A quantitative TaqMan MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague Aphanomyces astaci

Torstein Tengs

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A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*

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**1. Introduction**

The freshwater oomycete *Aphanomyces astaci* (Saprolegniaceae) is the causative agent of crayfish plague, a highly infectious disease lethal to freshwater crayfish of non-North American origin (Unestam, 1972). *Aphanomyces astaci* originated in North America and has co-evolved in a balanced parasite–host relationship with North American freshwater crayfish (Unestam, 1972; Dieguez-Uribeondo et al., 1995; Söderhäll and Cerenius, 1999). Introduction and spread of North American carrier crayfish in Europe has therefore caused massive ecological and economical damage, and resulted in constant crayfish plague infection reservoirs (Taugbøl et al., 1993; Souty-Grosset et al., 2006). The World Organization for Animal Health (OIE) previously required that the diagnosis of crayfish plague should be based on isolation of *A. astaci* in pure culture (OIE, 2003). This approach is time consuming and commonly fails since the agent is slow growing, easily killed and easily overgrown by other, faster growing secondary invaders of oomycetes and fungi (Oidtmann et al., 2004). When rapid actions need to be taken to restrict or prevent further spread of the plague, the culture dependent approach is unsuitable. A recent PCR method for detection of *A. astaci* (Oidtmann et al., 2006) has therefore been juxtaposed to the cultivation approach for diagnostics of crayfish plague.
However, conventional PCR assays may fail to discriminate between closely related taxa (Oidtmann et al., 2006; Ballesteros et al., 2007), and may also detect false positives as a result of carry-over contamination. It is therefore recommended to contrast PCR results to histology and disease history (Ballesteros et al., 2007), and to confirm PCR products by sequencing (OIE, 2006). Real-time PCR (RT-PCR) assays for direct detection and quantification of biological agents provide higher specificity and reduced contamination risk, are faster than methods relying on post-PCR analyses, and are therefore increasingly used for species specific detection of numerous pathogens and parasites (Monis and Giglio, 2006). Here we present the development and first validation of a TaqMan® MGB RT-PCR assay for quantitative and species specific detection of A. astaci in susceptible- and carrier crayfish.

2. Materials and methods

2.1. Sample materials

The crayfish samples included in the present study are listed in Table 1. For DNA extraction, standardised pieces of crayfish tissues were taken in duplicates (sub-samples a and b) from three different tissues. For susceptible noble crayfish (Astacus astacus), these were soft abdominal cuticle (Ca; Cb; 10–20 mg fresh weight [fw] per sub-sample), muscle tissue (Ma; Mb; 50–100 mg fw) and eyes including eyestalks (Ea; Eb; 30–40 mg fw). For carrier signal crayfish (Pacifastacus leniusculus), the selected tissues were soft abdominal cuticle (Ca; Cb; 10–20 mg fw), the core joint of the former walking leg (limb; La; Lb; 20–30 mg fw) and (if present) melanised spots on the exoskeleton (Sa; Sb; 1–2 mg fw). DNA from the pure culture isolates (Table 2) was used for development and validation of the RT-PCR assay. Approximately 2–5 mg lyophilized pure culture mycelium was used for DNA extraction.

2.2. DNA extraction

DNA extraction and RT-PCR were performed separately for the duplicate sub-samples, and pure culture isolates and crayfish tissue samples were never subjected to DNA extraction simultaneously. The a sub-samples were run in duplicate RT-PCR analyses. DNA was extracted from lyophilized mycelium and crayfish tissues using a modified version of the miniprep adaptation published by Gardes and Bruns (1993). Mycelium or crayfish tissues in 1.5 ml eppendorf tubes were initially frozen (−80 °C, minimum 15 min). Then 700 μl pre-heated (65 °C) CTAB buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris–HCl, 20 mM Na2EDTA) was added and the material was ground in the buffer. Initially, grinding was performed manually with a sterile pestle in 1.5 ml eppendorf tubes until the material was completely crushed. This was done for the A. astacus a sub-samples and pure culture mycelium. To reduce the labour and work load, and to standardise the protocol, we later applied mechanical grinding for the crayfish tissue samples using disposable 2 ml tubes with steel beads (Kit Precellys MK28) on a Precellys® 24 lysis and homogenization automated equipment (Bertin technologies, Montigny, France). This was done for the A. astacus b sub-samples and all P. leniusculus samples. Mechanical grinding applied the following program: 6500 rpm for 1 min × 3 sessions with 2 min rest time between the sessions. After grinding, the samples were frozen at −80 °C (minimum 10 min), heated at 65°C (5–10 min), and centrifuged (5 min) to remove potential bubbles or vapour. Ten microliters RNase solution (10 mg/ml) was added, followed by mixing and incubation on a heating block (65°C, 30 min). Subsequently 10 μl proteinase K solution (20 mg/ml) was added, followed by mixing and incubation (65°C, 30 min). The tubes were vortexed and then centrifuged for 5 min at 12,000 × g, and 600 μl of the supernatants were transferred to new 1.5 ml eppendorf tubes. Chloroform (600 μl) was added and mixed with the samples by vortexing. The tubes were centrifuged for 15 min at ~18,000 × g, and 400 μl of the aqueous phase was carefully transferred to new tubes. Ice cold isopropanol (300 μl) was added and the liquids mixed by careful inversion. The tubes were incubated for 20 min at room temperature, centrifuged at ~18,000 × g for 10 min to pellet the DNA, and the supernatants were drained off without disturbing the DNA pellet. The pellet was rinsed in 300 μl ice cold 70% ethanol and centrifuged at ~18,000 × g for 5 min. The supernatant was carefully discarded, and DNA pellets were dried for 15–30 min (vacuum dryer or heating block ~50 °C) before re-suspension in sterile TE-buffer. The DNA templates were left at room temperature for minimum 1 h, and carefully mixed and centrifuged (30 s) before RT-PCR setup. In order to control for potential carry-over contamination, environmental control and extraction blank control were always included in the DNA-isolation step and the subsequent PCR analyses. The environmental control consisted of a tube with 200 μl Milli-Q water left open on the laboratory work-bench during the isolation process, and the extraction blank control consisted of an eppendorf tube with 700 μl template free CTAB-buffer that was processed as a sample throughout the extraction process.

2.3. PCR amplification for DNA sequencing

The primer pair ITS1/ITS4 (White et al., 1990) was used for amplification and subsequent sequencing of the nuclear ribosomal DNA (nrDNA) of the internal transcribed spacer (ITS). PCR amplification was conducted on a PTC-200 (Peltier Thermal Cycler, MJ Research, Waltham, MA) using 1.7 μM of each primer, 2 μl genomic DNA, PuReTaq Ready-To-GoTM PCR Beads (Amersham Biosciences, UK) and Milli-Q water to a final reaction volume of 25 μl. The PCR-program included initial denaturation (95 °C/10 min), 38 cycles of 95°C/1 min, 55°C/45 s, 72°C/1 min, and final elongation (72 °C/5 min). PCR products were visualized by gel electrophoresis on 1% agarose gels with ethidium bromide. Fragment sizes were determined by comparison to pUC Mix Marker, 8, ready-to-use (19–1118 bp; Fermentas; USA).

2.4. DNA sequencing

The PCR amplicons were purified with ExoZapTM (Amersham Biosciences, Buckinghamshire, UK) following...
Table 1
Crayfish material, infection status and RT-PCR results.

<table>
<thead>
<tr>
<th>Crayfish number (record number)</th>
<th>Infection status</th>
<th>RT-PCR results from tissue sub-samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-infected Astacus astacus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (05-09-228-K6)</td>
<td>Non-infected</td>
<td>$A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$</td>
</tr>
<tr>
<td>2 (05-09-228-K7)</td>
<td>Non-infected</td>
<td>$A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$</td>
</tr>
<tr>
<td>3 (05-09-280-K1)</td>
<td>Non-infected</td>
<td>$A_0$ $A_0$ NT $A_0$ $A_0$ NT $A_0$ $A_0$ $A_0$</td>
</tr>
<tr>
<td>4 (05-09-342-K1)</td>
<td>Non-infected</td>
<td>$A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$</td>
</tr>
<tr>
<td>5 (Control crayfish)</td>
<td>Non-infected</td>
<td>$A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$ $A_0$</td>
</tr>
<tr>
<td><strong>Infected Astacus astacus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (05-09-229-K1)</td>
<td>Infected</td>
<td>$A_6$ $A_6$ $A_6$ $A_5$ $A_5$ $A_5$ $A_5$ $A_0$</td>
</tr>
<tr>
<td>7 (05-09-257-K2)</td>
<td>Infected</td>
<td>$A_5$ $A_5$ NT $A_4$ $A_4$ $A_4$ $A_4$ NT $A_0$</td>
</tr>
<tr>
<td>8 (05-09-292-K1)</td>
<td>Infected</td>
<td>$A_1$ $A_1$ $A_4$ $A_3$ $A_3$ $A_3$ $A_0$ $A_0$ $A_5$</td>
</tr>
<tr>
<td>9 (05-09-293-K1)</td>
<td>Infected</td>
<td>$A_3$ $A_3$ $A_5$ $A_5$ $A_5$ $A_6$ $A_2$ $A_2$ $A_0$</td>
</tr>
<tr>
<td>10 (05-09-312-K1)</td>
<td>Infected</td>
<td>$A_5$ $A_5$ $A_6$ $A_5$ $A_5$ $A_5$ $A_5$ $A_0$ $A_0$</td>
</tr>
<tr>
<td><strong>Pacifastacus leniusculus (carrier)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 (06-09-538-K1)</td>
<td>Symptom free</td>
<td>$A_2$ $A_2$ $A_2$ $A_2$ $A_2$ $A_2$ NA NA NA</td>
</tr>
<tr>
<td>12 (06-09-538-K2)</td>
<td>Symptom free</td>
<td>$A_1$ $A_1$ $A_1$ $A_2$ $A_2$ $A_2$ NA NA NA</td>
</tr>
<tr>
<td>13 (06-09-538-K3)</td>
<td>Symptom free</td>
<td>$A_2$ $A_2$ $A_2$ $A_2$ $A_2$ $A_2$ NA NA NA</td>
</tr>
<tr>
<td>14 (06-22-1182-K3)</td>
<td>Clinical signs</td>
<td>$A_3$ $A_3$ $A_2$ $A_2$ $A_2$ $A_2$ NT</td>
</tr>
<tr>
<td>15 (06-22-1185-K3)</td>
<td>Clinical signs</td>
<td>$A_3$ $A_3$ $A_2$ $A_2$ $A_2$ $A_2$ NT</td>
</tr>
</tbody>
</table>

1. 15 crayfish were selected for this study. The actual record number at the National Veterinary Institute (NVI) is given in parentheses. The condition of each crayfish upon arrival at the NVI is indicated by the symbols: $\blacklozenge$ = living, $\blacksquare$ = dead, $\blacklozenge$ = frozen, $\triangle$ ethanol fixed.

2. The listed crayfish are taken as examples from submissions where a diagnosis is given and the disease history known. The crayfish originate from lakes and watercourses (wc) in the South-Eastern part of Norway. Individuals listed as non-infected are from submissions where crayfish plague could not be detected and where the areas have remained disease free (1–2: Raudsjøbekken, Glomma wc, Akershus; 3: Urvannet, Drammen wc, Buskerud; 4: Lysen, Glomma wc, Akershus) or control crayfish (5: Einavann, Oppland). Individuals listed as infected are from submissions where crayfish plague was diagnosed and 100% mortality observed (6: Glomma wc, Sørum, Akershus; 7: Aremarkssjøen, Halden wc, Østfold; 8: Svartfossen, Glomma wc, Hedmark; 9–10: Øymarksjøen, Halden wc, Østfold). Signal crayfish are from one Norwegian locality (11–13: Dammane, Telemark; Johnsen et al., 2007) and from two Swedish locations in Värmland (14: River Högsäterälven; 15: Lake Store Le). Clinical signs = melanised spots.

3. Results for tissue sub-samples analysed from each crayfish individual using the *A. astaci* specific RT-PCR assay. For *A. astacus*, tested tissues include soft cuticle (C), muscle (M) and eyes including eyestalks (E); for *P. leniusculus* soft cuticle (C), limbs (L) and melanised spots (S) of exoskeleton. Agent levels $A_0$–$A_7$ refer to semi-quantitative categories based on the numbers of observed PCR forming units (PFUobs) in the PCR. Agent level 0 and 1 ($A_0$ and $A_1$) covers negative sub-samples and any detection below LODabs (PFUobs < 5 PFU), respectively. $A_2$ covers LODabs $\leq$ PFUobs $<$ LOQabs $= 50$ PFU. $A_3$ covers $LODabs \leq$ PFUobs $< 10^3$ PFU. $A_4$ covers $10^3$ PFU $\leq$ PFUobs $< 10^4$ PFU. $A_5$ covers $10^4$ PFU $\leq$ PFUobs $< 10^5$ PFU. $A_6$ covers $10^5$ PFU $\leq$ PFUobs $< 10^6$ PFU, and $A_7$ covers PFUobs $\geq 10^6$ PFU. NT = not tested. NA = not applicable, as samples are from symptom free carrier crayfish.
the manufacturer's instructions, sequenced in both directions with the PCR primers using DYEEnamic™ ET dye terminator chemistry (Amersham Biosciences), purified on AutoSeq96 (Amersham Biosciences) plates, diluted with 10 µl Milli-Q water and subsequently analyzed on a MEGABACE 1000™ (Amersham Biosciences) with the settings 8 V and 35 s injection time. Assembly and manual editing of the sequence chromatograms was conducted in Contig Express, Vector NTI Advance™ (Invitrogen, MD) or in BioEdit version 7.0.1 (Hall, 1999). Sequences were subject to similarity analyses using the NCBI nucleotide BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi; Altschul et al., 1997).

2.5. Design of real-time PCR primers and probe

Sequences from the ITS nrDNA of species of the Saprolegniaceae sequenced in-house or retrieved from the EMBL/GenBank sequence databases were aligned in BioEdit, initially using the automated ClustalW alignment option followed by manual adjustments (see Appendix A). Sequence motifs suitable for design of species specific primers and a TaqMan® MGB-probe were identified by manual inspection of the alignment. The selected motifs were successively used as basis for design of the following primers and probe with the Primer Express 2.0 software (Applied Biosystems, Foster City, CA): Forward primer AphAstITS-39F (5′-AAGGCGTTTGCTGGGATGTT-3′), reverse primer AphAstITS-97R (5′-CTTCTTGGCAACCTTCTGGTA-3′) and TaqMan® MGB probe AphAstITS-60T (5′-6-FAM-TTCGGGACGACCC-MGBNFQ-3′) labelled with the fluorescent reporter dye FAM at the 5′-end and a non-fluorescent quencher MGBNFQ at the 3′-end. The primers were purchased from DNA Technology (Aarhus, Denmark) and the probe from Applied Biosystems.

2.6. Real-time PCR operational procedure

Real-time PCR amplifications were performed in a total volume of 25 µl containing 12.5 µl Universal PCR Master Mix (Applied Biosystems) with the passive reference dye ROX, 500 nM of the forward (AphAstITS-39F) and reverse (AphAstITS-97R) primers, 200 nM of the MGB probe (AphAstITS-60T), 1.5 µl sterile Milli-Q water and 5 µl template DNA (undiluted and 10-fold diluted). Amplification and detection was performed in 96-well Optical Reaction Plates (Applied Biosystems) sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems) on an ABI Prism 7900HT Sequence Detection System (SDS) real-time thermal cycler (Applied Biosystems). The PCR program consisted of an initial decontamination step of 2 min at 50 °C to allow optimal UNG enzymatic activity, followed by 10 min at 95 °C to activate the DNA polymerase, deactivate the UNG and denature the template DNA, and successively 50 cycles of 15 s at 95 °C and 60 s at 58 °C. As part of the RT-PCR operational procedure, the environmental controls and the extraction blank controls from the DNA extraction were included along with a no template PCR control (template DNA replaced with sterile Milli-Q water). Data were collected and analysed with the SDS software 2.2.1 (Applied Biosystems). In all analyses, the software defined baseline was automatically set to 0.2, provided that ROX was selected as passive reference.
2.7. Real-time PCR assay specificity

Initially, specificity tests for each primer and probe were conducted using BLAST as described above probing with the oligonucleotide sequence. Secondly, aligned oligonucleotide and ITS1 sequences of Saprolegniaceae were inspected to assess the degree of similarity and mismatch between the oligonucleotides and the ITS1 of taxa closely related to *A. astaci* (cf. Appendix A). Thirdly, genomic DNA from pure culture isolates (Table 2) including reference strains of the four different *A. astaci* genotypes (Huang et al., 1994; Söderhäll and Cerenius, 1999), four Norwegian isolates of *A. astaci*, as well as other available species of *Aphanomyces* and *Saprolegnia* was used as templates to test the specificity of the RT-PCR assay. Pure culture material or DNA from *A. invadans* assay. Pure culture material or DNA from *A. invadans* was unavailable, despite repeated efforts to use as templates to test the specificity of the RT-PCR assay. DNA from *A. frigidophilus* was also unavailable, despite repeated efforts to obtain such material. Hence, a synthetic oligo representing the relevant parts of the published ITS1 sequences of these taxa was used as substitute DNA. Since their sequence motifs are invariant, the oligo AphInPiITS-29-97 (5’-TATCTTTTATAAGGCCTGTGACGTCACTCGG-CTAGCCAGGTTTCCGCAAGAACGATGTACT-3’) represented both species. A corresponding oligo representing the published ITS1 sequence motif of *A. frigidophilus* (AY647192; AphFriITS-29-97: 5’-TATCTTTTATAAGGCCTGTGACGTCACTCGG-CTAGCCAGGTTTCCGCAAGAACGATGTACT-3’) was also tested in addition to genuine DNA of two other *A. frigidophilus* isolates (Table 2). The synthetic oligos represent a 69 bp sequence motif of the respective species, and cover the RT-PCR target sequence motif in addition to 10 flanking bp on each side of the primer annealing sites. Undiluted, 10-fold and 100-fold diluted templates of genomic DNA from pure culture isolates (~20–50 ng/μl) were tested, while synthetic oligos (50 μM) were tested with undiluted, 10-, 100- and 1000-fold diluted templates.

2.8. First validation of the real-time PCR assay

In order to determine the sensitivity and the dynamic range of the assay, a calibration curve (or standard curve) was established based on genomic DNA from a pure culture isolate of *A. astaci* (VI 03628), plotting mass of DNA against observed cycle threshold (Ct) values. A NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to measure the DNA concentration in ng/μl (mass). Low concentrations were established on the basis of dilutions of more concentrated DNA. The calibrant material was four-fold serially diluted from a measured starting concentration of 80 ng/μl of *A. astaci* stock DNA. The first four-fold calibrant dilution corresponded therefore to 20 ng DNA/μl. Altogether 13 calibration points (4, 4–2, 4–12, 4–13 dilutions) were included to establish the standard curve (cf. Table 3). Each calibrant standardized was run in 30 replicates with the RT-PCR assay in order to test the repeatability of the quantification. The three most diluted concentrations (4, 4–2, 4–13) were run in 40 replicates in total in order to estimate the number of PCR forming units (PFU) or number of amplifiable ITS copies per standard dilution on the basis of single molecule quantification (SIMQUANT; Berdal et al., 2008). The initial standard curve for *A. astaci* that was generated by plotting the mean Ct-values for each dilution (n = 30) against the mass of *A. astaci* DNA, was successively transformed by converting mass to estimated PFU numbers. A separate experiment was set up in order to estimate the impact of exogenous background DNA and to mimic the situation in real samples. In this experiment a background of 100 ng non-infected control crayfish DNA

### Table 3
Calibrant material.

<table>
<thead>
<tr>
<th>Standards/dilutions</th>
<th>Mean Ct-values (n = 30)b</th>
<th>% detectionc</th>
<th>DNA (ng/μl) in calibrantsd</th>
<th>ng DNA (5 μl) in PCRd</th>
<th>Estimated PFU in PCRd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4–1 dilution</td>
<td>16.76 (±0.26)</td>
<td>100</td>
<td>20</td>
<td>100</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>2/4–2 dilution</td>
<td>18.85 (±0.40)</td>
<td>100</td>
<td>5</td>
<td>25</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>3/4–3 dilution</td>
<td>21.07 (±0.27)</td>
<td>100</td>
<td>1.25</td>
<td>6.25</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>4/4–4 dilution</td>
<td>23.33 (±0.20)</td>
<td>100</td>
<td>3.13 × 10⁻¹</td>
<td>1.56</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>5/4–5 dilution</td>
<td>25.67 (±0.41)</td>
<td>100</td>
<td>7.8 × 10⁻²</td>
<td>3.9 × 10⁻¹</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>6/4–6 dilution</td>
<td>28.31 (±0.42)</td>
<td>100</td>
<td>2 × 10⁻²</td>
<td>1 × 10⁻¹</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>7/4–7 dilution</td>
<td>30.52 (±0.52)</td>
<td>100</td>
<td>4.9 × 10⁻³</td>
<td>2.4 × 10⁻²</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>8/4–8 dilution</td>
<td>32.80 (±0.58)</td>
<td>100</td>
<td>1.2 × 10⁻³</td>
<td>6.1 × 10⁻³</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>9/4–9 dilution</td>
<td>35.10 (±0.83)</td>
<td>100</td>
<td>3.1 × 10⁻⁴</td>
<td>1.5 × 10⁻³</td>
<td>3 × 10⁴</td>
</tr>
<tr>
<td>10/4–10 dilution</td>
<td>37.21 (±0.95)</td>
<td>100</td>
<td>7.6 × 10⁻⁵</td>
<td>3.8 × 10⁻⁴</td>
<td>3 × 4</td>
</tr>
<tr>
<td>11/4–11 dilution</td>
<td>39.66 (±1.69)</td>
<td>90</td>
<td>1.9 × 10⁻⁵</td>
<td>9.5 × 10⁻⁵</td>
<td>3</td>
</tr>
<tr>
<td>12/4–12 dilution</td>
<td>40.85 (±1.38)</td>
<td>50</td>
<td>4.8 × 10⁻⁶</td>
<td>2.4 × 10⁻⁵</td>
<td>3 × 10⁻⁴</td>
</tr>
<tr>
<td>13/4–13 dilution</td>
<td>41.64 (±0.82)</td>
<td>27.5</td>
<td>1.2 × 10⁻⁶</td>
<td>6 × 10⁻⁶</td>
<td>3 × 10⁻²</td>
</tr>
</tbody>
</table>

a The standard curve is established from several calibrant points. A total of 13 calibrants (“diluted standards”) were made from a four-fold dilution series of a DNA stock with a measured concentration of 80 ng genomic DNA/μl. The DNA stock was extracted from lyophilized mycelium of the pure culture isolate VI 03628 of *A. astaci* (cf. Table 2).

b Mean Ct-values are based on the RT-PCR replicates of each standard. The first nine standards were run in 30 replicates and the latter three in 40 replicates. The replicates include RT-PCR runs of two different dilution series made from the same DNA stock, and the runs were performed by four different members of our staff.

c The percentage of RT-PCR replicates yielding positive results (detection) for each standard.

d Theoretical content of DNA in ng/μl for each standard calculated from the concentration assigned to the DNA stock (80 ng/μl).

e Quantity of template DNA in each RT-PCR replicate (5 μl template multiplied by assigned concentration per μl).

f Number of PCR forming units (PFU) in each PCR replicate (5 μl template DNA) estimated on the basis of application of single molecule quantification (SIMQUANT; Berdal et al., 2008).
per 5 μl template DNA was added to the templates of each of the standard calibration points.

2.9. Real-time PCR analysis of crayfish samples

DNA-templates from all tissue sub-samples of infected and non-infected noble crayfish or signal crayfish (Table 1) were subject to RT-PCR analysis, with two concentrations per sub-sample (undiluted and 10-fold diluted original DNA extract) to assess for the possible presence of PCR inhibitors that may affect the PCR efficiency and reduce the detectability. The Ct-values for positive samples were compared, and if the difference between the Ct-values (ΔCt) was less than 3, this was interpreted as evidence of inhibition of the PCR for the most concentrated DNA. Six to eight standards covering the range from ~50 PFU to ~3 × 10^10 PFU (standards, corresponding to Ct values from ~35 to ~17) were included in duplicates in each RT-PCR run to produce a standard curve for quantitative purposes. The standard curve was established with the SDS software by plotting the assigned PFU number for each standard against the Ct-value observed in the RT-PCR analysis. The concentration of A. astaci DNA in the crayfish tissue samples was estimated by extrapolating the observed Ct-value for the sub-sample to a PFU number based on the standard curve, successively translated into comprehensible semi-quantitative agent levels (cf. Fig. 1) where A₀ = no agent DNA observed in sub-sample, A₁ = possible presence of the agent, but result correspond to sub-LOD concentration of A. astaci DNA, A₂ = agent present in sub-sample at a very low level (A. astaci DNA at sub-LOQ concentration), A₃ = low levels of the agent in sub-sample, A₄ = moderate levels, A₅ = high levels, A₆ = very high levels, and A₇ = exceptionally high levels of the agent in sub-sample.

3. Results

3.1. Real-time PCR assay design

The primer pair AphAstITS-39F and AphAstITS-97R was designed to amplify a 59 bp unique sequence motif found in the ITS1 nrDNA of A. astaci. For confirmation of the specificity of the amplified product, the MGB probe AphAstITS-60T was designed for inclusion in the RT-PCR assay. More than half of the probe sequence included an insertion–deletion motif that is unique to the presently known A. astaci genotypes and located in the most variable part of the ITS1. The position of the primers and probe are visualized in Appendix A.

3.2. Specificity of the real-time PCR assay

The BLAST searches probing with the AphAstITS-60T probe sequence motif did not retrieve any ITS sequence

Fig. 1. The standard curve based on mean Ct values (n = 30) of the quantitative calibrant dilutions (the standards 1–9 in Table 3) of the four-fold dilution series of A. astaci calibrant DNA plotted against assigned numbers of PCR forming units (PFU) in the reaction volume. The four latter calibration points (n = 40 for the latter three) referred as non-quantitative calibrant dilutions are visualized as a separate graph in the figure and excluded from the quantitative standard curve. The absolute limits of detection (LOD abs) = 5 PFU and quantification (LOQ abs) = 50 PFU are indicated. The agent levels (standards, corresponding to Ct values from 35 to 17) were included in duplicates in each RT-PCR run to produce a standard curve for quantitative purposes. The standard curve was established with the SDS software by plotting the assigned PFU number for each standard against the Ct-value observed in the RT-PCR analysis. The concentration of A. astaci DNA in the crayfish tissue samples was estimated by extrapolating the observed Ct-value for the sub-sample to a PFU number based on the standard curve, successively translated into comprehensible semi-quantitative agent levels (cf. Fig. 1) where A₀ = no agent DNA observed in sub-sample, A₁ = possible presence of the agent, but result correspond to sub-LOD concentration of A. astaci DNA, A₂ = agent present in sub-sample at a very low level (A. astaci DNA at sub-LOQ concentration), A₃ = low levels of the agent in sub-sample, A₄ = moderate levels, A₅ = high levels, A₆ = very high levels, and A₇ = exceptionally high levels of the agent in sub-sample.
other than *A. astaci* that shared an identical or similar sequence motif. This was also confirmed by manual inspection of the ITS1 alignment of available species of the Saprolegniaceae (see Appendix A). Importantly, the closest known related species to *A. astaci*, namely *A. frigidophilus*, *A. invadans* and *A. piscida* missed respectively 9, 6 and 6 bases of the 13 bp probe sequence motif. Experimentally, no cross-reactions were observed for any of the tested DNA templates of other *Aphanomyces* and *Saprolegnia* spp. (Table 2), or for the synthetic oligo that mimic the partial ITS1 of *A. invadans* and *A. piscida*. Selectivity was finally confirmed when all tested strains of *A. astaci* (Table 2) including the four presently known genotypes tested positive with the RT-PCR assay.

### 3.3. Calibrating the real-time PCR assay

The RT-PCR assay was calibrated on the basis of a four-fold dilution series starting from ~100 ng per reaction (5 µL of the 4⁻¹ standard dilution) going down to ~6 fg (5 µL of the 4⁻¹¹ standard dilution) per reaction, scoring the mean Ct-values, SD of Ct-values and the number of positives and negatives of replicates per dilution point (Table 3). According to the SIMQUANT approach the observed ratios of positive and negative reactions are used to estimate the target concentration, and a template concentration of approximately 1 PFU per PCR volume will yield a positive:negative ratio of 7:3 (70% detection) (Berdal et al., 2008). From the standards representing the 4⁻⁰¹¹, 4⁻¹² and 4⁻¹³ fold dilutions (standard dilutions run in 40 replicates) we observed a positive:negative ratio of 36:4 (90% detection), 20:20 (50% detection), and 11:29 (27.5% detection). Based on this observation, we roughly estimated that the calibrant material contained 1 PFU per ~31.7 fg DNA (corresponding to 3 PFU in the 4⁻¹¹ standard dilution that contains ~95 fg DNA; Table 3). The standard curve mass estimates were successively transformed into estimated PFU concentrations (Table 3). The experiment testing the impact of exogenous background DNA showed that crayfish DNA did not significantly affect the observed Ct-values, i.e. no inhibitory effect was observed.

### 3.4. Limit of detection (LOD)

The limit of detection (LOD) for an analytical procedure has more than one definition, but is for an RT-PCR assay generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the times (Burns and Valdivia, 2008), or the lowest concentration that yields a probability of false negatives < 5%. If the target DNA PFU follow a Poisson distribution in the template DNA solution, then the probability of including at least one target PFU in a PCR (this will by definition of PFU yield a positive PCR) is > 95% when the expected number of PFU per PCR is 5 (Berdal and Holst-Jensen, 2001). Theoretically, this would therefore correspond to the absolute LOD (LODabs) of our assay. This LOD (5 PFU per PCR) was corroborated by the observed ratio of positive and negative reactions for the standard dilutions (Table 3).

### 3.5. Limit of quantification (LOQ)

The limit of quantification (LOQ) for an assay is normally 5- to 10-fold higher than the LOD in complex samples (Berdal and Holst-Jensen, 2001). The definition of the LOQ is highly dependent on the degree of measurement uncertainty accepted. Since the observed measurements with the RT-PCR assay are Ct-values, successively translated into PFU, we chose to use the observed SDs for Ct-values of the standards (Table 3) as a basis for establishing the LOQ of the assay. A ΔCt = 0.6 theoretically correspond to approximately 33–50% difference in PFU concentration. The standard RT-PCR replicates presented here were obtained from runs performed by four different members of our staff, and include RT-PCR runs of two different dilution series made from the same starter concentration. The results demonstrate satisfactory repeatability. Based on the observed SD for the Ct-values of the standards (±0.5 for the first eight standards, ±0.8 for the ninth; Table 3) we conclude that an acceptable relative standard deviation R.S.D.r of ≤50% correspond to a LOQabs of approximately 50 PFU per PCR. In Fig. 1 we present data based on all the standard RT-PCR replicates and indicate the LODabs and LOQabs. We also introduce semi-quantitative agent levels (A₁–A₇) that is comprehensible for interpretation of analytical results in real samples.

### 3.6. Application of the real-time PCR assay on clinical samples of crayfish

Fifteen crayfish individuals (Table 1) were tested in the present study, five infected susceptible crayfish (*Astacus astacus*) from localities with manifest crayfish plague, five non-infected from disease free localities, and five presumed infected carrier crayfish (*Pacifastacus leniusculus*). No false positives were detected for the non-infected crayfish sub-samples (Table 1). Another 20 non-infected control crayfish originating from a lake with a disease free historical record (same location as crayfish 5; Table 1), were tested without detection of false positives (data not included). For all tested tissue sub-samples, the duplicate analyses of the a sub-samples always yielded the same agent level, while the b sub-samples of cuticle and muscle tissue in the majority of sub-samples yielded similar results as the a sub-samples (same or neighbouring agent level). The eye tissue sub-samples were the most remarkable exception. The tissue samples of the five infected noble crayfish commonly contained moderate to exceptionally high levels of *A. astaci* DNA (agent level A₄–A₇). This was contrasted by the observations from carrier crayfish where tissue sub-samples of the symptom free carrier crayfish contained very low to low levels of *A. astaci* DNA (agent level A₂–A₃) with frequent observations of detection below LOD (agent level A₁; Table 1). Only one carrier crayfish yielded high agent level results (agent level A₅), and in this case the sub-samples were derived from a large melanised spot, probably representing a clinical sign of infection by *A. astaci*. 

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4. Discussion

Crayfish plague diagnostics have traditionally been complicated based on morphological and culturing techniques. Numerous reports of suspected crayfish plague in Europe have therefore never been confirmed (Edgerston et al., 2004). Molecular detection methods hold a potential to significantly improve the reliability and speed of crayfish plague diagnostics, but it is crucial to take precautionary measures to reduce the risk of basing the diagnosis on false positives. Possible pitfalls include laboratory introduced carry-over contamination, insufficient primer specificity and insufficient knowledge on the diversity, pathology and phylogeny of Aphanomyces spp. Ballesteros et al. (2007) have pointed out that molecular results should be contrasted with pathology and disease history whenever possible. This is particularly valuable for posterior evaluation of the predictability of the molecular diagnostics. However, in an acute situation of possible crayfish plague, any delay in the diagnostic decision making will reduce the likelihood of preventing further spread of the disease. In such cases, molecular diagnostic methods that minimize the risk of carry over contamination and maximize the assay specificity should be preferred. In this respect, RT-PCR has important advantages above conventional PCR. The risk of carry-over contamination is reduced to a minimum both due to use of UNG decontamination reagents and since amplified DNA is monitored in closed systems without handling of PCR products in the laboratory. Probe based RT-PCR provides increased specificity by combining specific primers with a specific probe. Hence, putatively specific PCR products are detected only if the amplified sequence contains the complementary probe motif. The short TaqMan® MGB probes are particularly useful for design of unique diagnostic sequence motifs (Andersen et al., 2006 and references therein) and extremely sensitive to mismatches at the annealing site (Yao et al., 2006). In contrast, conventional PCR frequently permit some mismatches in the priming site without significantly affecting the amplification efficiency (Yao et al., 2006). Unspecific amplification products will subsequently act as perfect templates for further PCR cycles since the primers are incorporated into the products. This is avoided by RT-PCR where the probe is not incorporated into the amplification product.

The A. astaci specific assay described here is rapid, reliable and affordable. It is not only useful for diagnosis of crayfish plague, but also for quantifying agent levels both in susceptible crayfish and carrier crayfish (Table 1). The assay specificity has been demonstrated experimentally with no cross reactions observed for the putatively closest relatives of A. astaci (Table 2), and theoretically by BLAST similarity analyses and visual inspection of aligned DNA sequences from relevant species of the Saprolegniaceae (Appendix A). The 13 bp AphAstITS-60T MGB probe was specifically designed to include a sequence motif that is largely missing in all other known Saprolegniacean species. Hence, if the primer pair AphAstITS-39F/AphAstITS-97R would yield weak unspecific amplification products, the highly specific MGB probe will ensure detection of A. astaci only. Ideally, the specificity testing should have covered a broader range of oomycetes than those included. However, judging from the sequence alignment (Appendix A) and the tests of genuine A. stellatus, A. repetans, A. laevis and A. frigidophilus DNA and artificial A. piscicida/A. invadans DNA, the most relevant known species are in our view covered.

All included strains of A. astaci (Table 2) tested positive with the RT-PCR assay, and all strains of A. astaci sequenced as part of this study share 100% sequence identity in the target sequence motif. If the minor differences seen in the EMBL/GenBank derived sequences of the isolates FDL457, M96/1 and Pc (Appendix A) represents true intraspecific sequence variation and not sequencing-or reading errors, this variation count only for one single nucleotide change within one primer for each involved isolate. Since one mismatch should not affect the amplification efficiency for conventional PCR primers (Yao et al., 2006), we assume the assay will detect and quantify all presently known A. astaci strains.

The target sequence copy number in calibrant materials for real-time PCR is often estimated on the basis of the target copy number per haploid genome (copy number per 1C) and the size (mass) per 1C (Chaouachi et al., 2007). Unfortunately, target copies of the template may fail to amplify, e.g. due to strand breaks not detected in mass based quantity determinations, or because the target copies may not be independently distributed. An example of the latter is the potentially clustered distribution of sequence copies that are organized as part of a tandem repeat, such as the nuclear ribosomal gene cluster. We therefore adopted the term PCR forming units (PFU) proposed by Holst-Jensen and Berdal (2004) as a more suitable term for referring to the amplifiable target copies. The quantitative range of the assay was demonstrated from approximately 50 PFU to ~10^6 PFU per PCR (Fig. 1). Absolute quantification is therefore possible, but requires accuracy of the standard curve calibration, volumes added and serial dilutions of unknown DNA in order to correct for potential inhibition. For crayfish plague diagnostics, the classification of simpler semi-quantitative categories (agent levels; Fig. 1) is therefore robust, convenient and more comprehensible, not only to stakeholders using the analytical results as a basis for deciding on appropriate actions, but also for the laboratories performing the diagnostic work. Results from the performance of the assay on a small number of natural crayfish samples were therefore reported in terms of agent levels (Table 1). We never obtained positive results from the presumed non-infected crayfish representing crayfish from disease free locations or from any internal controls (DNA-extraction control, PCR control and environmental control). This indicates that the risk of detecting false positives is minimized. On the other hand, clear positives were repeatedly detected for all tested tissue types of presumed infected noble crayfish from diseased locations as well as from signal crayfish with presumed carrier status. For noble crayfish, agent levels corresponding to 10^4–10^6 PFU (A4–A6) were with one exception found in all three tissue types tested. When parallel tissue sub-samples yielded divergent results (Table 1), this may be explained by varying infection routes and uneven distribution of the
agent in different subsections of the same tissue due to a gradual invasion of the tissue from the infection site. As expected, analysed material of symptom free carrier crayfish yielded much lower levels of agent DNA. For these sub-samples, agent levels corresponded to a range from 5 to $10^6$ PFU ($A_1$–$A_5$). The very few samples tested for melanized spots showed varying results ($A_5$–$A_8$).

The present assay was experimentally demonstrated to be very sensitive with LOD$_{abs}$ ≈ 5 PFU corresponding to approximately $\sim 160$ fg genomic $A$. astaci (Table 3). Since the genome size (1C value) and the ITS rDNA copy number per 1C are unknown parameters for $A$. astaci, our PFU results could not be accurately compared to the target copy number per 1C. However, according to a rough extrapolation used by Oidtmann et al. (2006), 100 fg genomic $A$. astaci DNA corresponds to $\sim 0.4$–0.5 genomic units when we assume that data provided for the genome size of other oomycetes (Kamoun, 2003) also apply for $A$. astaci. According to this extrapolation, the LOD of our assay corresponds to $\sim 0.5$–$0.6$ genomic unit of $A$. astaci. Detecting less than one genomic unit is therefore possible and seems biological meaningful as the nuclear ribosomal DNA gene cluster including the ITS-region is present in several copies per 1C. Notably, ITS copies may have a clustered distribution since they are organized as part of a tandem repeat. The observed LOD for this assay is comparable to the LOD reported for the published $A$. astaci PCR assays (Oidtmann et al., 2004, 2006), although we are not sure if the LOD of these assays were tested statistically (95% probability of detection). Our observed LOD is further comparable to reported LODs for other real-time PCR assays in the published literature, including a recent study where the LOD in RT-PCR was estimated by computer modelling (Burns and Valdivia, 2008 and references therein). With increased demands for high throughput bio-analytical methods and increased interest in detection of trace levels of target DNA, the sensitivity of RT-PCR assays gained increased attention. With decreased probability of detection, our assay also detects $A$. astaci at levels below LOD. At levels corresponding to 0.03–0.04 genomic unit (6 fg $A$. astaci DNA), we observed detection in 27% of the PCR reactions (Table 3). In some assays, a “Ct cut-off value” is chosen in order to reduce the risk of interpreting a false positive signal as a true positive. If used, a Ct cut-off value should be carefully evaluated for individual assays and RT-PCR thermal cyclers in order to minimize the number of false negatives and positives (Burns and Valdivia, 2008). For our assay, a Ct cut-off corresponding to the observed LOD (Ct-value > 39; Fig. 1) may be justified. However, we prefer to classify such test results as agent level 1 ($A_1$) and use confirmatory analyses to determine if $A_1$ results represent false positives or trace amounts of target DNA. Hence, instead of categorically rejecting such results as false positives, more material is preferably collected and analysed. We have experienced that trace levels ($A_1$) sometimes are detected in crayfish a few weeks ahead of extensive crayfish mortality and confirmed crayfish plague outbreaks (unpublished data). In this way, detection of trace levels of $A$. astaci may become useful, e.g. as early warnings of sub-clinical infections in crayfish populations where rapid and appropriate actions may secure neighbouring populations and prevent further spread of the disease. The high assay sensitivity may prove useful also for other purposes. In an on-going study we are testing the power of the assay to confirm suspected infections on degraded, historical material. In the near future, we hope to apply the assay for direct detection of $A$. astaci from water and environmental samples.

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Appendix A. Supplementary data


References


