transmission mechanisms

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, as may occur during movements of finfish, or 3) through colonisation of habitats by North American crayfish species.

Transmission from crayfish to crayfish occurs through the release of zoospores from an infected animal and attachment of the zoospores to naïve crayfish. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (Cerenius & Söderhäll, 1984a).

The main route of spread of *A. astaci* in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms (Alderman, 1996; Dehus et al., 1999). Spread now mainly occurs through expanding populations of North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (Edsman, 2004).

Colonisation of habitats, initially occupied by highly susceptible species, by North American crayfish species carrying *A. astaci* is likely to result in an epizootic among the highly susceptible animals. The rate of spread will depend, among other factors, on the prevalence of infection in the population of North American crayfish.

Finfish transports may facilitate the spread of *A. astaci* in a number of ways, such as through the presence of spores in the transport water, co-transport of infected crayfish specimens, or a combination of both (Alderman et al., 1987; Oidtmann et al., 2002). There is also circumstantial evidence of spread by contaminated equipment (nets, boots clothing, etc.) (Alderman et al., 1987).

2.3.2. Prevalence

In the highly susceptible European crayfish species, exposure to *A. astaci* spores usually leads to infection and eventually to death. The minimum infectious dose has still not been established, but it may be as low as a single spore per animal (B. Oidtmann, unpublished data). Prevalence of infection within a population in the early stage of an outbreak may be low (a few animals in a river population may be affected). However, the pathogen is amplified in affected animals and subsequently released into the water; usually leading to 100% mortality in a contiguous population. The rate of spread from initially affected animals depends on several factors, one being water temperature. Therefore, the time from first introduction of the pathogen into a population to noticeable crayfish mortalities can vary greatly and may range from a few weeks to months. Prevalence of infection will gradually increase over this time and usually reach 100%. Data from a Noble crayfish population in Finland that experienced an acute mortality event due to infection with *A. astaci* in 2001 suggest that in sparse Noble crayfish populations, spread of disease throughout the host population may take several years (Viljamaa-Dirks et al., 2011).

Prevalence levels in North American crayfish appear to vary greatly. Limited studies suggest prevalence levels ranging from anywhere between 0 and 100% are possible (Oidtmann et al., 2006).

2.3.3. Geographical distribution

In Europe reports of large mortalities of crayfish go back to 1860 in Italy (Ninni, 1865; Seligo, 1895). These were followed by further reports of crayfish mortalities, where no other aquatic species were affected, in the Franco-German border region in the third quarter of the 19th century. From there a steady spread of infection occurred, principally in two directions: down the Danube into the Balkans and towards the Black Sea, and across the North German plain into Russia and from there south to the Black Sea and north-west to Finland and, in 1907, to Sweden. In the 1960s, the first outbreaks in Spain were reported, and in the 1980s further extensions of infection to the British Isles, Turkey, Greece and Norway followed (Alderman, 1996). The reservoir of the original infections in the 19th century was never established; *Orconectes* spp. were not known to have been introduced into Europe until the 1890s, but the post-1960s extensions are largely linked to more recent introductions of North American crayfish farming (Alderman, 1996). Escapes of the introduced species occurred and *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

Australia and New Zealand have never experienced any outbreaks of infection with *A. astaci* and are currently considered free of the disease (OIE WAHID website, accessed June 2011).

2.3.4. Mortality and morbidity

When the infection first reaches a naïve population of highly susceptible crayfish species, high levels of mortality are usually observed within a short space of time, so that in areas with high crayfish densities the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread quickly from the initial outbreak site downstream, whereas upstream spread is slower. Where population densities of susceptible crayfish are low fewer zoospores will be produced, the spread of infection will be slower and evidence of mortality less dramatic. Water temperature has some effect on the speed of spread and this is most evident in low-density crayfish populations where animal-to-animal spread takes longer and challenge intensity will be lower. Lower water temperatures and reduced numbers of zoospores are associated with slower mortalities and a greater range of clinical signs in affected animals (Alderman et al., 1987). Observations from Finland suggest that at low water temperatures, noble crayfish can be infected for several months without the development of noticeable mortalities (S. Viljamaa-Dirks, unpublished data).

On rare occasions, single specimens of the highly susceptible species have been found after a wave of infection with *A. astaci* has gone through a river or lake. This is most likely to be due to lack of exposure of these animals during an outbreak (animals may have been present in a tributary of a river/lake or in a part of the affected river/lake that was not reached by spores, or crayfish may have stayed in burrows during the epizootic). However, low-virulent strains of *A. astaci* have been described to persist in a water way, kept alive by a weak infection in the remnant population (Viljamaa-Dirks et al., 2011). Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now heavily diminished (Alderman, 1996; Souty-Grosset et al., 2006). Populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with infection, new outbreaks of infection with *A. astaci* and large-scale mortalities will occur.

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows slightly varies depending on the strain. In a study, which compared a number of *A. astaci* strains that had been isolated from a variety of crayfish species, mycelial growth was observed between 4 and 29.5°C, with the strain isolated from *Procambarus clarkii* growing better at higher temperatures compared to the other strains. Sporulation efficiency was similarly high for all strains tested between 4 and 20°C, but it was clearly reduced for the non-*P. clarkii* strains at 25°C and absent at 27°C. In contrast, sporulation still occurred in the *P. clarkii* strain at 27°C. The proportion of motile zoospores (out of all zoospores observed in a zoospore suspension) was almost 100% at temperatures ranging from 4–18°C, reduced to about 60% at 20°C and about 20% at 25°C in all but the *P. clarkii* strain. In the *P. clarkii* strain, 80% of the zoospores were still motile at 25°C, but no motile spores were found at 27°C (Diéguez Uribeondo et al., 1995).

Field observations show that outbreaks of infection with *A. astaci* occur at a wide temperature range, and at least in the temperature range from 4–20°C. The rate of spread within a population depends on several factors, including water temperature. In a temperature range between 4 and 16°C, the speed of an epizootic is enhanced by higher water temperatures. At low water temperatures, the epizootic curve can increase very slowly and the period during which mortalities are observed can be several months (B. Oidtmann, unpublished data).

In buffered, redistilled water, sporulation occurs between pH 5 and 8, with the optimal range being pH 5–7. The optimal pH range for swimming of zoospores appears to be in a pH range from 6.–7.5, with a maximum range between pH 4.5 and 9.0 (Unestam, 1966).

Zoospore emergence is influenced by the presence of certain salts in the water. $CaCl_2$ stimulates zoospore emergence from primary cysts, whereas $MgCl_2$ has an inhibitory effect. In general, zoospore emergence is triggered by transferring the vegetative mycelium into a medium where nutrients are absent or low in concentration (Cerenius & Söderhäll, 1984b).

2.4. Control and prevention

Once *A. astaci* has been introduced into a population of highly susceptible crayfish species in the wild, the spread within the affected population cannot be controlled. Therefore, prevention of introduction is essential. To avoid the main pathways of introduction, the following measures are necessary:

4. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species should be prevented.

5. When transfers of finfish are being planned, the water source should be assessed for the likelihood that itmay harbour infected crayfish (including North American carrier crayfish).

6. Any movements of finfish from the site of a current epizootic of infection with *A. astaci* carries a high risk of spread and should generally be avoided.

7. If movements of finfish from a source containing North American crayfish are being planned, fish harvest methods at the source site need to ensure that: a) crayfish are not accidentally co-transported; b) the transport water does not carry *A. astaci* spores, and, c) equipment is disinfected between use; d) the consignment does not become contaminated during transport.

8. The release of North American crayfish into the wild in areas where any of the highly susceptible species are present should be prevented. Once released, North American crayfish tend to spread, sometimes over long distances. If release into the wild in planned then a risk assessment should be conducted to estimate the long-term potential consequences of such a release. Highly susceptible crayfish populations at a distance from the release site may eventually be affected.

9. Biosecurity measures to completely guarantee that crayfish do not escape from aquaculture facilities are extremely difficult to implement. Therefore, a risk assessment needs to be conducted to determine whether these facilities should be established.

Certain pathways of introduction, such as the release of North American crayfish by private individuals are difficult to control.

2.4.1. Vaccination

Currently, there is no evidence that vaccines offer long-term protection in crustaceans and even if this were not to be the case, vaccination of natural populations of crayfish is not practical.

2.4.2. Chemotherapy

No treatments are currently known that can successfully treat the highly susceptible crayfish species, once infected.

2.4.3. Immunostimulation

No immunostimulants are currently known that can successfully protect the highly susceptible crayfish species against infection and consequent disease due to *A. astaci* infection.

2.4.4. Breeding for resistance

In the 125 years since infection with *A. astaci* first occurred in Europe, there is little evidence of resistant populations of European crayfish. However, the fact that North American crayfish generally do not develop clinical disease suggests that selection for resistance may be possible and laboratory studies using *A. astaci*

strains attenuated for virulence might be successful. However, there are currently no published data from such studies.

2.4.5. Restocking with resistant species

North American crayfish have been used in various European countries to replace the lost stocks of native crayfish. However, as North American crayfish is considered a susceptible species for *A. astaci*, restocking with North American crayfish may provide a reservoir of *A. astaci*. This would minimise the chances for success of re-introduction of indigenous species. A risk assessment should be conducted to assist in decisions on restocking.

2.4.6. Blocking agents

No data available.

2.4.7. Disinfection of eggs and larvae

Limited information is available on the susceptibility of crayfish eggs to infection with *A. astaci*. Unestam & Söderhäll mention that they experimentally exposed *Astacus astacus* and *P. leniusculus* eggs to zoospore suspensions and were unable to induce infection (Unestam & Söderhäll, 1977). However, the details of these studies have not been published.

Although published data are lacking, disinfection of larvae, once infected, is unlikely to be successful, since *A. astaci* would be protected from disinfection by the crayfish cuticle, in which it would be present.

2.4.8. General husbandry practices

If a crayfish farm for highly susceptible species is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or whether North American crayfish populations may be present upstream (for sites that are “online” on a stream or abstracting water from a stream), even if at a great distance upstream. If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

In an endemic area, where the highly susceptible species are being farmed, the following biosecurity recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

1. General biosecurity measures should be in place (e.g. controlled access to premises; disinfection of boots when entering the site; investigation of mortalities if they occur; introduction of live animals (crayfish, finfish) only from sources known to be free from infection with *A. astaci*).

2. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species must be prevented.

3. If transfers of finfish or crayfish are being planned, these must not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of infection with *A. astaci* or North American carrier crayfish).

4. North American crayfish must not be brought onto the site.

5. Finfish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of infection with *A. astaci* may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment that will kill *A. astaci* (see Section 2.1.3).

6. Any equipment that is brought onto site should be disinfected.

3. Sampling

3.1. Selection of individual specimens

In the case of a suspected outbreak of infection with *A. astaci* in a population of highly susceptible crayfish species, the batch of crayfish selected to identify *A. astaci* should ideally consist of: a) live crayfish showing signs of disease,

and b) live crayfish appearing to be still healthy. Dead crayfish may also be suitable, although this will depend on their condition.

Live crayfish should be transported using polystyrene containers equipped with small holes to allow aeration, or an equivalent container. The temperature in the container should not exceed 16°C.

The container should provide insulation against major temperature differences outside the container. In periods of hot weather, freezer packs should be used to ensure that temperatures are not deleterious to the animals. These can be attached at the inside bottom of the transport container. However, the crayfish must be protected from direct contact with freezer packs. This can be achieved using, for instance, cardboard or a several layers of newspaper.

Crayfish should be transported in a moist atmosphere, for example using moistened wood shavings/wood wool, newspaper or grass/hay. Unless transport water is sufficiently oxygenated, live crayfish should not be transported in water, as they may suffocate.

The time between sampling of live animals and delivery to the investigating laboratory should not exceed 24 hours.

Should only dead animals be found at the site of a suspected outbreak, these might still be suitable for diagnosis. Depending on the condition they are in, they can either be: a) transported chilled (if they appear to have died only very recently), or, b) placed in non-methylated ethanol (minimum concentration 70%; see Section 3.2).

Animals showing advanced decay are unlikely to give a reliable result, however, if no other animals are available, these might still be tested.

3.2. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If transport of recently dead or moribund crayfish cannot be arranged, crayfish may be fixed in ethanol (minimum 70%). However, fixation may reduce test sensitivity. The crayfish:ethanol ratio should ideally be 1:10 (1 part crayfish, 10 parts ethanol).

3.3. Pooling of samples

Not recommended.

3.4. Best organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended.

3.5. Samples/tissues that are not suitable

Autolytic material is not suitable for analysis.

4. Diagnostic methods

Large numbers of dead crayfish of the highly susceptible species with the remaining aquatic fauna being unharmed gives rise to a suspicion that the population may be affected by infection with *A. astaci*. Clinical signs of infection with *A. astaci* include behavioural changes and a range of visible external lesions. However, clinical signs are of limited diagnostic value. The main available diagnostic methods are polymerase chain reaction (PCR) and isolation of the pathogen in culture media followed by confirmation of its identity. Isolation can be difficult and requires that samples are in good condition when they arrive at the diagnostic laboratory (Oidtmann et al., 1999). Molecular methods are less dependent on speed of sample delivery and can deal with a greater range of samples compared with methods relying on agent isolation (Oidtmann et al., 2006; Vrålstad et al., 2009).

4.1. Field diagnostic methods

4.1.1. Clinical signs

Highly susceptible species

Gross clinical signs are variable and depend on challenge severity and water temperatures. The first sign of an epizootic may be the appearance of crayfish during daylight (crayfish are normally nocturnal), some of which may show loss of co-ordination, falling onto their backs and remaining unable to right themselves. Often, however, the first sign of an outbreak may be the presence of large numbers of dead crayfish in a river or lake (Alderman et al., 1987).

In susceptible species where crayfish numbers are sufficient to allow rapid disease spread, particularly at summer water temperatures, all the highly susceptible native crayfish within stretches of over 50 km may die within 21 days or less from the first observed mortality (D. Alderman, pers. comm.). Infection with *A. astaci* has unparalleled severity as infected susceptible crayfish generally do not survive. It must be emphasised, however, that the presence of large numbers of dead crayfish is not on its own sufficient for diagnosis. The general condition of other aquatic fauna must be assessed. Mortality or disappearance of other aquatic invertebrates, as well as crayfish, even though fish survive, may indicate pollution (e.g. insecticides such as cypermethrin have been associated with initial misdiagnoses).

North American crayfish species

Melanised cuticle has sometimes been suggested as a sign of infection with *A. astaci*. However, melanisation can have a wide variety of causes and is not a specific sign of Infection with *A. astaci*.

4.1.2. Behavioural changes

Highly susceptible species

Infected crayfish of the highly susceptible crayfish species may leave their hides during daytime (which is not normally seen in crayfish), have a reduced escape reflex, and progressive paralysis. Dying crayfish are sometimes found lying on their backs. The animals are often no longer able to upright themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

North American crayfish species

Infected North American crayfish do not show any behavioural changes (B. Oidtmann, unpublished data).

4.2. Clinical methods

4.2.1. Gross pathology

Highly susceptible species

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and abdomen, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example the soft abdominal cuticle. However, these melanisations can be caused by mechanical injuries or infections with other water moulds and are unspecific.

4.2.2. Clinical chemistry

No suitable methods available.

4.2.3. Microscopic pathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used. However, such material does not prove that any hyphae observed are those of *A. astaci*.

See also Section 4.2.4

4.2.4. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 4.2.1) and examined under a compound microscope using low to medium power will confirm the presence of aseptate fungal hyphae 7–9 µm wide. The hyphae can usually be found pervading the whole thickness of the cuticle, forming a three-dimensional network of hyphae in heavily affected areas of the cuticle. The presence of host haemocytes and possibly some melanisation closely associated with and encapsulating the hyphae give good presumptive evidence that the hyphae represent a pathogen rather than a secondary opportunist invader. In some cases, examination of the surface of such mounted cuticles will demonstrate the presence of characteristic *A. astaci* sporangia with clusters of encysted primary spores (see Section 4.3).

4.2.5. Smears

Not suitable.

4.2.6. Fixed sections

See section 4.2.3

4.2.7. Electron microscopy/cytopathology

Not suitable.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

As indicated above (Section 4.2.4), presumptive identification of *A. astaci* may be made from i) the presence of hyphae pervading the cuticle and ii) sporangia of the correct morphological types (see below) on the surface of crayfish exoskeletons.

4.3.1.1.2. Smears

Not suitable.

4.3.1.1.3. Fixed sections

See Section 4.2.3

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Highly susceptible species

Care should be taken that animals to be used for isolation of *A. astaci* via culture are not exposed to desiccation.

Isolation methods have been described by Benisch, 1940; Nyhlén & Unestam, 1980; Alderman & Polglase, 1986; Cerenius et al., 1988; Oidtmann et al., 1999and Viljamaa-Dirks & Heinikainen, 2006.

Isolation medium (IM) according to Alderman & Polglase, 1986: 12.0 g agar; 1.0 g yeast extract; 5.0 g glucose; 10 mg oxolinic acid; 1000 ml river water; and 1.0 g penicillin G (sterile) added after autoclaving and cooling to 40°C. River water is defined as any natural river or lake water, as opposed to demineralised water.

Any superficial contamination should first be removed from the soft intersternal abdominal cuticle or any other areas from which cuticle will be excised by thoroughly wiping the cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. Simple aseptic excision of infected tissues, which are then placed as small pieces (3–5 mm^2) on the surface of isolation medium plates, will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 hours) animals. Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low-power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.

Provided that care is taken in excising infected tissues for isolation, contaminants need not present significant problems. Small pieces of cuticle and muscle may be transferred to a Petri dish of sterile water and there further cut into small pieces with sterile instruments for transfer to isolation medium (IM). Suitable instruments for such work are scalpels, fine forceps and scissors.

To reduce potential contamination problems, disinfection of the cuticle with ethanol and melting a sterile glass ring 1–2 mm deep into the isolation medium can improve isolation success (Nyhlén & Unestam, 1980; Oidtmann et al., 1999). The addition of potassium tellurite into the area inside the glass ring has been described (Nyhlén & Unestam, 1980).

Inoculated agar can be incubated at temperatures between 16°C and 24°C. The Petri dishes should be sealed with a sealing film (e.g. Parafilm[1]) to avoid desiccation.

On IM agar, growth of new isolates of *A. astaci*is almost entirely within the agar except at temperatures below 7°C, when some superficial growth occurs. Colonies are colourless. Dimensions and appearance of hyphae are much the same in crayfish tissue and in agar culture. Vegetative hyphae are aseptate and (5)7–9(10) µm in width (i.e. normal range 7–9 µm, but observations have ranged between 5 and 10 µm). Young, actively growing hyphae are densely packed with coarsely granular cytoplasm with numerous highly refractile globules (Alderman & Polglase, 1986). Older hyphae are largely vacuolate with the cytoplasm largely restricted to the periphery, leaving only thin strands of protoplasm bridging the large central vacuole. The oldest hyphae are apparently 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 thalli or portions of thalli from broth or agar culture are transferred to river water (natural water with available cations encourages sporulation better than distilled water), sporangia form readily in 20–30 hours at 16°C and 12–15 hours at 20°C. Thalli transferred from broth culture may be washed with sterile river water in a sterile stainless steel sieve, before transfer into fresh sterile river water for induction of sporulation. Thalli in agar should be transferred by cutting out a thin surface sliver of agar containing the water mould so that a minimum amount of nutrient-containing agar is transferred. Always use a large volume of sterile river water relative to the amount of water mould being transferred (100:1). Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. The 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. The cytoplasm of such developing discharge tubes is noticeably dense, and these branches are slightly wider (10–12 µm) than ordinary vegetative hyphae. Sporangia are delimited by a single basal septum in the case of terminal sporangia and by septa at either end of the sporangial segment in intercalary sporangia. Such septa are markedly thicker than the hyphal wall and have a high refractive index. Successive sections of vegetative hypha may develop into sporangia, and most of the vegetative thallus is capable of developing into sporangia.

1 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

Within developing sporangia, the cytoplasm cleaves into a series of elongate units (10–25 × 8 µm) that are initially linked by strands of protoplasm. Although the ends of these cytoplasmic units become rounded, they remain elongate until and during discharge. Spore discharge is achlyoid, that is, the first spore stage is an aplanospore that encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been found for the existence of a flagellated primary spore, thus, in this description, the terms 'sporangium' not 'zoosporangium' and 'primary spore' not 'primary zoospore' have been used. Discharge is fairly rapid (<5 minutes) and the individual primary spores (=cytoplasmic units) pass through the tip of the sporangium and accumulate around the sporangial orifice. The speed of cytoplasmic cleavage and discharge is temperature dependent. At release, each primary spore retains its elongate irregularly amoeboid shape briefly before encystment occurs.

Encystment is marked by a gradual rounding up followed by the development of a cyst wall, which is evidenced by a change in the refractive index of the cell. The duration from release to encystment is 2–5 minutes. Some spores may drift away from the spore mass at the sporangial tip and encyst separately. Formation of the primary cyst wall is rapid, and once encystment has taken place the spores remain together as a coherent group and adhere well to the sporangial tip so that marked physical disturbance is required to break up the spore mass.

Encysted primary spores are spherical, (8)9–11(15) µm in diameter, and are relatively few in number, (8)15–30(40) per sporangium in comparison with other *Aphanomyces* spp. Spores remain encysted for 8–12 hours. Optimum temperatures for sporangial formation and discharge for the majority of European isolates of *A. astaci* are between 16 and 24°C (Alderman & Polglase, 1986). For some isolates, particularly from Spanish waters, slightly higher optimal temperatures may prevail (Dehus et al., 1999). The discharge of secondary zoospores from the primary cysts peaks at 20°C and does not occur at 24°C. In new isolates of *A. astaci*, it is normal for the majority of primary spore cysts to discharge as secondary zoospores, although this varies with staling in long-term laboratory culture. Sporangial formation and discharge occurs down to 4°C. *A. astaci* does not survive at –5°C and below for more than 24 hours in culture, although –20°C for >48 hours may be required in infected crayfish tissues, nor does it remain viable in crayfish tissues that have been subject to normal cooking procedures (Alderman, 2000; Oidtmann et al., 2002).

In many cases, some of the primary spores are not discharged from the sporangium and many sporangia do not discharge at all. Instead, the primary spores appear to encyst *in situ* within the sporangium, often develop a spherical rather than elongate form and certainly undergo the same changes in refractive index that mark the encystment of spores outside the sporangium. This within-sporangial encystment has been observed on crayfish. Spores encysted in this situation appear to be capable of germinating to produce further hyphal growth.

Release of secondary zoospores is papillate, the papilla developing shortly before discharge. The spore cytoplasm emerges slowly in an amoeboid fashion through a narrow pore at the tip of a papilla, rounds up and begins a gentle rocking motion as a flagellar extrusion begins and the spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral (Scott, 1961); zoospores are typical saprolegniaceous secondary zoospores measuring 8 × 12 µm. Active motility takes some 5–20 minutes to develop (dependent on temperature) and, at first, zoospores are slow and uncoordinated. At temperatures between 16 and 20°C, zoospores may continue to swim for at least 48 hours (Alderman & Polglase, 1986).

Test sensitivity and specificity of the cultivation method can be very variable depending on the experience of the examiner, but in general will be lower than the PCR.

North American crayfish species

Isolation of *A. astaci* by culture following the methods described for the highly susceptible species usually fails. Currently, the recommended method for detecting infection in such species is by PCR.

4.3.1.2.2. Antibody-based antigen detection methods

None available.

4.3.1.2.3. Molecular techniques

Animals

In the case of a suspected outbreak of the disease in highly susceptible crayfish species, moribund or recently dead (<24 hours) crayfish are preferably selected for DNA extraction. Live crayfish can be killed using chloroform. If the only animals available are animals that have died a few days prior to DNA extraction, they can be tested, but a negative PCR result must be interpreted with caution as DNA degradation may have occurred. Endogenous controls can be used to assess whether degradation may have occurred. These should preferably use host tissues richer in host cells compared to the cuticle; cuticle itself contains very few host cell nuclei. If circumstances prevent delivery of crayfish to the specialist laboratory within 24 hours, fixation in 70% ethanol (≥ 10:1 ethanol to crayfish tissue) is possible, but may result in a reduction of the DNA yield.

DNA extraction

Where animals of the highly susceptible species are analysed, the soft abdominal cuticle is the preferred sample tissue for DNA extraction. Any superficial contamination should first be removed by thoroughly wiping the soft abdominal cuticle with a wet (using autoclaved H_2O) clean disposable paper towel. The soft abdominal cuticle is then excised and 30–50 mg ground in liquid nitrogen to a fine powder using a pestle and mortar (alternative grinding techniques may be used, but should be compared with the liquid nitrogen method before routine use). For carrier identification, 30–50 mg tissue from each soft abdominal cuticle, and telson and uropods are sampled and processed separately. DNA is extracted from the ground cuticle using a proteinase K-based DNA extraction method (e.g. DNeasy tissue kit; Qiagen, Hilden, Germany; protocol for insect tissue) following the manufacturer's instructions (Oidtmann et al., 2006) or using a CTAB (cetyltrimethylammonium bromide-based)-based assay (Vrålstad et al., 2009). Negative controls should be run alongside the samples. Shrimp tissues may be used as negative controls.

4.3.1.2.3.1. PCR

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here that have proven highly sensitive and specific. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome. As should be standard for any PCR-based diagnostic tests, negative controls should be run alongside the samples to control for potential contamination. Environmental controls (using for example shrimp tissue as described above) and extraction blank controls from the DNA extraction should be included along with 'no template' PCR controls (template DNA replaced with molecular grade water). The no template PCR controls should include an environmental PCR control left open during pipetting of sample DNA.

Method 1:

This conventional PCR assay uses species-specific primer sites located in the ITS1 and ITS2 regions. Forward primer (BO 42) 5'-GCT-TGT-GCT-GAG-GAT-GTT-CT-3' and reverse primer (BO 640) 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3'. The PCR is carried out in a 50 µl reaction volume containing 1 × PCR buffer 75 mM Tris/HCl, pH 8.8, 20 mM $[NH_4]_2SO_4$, 0.01% (v/v) Tween 20), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 0.5 µM of each primer, and 1.25 units of DNA polymerase (e.g. Thermoprime Plus DNA Polymerase; AB Gene, Epsom, UK) or equivalent Taq polymerase and 2 µl DNA template. The mixture is denatured at 96°C for 5 minutes, followed by 40 amplification cycles of: 1 minute at 96°C, 1 minute at 59°C and 1 minute at 72°C followed by a final extension step of 7 minutes at 72°C. Amplified DNA is analysed by agarose gel electrophoresis. The target product is a 569 bp fragment. Confirmation of the identity of the PCR product by sequencing is recommended. The assay consistently detects down to 500 fg of genomic target DNA or the equivalent amount of ten zoospores submitted to the PCR reaction (Tuffs & Oidtmann, 2011).

Method 2:

This assay is a TaqMan minor groove binder (MGB) real-time PCR assay that targets a 59 bp unique sequence motif of *A. astaci* in the ITS1 region. Forward primer AphAstITS-39F (5'-AAG-GCT-TGT-GCT-GGG-ATG-TT-3'), reverse primer AphAstITS-97R (5'-CTT-CTT-GCG-AAA-CCT-TCT-GCT-A-3') and TaqMan MGB probe AphAstITS-60T (5'-6-FAM-TTC-GGG-ACG-ACC-CMG-BNF-Q-3') labelled with the fluorescent reporter dye FAM at the 5'-end and a non-fluorescent quencher MGBNFQ at the 3'-end. Real-time PCR amplifications are performed in a total volume of 25 µl containing 12.5 µl PCR Master Mix (e.g. Universal PCR Master Mix or Environmental PCR Master Mix, Applied Biosystems), 0.5 µM of the forward (AphAstITS-39F) and

reverse (AphAstITS-97R) primers, 0.2 µM 200 nM of the MGB probe (AphAstITS-60T), 1.5 µl molecular grade water and 5 µl template DNA (undiluted and tenfold diluted). Amplification and detection is performed in Optical Reaction Plates sealed with optical adhesive film or similar on a real-time thermal cycler. The PCR programme consists of an initial decontamination step of 2 minutes at 50°C to allow optimal UNG enzymatic activity, followed by 10 minutes at 95°C to activate the DNA polymerase, deactivate the UNG and denature the template DNA, and successively 50 cycles of 15 seconds at 95°C and 60 seconds at 58°C. A dilution series with reference DNA of known DNA content needs to be run alongside with the samples.

The absolute limit of detection of this assay was reported as approximately 5 PCR forming units (= target template copies), which is equivalent to less than one *A. astaci* genome (Vrålstad et al., 2009). Another study reported consistent detection down to 50 fg using this assay (Tuffs & Oidtmann, 2011).

The diagnostic test sensitivity of either assay largely depends on the quality of the samples taken. Where an outbreak is investigated, the test sensitivity in animals that had died of infection with *A. astaci* 12 hours or less prior to sampling or in live crayfish showing clear clinical signs of disease is expected to be high. Studies to investigate the effect of sensitivity loss caused by deteriorating sample quality (for instance because of delayed sampling, processing or unsuitable storage of samples) have not been carried out. It is recommended that multiple (5–10) crayfish be tested, to compensate for variations in sample quality and invasion site of the pathogen.

Analytical test specificity has been investigated (Oidtmann et al., 2006; Tuffs & Oidtmann, 2011; Vrålstad et al., 2009) and no cross reaction was observed. However, owing to the repeated discovery of new *Aphanomyces* strains, sequencing is recommended to confirm diagnosis. In the case of the real-time PCR assay, this requires separate amplification of a PCR product, either using the primers as described in method 1, or using primers ITS 1 and ITS4 (see section 'sequencing' below).

4.3.1.2.3.2. Sequencing

A PCR product of 569 bp can be amplified using primers BO42 and BO640. The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel (e.g. using the Freeze n' Squeeze DNA purification system, Anachem, Luton, UK). Both DNA strands must be sequenced using the primers used in the initial amplification. The consensus sequence is generated using sequence analysis software and compared with published sequences using an alignment search tool such as BLAST. If 100% identify between the submitted sequence and the published sequences is found, then the amplified product is *A. astaci*. If the sequence is not 100% identical, further sequencing should be performed using primers ITS-1 (5'-TCC-GTA-GGT-GAA-CCT-GCG-G-3') and ITS-4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3') (White et al., 1990), which generate an amplicon of 757 bp that provides sequence data in the same region, but expanded at both ends relative to the sequence generated by primers BO42 and BO640. This expanded sequence should confirm the identity of the pathogen to the species level.

Highly susceptible species

PCR (conventional or real-time) is a suitable method to investigate suspected outbreaks of infection with *A. astaci* (see Section 7.1). Where the conditions of a suspect case are fulfilled, amplification of a PCR product of the expected size using conventional PCR or real-time PCR can be considered sufficient as a confirmatory diagnosis, if a high level of template DNA is detected. Where low levels of template DNA are detected (weak amplification) or the samples are investigated from a site not meeting the conditions of a suspect case, it is recommended to sequence PCR products generated as described under the section sequencing to confirm the diagnosis.

4.3.1.2.4. Agent purification

Not available.

4.3.2. Serological methods

None available.

5. Rating of tests against purpose of use

The methods currently available for diagnosis of clinical diseases resulting from infection with *Aphanomyces astaci* in highly susceptible species are listed in Table 5.1.Methods for targeted surveillance to demonstrate freedom from infection with *A. astaci* in highly susceptible species are displayed in Table 5.2.

Clinical disease is extremely rare in North American crayfish. Therefore a rating of methods for diagnosing clinical disease in these species is not provided. However, methods for targeted surveillance to demonstrate freedom from infection in North American crayfish are listed in Table 5.3.

The designations used in the tables 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. *Dagnostic methods for infection with A. astaci in highly susceptible crayfish species*

Method	Presumptive diagnosis	Confirmatory diagnosis
Gross and microscopic signs	b	d
Isolation and culture	b	d
Histopathology	d	d
PCR	a	b or a[1]
qPCR	a	b or a[1]
Sequencing of PCR products	n/a	a
Transmission EM	n/a	n/a
Antibody-based assays	n/a	n/a
***In situ* DNA probes**	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative PCR; EM = electron microscopy; n/a = not applicable or not available;
[1] = see definitions of confirmed case in Section 7.1

Table 5.2. *Methods for targeted surveillance in highly susceptible crayfish species to declare freedom from infection with A. astaci*

Method	Screening method	Confirmatory method
Inspection for gross signs and mortality	a	c
Microscopic signs (wet mounts)	c	c
Isolation and culture	c	b
Histopathology	d	d
PCR	a	b, possibly a[1]
qPCR	a	b, possibly a[1]
Sequencing of PCR products	n/a	a

Table 5.2. *Methods for targeted surveillance in highly susceptible crayfish species to declare freedom from infection with A. astaci*

Method	Screening method	Confirmatory method
Transmission EM	n/a	n/a
Antibody-based assays	n/a	n/a
***In situ* DNA probes**	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative PCR; EM = electron microscopy; n/a = not applicable or not available;
[1] = see definitions of confirmed case in Section 7.1

Table 5.3. *Methods for targeted surveillance in North American crayfish species to declare freedom from infection with A. astaci*

Method	Screening method	Confirmatory method
Gross and microscopic signs	d	d
Isolation and culture	c	c
Histopathology	d	d
PCR	a	b
qPCR	a	b
Sequencing of PCR products	n/a	a
Transmission EM	n/a	n/a
Antibody-based assays	n/a	n/a
***In situ* DNA probes**	n/a	n/a

PCR = polymerase chain reaction; qPCR = quantitative PCR; EM = electron microscopy; n/a = not applicable or not available

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Aphanomyces astaci*

6.1. Highly susceptible species

Crayfish farms keeping susceptible crayfish would need to be inspected at a frequency outlined in Chapter 2.2.0. A history of no mortalities (this does not include losses due to predation) occurring within the population over a period of at least 12 months combined with absence of clinical signs, as well as gross and microscopic pathology at the time of inspection are suitable methods for this purpose. Surveillance of wild crayfish stocks presents greater problems, especially where the species concerned is endangered. As movements of both finfish and crayfish stocks from infected waters present a risk of disease transmission, monitoring the status of crayfish populations to confirm that they remain healthy, is necessary.

In a crayfish farm setting, infection with *A. astaci* would be noticed relatively quickly, due to the rapid onset of mortalities in the farmed population.

To undertake targeted surveillance, regular inspections are recommended, where samples are collected if there is any suspicion of mortality or disease. If moribund or dead animals are found, it is recommended that samples are analysed by PCR and if PCR returns a positive result, that PCR products generated using primers 42 and 640, or ITS-1 and -4 are sequenced and the sequences analysed.

6.2. North American crayfish species

In North American crayfish species, animals would need to be sampled and analysed using one of the PCR assays described above. For reasons of higher sensitivity, the real-time PCR assay is the preferred method. This applies to both farmed and naturalised stocks, and surveillance programmes need to take into account the risks of indirect transmission by movements of fish.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

In highly susceptible crayfish species, infection with *A. astaci* is suspected if at least one of the following criteria is met:

i) Any extensive mortality solely of the highly susceptible species of freshwater crayfish where all other aspects of the flora and fauna, particularly other aquatic crustaceans, are normal and healthy.

ii) The presence of gross and microscopic signs consistent with infection with *A. astaci.*

iii) Isolation and culture of a water mould consistent with *A. astaci*.

iv) A positive result for *A. astaci* by PCR.

v) A positive result for *A. astaci* by real-time PCR.

North American crayfish species

In North American crayfish species, infection with *A. astaci* is suspected if at least one of the following criteria is met:

i) A positive result for *A. astaci* by PCR.

ii) A positive result for *A. astaci*by real-time PCR.

7.2. Definition of confirmed case

In highly susceptible crayfish species, infection with *A. astaci* is confirmed if at least two of the following criteria are met:

i) Isolation and culture of a water mould consistent with *A. astaci.*

ii) A positive result for *A. astaci* by PCR.

iii) A positive result for *A. astaci* by real-time PCR.

iv) Sequenced PCR products that match known sequences of *A. astaci*.

If the investigated suspect case is in a country or zone previously considered free from infection with *A. astaci,* sequencing of PCR products should be conducted for confirmation.

In North American crayfish species, infection with *A. astaci* is confirmed if at least two of the following criteria are met:

i) A positive result for *A. astaci* by PCR.

ii) A positive result for *A. astaci* by real-time PCR.

iii) Sequenced PCR products that match known sequences of *A. astaci*.

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NB: There are OIE Reference Laboratories for Infection with *Aphanomyces astaci*
(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/).
Please contact the OIE Reference Laboratories for any further information on Infection with *Aphanomyces astaci*
(Crayfish plague)

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2017.