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Diagnostic protocols for the detection of *Acheta domesticus* densovirus (AdDV) in cricket frass

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ABSTRACT

The European house cricket (*Acheta domesticus*) is a species of interest for the emerging insect-as-food industry. *Acheta domesticus* densovirus (AdDV) is a member of the Parvoviridae virus family which infects *A. domesticus*, causing widespread mortality and even extinction of local cricket populations. Despite the well-known detrimental effects of AdDV in commercial rearing of *A. domesticus* there are no optimized protocols to accurately and non-destructively detect and quantify the virus. This study establishes a new protocol for the detection of AdDV in faecal material from *A.domesticus*. The protocol includes methodological improvements, such as upgrading from conventional PCR to quantitative real-time PCR and is much more sensitive than previously published protocols. Moreover, this study shows that cricket faeces are a suitable, non-destructive sample substrate to infer reliably if a cricket population is infected with AdDV or not. Early detection of lethal or economic threats, such as disease-causing viruses, is an essential part of commercial cricket management as well as for monitoring the risk of spread to wild cricket populations or to (human) consumers.

1. Introduction

The European house cricket (*Acheta domesticus*) is currently used in insect physiological studies and is reared as feed for pets. It is also a species of interest for the emerging insect-as-food industry (Clifford and Woodring, 1990; Szelei et al., 2011; van Huis et al., 2013). *Acheta domesticus* densovirus (AdDV) is a member of the Parvoviridae virus family (Bergoin and Tijssen, 2008; Tijssen et al., 2011; Cotmore and Davison, 2015) and infects *A. domesticus* (Styer and Hamm, 1991; Szelei et al., 2011). The virus can also infect other cricket species, but has only been shown to be fatal to *A. domesticus* (Weissman and Gray, 2012). AdDV infection in cricket populations often results in widespread mortality and even extinction of local cricket populations (Maciel-Vergara and Ros, 2017; Szelei et al., 2011). Infected crickets show a range of symptoms, such as malnutrition, inhibited growth, reduced fecundity, paralysis and death (Liu et al., 2011; Szelei et al., 2011). Despite the detrimental effects of AdDV to wild *A. domesticus* populations and for the commercial rearing of the species, there are no well-developed and optimized protocols to accurately and non-destructively detect and quantify the virus. Such a tool would be invaluable for densovirus epidemiological studies and surveillance, which are essential for the sustainable rearing of *A. domesticus*, especially if this species will be mass reared for human consumption or as feed for fish and livestock (Berggren et al., 2018; Jansson and Berggren, 2015; van Huis et al., 2013). Since AdDV is spread through oral-fecal transmission (Szelei et al., 2011), cricket frass (faeces) is a promising sample type for non-destructive viral screening. Individual frass samples can also be used to determine virus prevalence in populations, and can easily be pooled for population-level analyses. A sensitive frass-based virus detection method would make it possible to detect infection in a cricket rearing facility at an early stage before clinical symptoms emerge, potentially minimizing disease spread between sub-populations. Thus, a non-destructive screening protocol for densovirus in cricket populations would be a major development in improving cricket rearing standards. The aim of this study was therefore to develop and optimize a quantitative assay for AdDV detection in cricket frass and to develop this into a sensitive, accurate and reproducible screening protocol.

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2. Material and methods

2.1. Origin of samples

Acheta domesticus used in this study were collected from a wild cricket population (hereafter 'wild') outside Uppsala, Sweden and a commercially-reared cricket population (hereafter 'reared'), bought in a pet shop in Uppsala. The wild and reared crickets were quarantined from each other in isolated cages, and each of these groups was further divided into two separate cages. Frass samples were collected from the bottom of each of the cages after 24 h and 1 week and stored in collection tubes at −20 °C until processed.

2.2. DNA extraction from crickets and frass

AdDV was confirmed to be present in the reared crickets, by assaying two dead and one live specimen according to the protocol of Szelei et al. (2011), with the following modifications: individual crickets were placed in a Bioreba mesh bag (Bioreba, Reinach, Switzerland), flash frozen with liquid nitrogen and ground to a powder using a pestle. The resulting powder was mixed with 2 mL nuclease-free water and further homogenized by centrifuging through a QIAshredder (Qiagen, Hilden, Germany). One hundred µL cricket homogenate was mixed with 180 µL Buffer ATL and 20 µL proteinase K (Qiagen, Hilden, Germany) and incubated at 56 °C for 3 h. DNA was purified from the homogenate by a QIAcube extraction robot (Qiagen, Hombrechtikon, Switzerland) following the Qiagen DNA extraction protocol for Tissues and Rodent tails, eluting in 200 µL AE buffer (Qiagen, Hilden, Germany). The DNA concentration was estimated using a NanoDrop 1000 instrument (NanoDrop, USA) and stored in −20 °C until further use.

The DNA extraction protocol for frass was adapted from the published protocol for purifying cricket DNA (Szelei et al., 2011) as follows: 0.1 g frass was homogenized in 0.5 mL nuclease-free water in a MixerMill 400 (Retsch Haan, Germany) at maximum speed (30 f/s) for 1 min using 10 glass beads (ø 3 mm). One hundred µL homogenate was mixed with 180 µL ATL buffer and 20 µL proteinase K (Qiagen, Hilden, Germany), incubated at 56 °C for 1–5 h and extracted using a QIAcube extraction robot (Qiagen, Hombrechtikon, Switzerland) following the Qiagen DNA extraction protocol for Tissues and Rodent tails, eluting in 200 µL buffer AE (Qiagen, Hilden, Germany). The DNA concentration was estimated using a NanoDrop 1000 instrument (NanoDrop, USA), diluted to a final concentration of 10 ng/µL and stored at −20 °C until further use.

2.3. Real-time quantitative PCR

The quantitative real-time PCR (qPCR) assays for AdDV detection were designed around the primers previously described for amplifying a 305 bp fragment (VP) located in the virus capsid protein gene cassette and a 357 bp fragment (NS) located in the non-structural region (Szelei et al., 2011; Supplementary Table I). A constant amount of template DNA was included in each reaction, to minimize template concentration-dependent bias in qPCR efficiency (Nolan et al., 2007; Forsgren et al., 2017). The qPCR reactions were run using the EvaGreen® SYBR Green kit (Bio-Rad, Singapore) containing 0.4 µL of each primer (10 µM) and 2 µL template DNA (20 ng) in 10 µL total volume. The qPCR reactions were run in a CFX-Connect thermal cycler (Bio-Rad), with following cycling conditions: initial enzyme activation step at 98 °C for 2 min followed by 40 cycles of denaturation at 98 °C for 10 s and annealing/extension at 58 °C (AdVP-primers) or 62 °C (AdNS-primers) for 30 s. The amplification was followed by a melting curve analysis starting with 65 °C for 5 s with 0.5 °C increments up to 95 °C. All assays were run in duplicate if not stated otherwise.

2.3.1. qPCR performance parameters

Each reaction plate contained positive and negative (non-template) assay controls. For each assay, a quantitative calibration curve was established through a 10-fold dilution series of a positive control (purified PCR product) of known concentration, covering 6 orders of magnitude. These positive controls were used for quantitative data conversion, establishing the reference melting curve profile of the amplicon and for estimating the qPCR performance statistics.

2.3.2. Annealing temperature optimization

The annealing-extension temperature was optimized for product specificity and detection sensitivity using a 55–69 °C temperature gradient for both assays, followed by narrower separate gradient intervals for each assay between 59–63 °C (AdNS) and 56–61 °C (AdVP), based on the data from the first gradient.

2.4. DNA extraction optimization

2.4.1. Optimization of Proteinase-K incubation

Three different proteinase-K incubation times (1, 3 and 5 h) were tested (n = 12 for each). This was done to establish the minimum necessary incubation time for proteinase K, with the minimum time being judged as the time beyond which no significant increase in PCR copies were detected, in either of the qPCR assays (AdNS and AdVP).

2.4.2. Evaluation of a post-homogenization centrifugation step

Frass homogenate is very thick and difficult to pipet, which can affect pipetting accuracy, and thus the variability of the assay. A test was therefore conducted to determine if centrifuging the homogenate at 8000*g* for 1 min and analyzing the supernatant instead of the whole homogenate would improve the robustness of the protocol without sacrificing sensitivity. Preliminary results (using the initial protocols of Szelei et al., 2011) showed that the reared crickets contained high levels of AdDV, whereas the wild crickets appeared to be free of AdDV. Four frass samples were prepared as follows: samples R1 and R2 came from 2 separate cages containing AdDV-infected reared crickets; sample W1 came from a cage containing wild crickets (determined previously to be AdDV-free); and sample R1-W1 consisted of 14% R1 frass and 86% W1 frass, in order to create a sample with intermediate AdDV levels (see Supplementary Table II). A single homogenate was prepared from each frass sample. The frass was weighed, 5 u L nuclease-free water was added per mg frass and the mixture was homogenized with a MixerMill 400 (Retsch Haan, Germany) and 10 glass beads as described above. This primary homogenate was split into 2 equal duplicate test homogenates. From each duplicate test homogenate, 100 µL was removed for direct DNA extraction while the remainder was centrifuged at 8000*g* for 1 min, after which 100 µL supernatant was removed for DNA extraction, as described above.

2.5. Limits of detection, LOD

2.5.1. LOD for the qPCR assay

The limit of detection (LOD) of the qPCR assays was determined through two replicate 10-fold serial dilution series of an AdDV-positive frass DNA sample with a 10 ng/µL starting concentration. Each assay was run 8 times at each dilution level, for both dilution series. The LOD is defined here as the estimated amount of target DNA, as determined by qPCR, at the highest dilution level where the target was detected by all 16 replicate reaction assays in both dilution series.

2.5.2. LOD for the entire protocol

The entire protocol LOD was tested similarly, but starting with a crude virus-positive frass homogenate diluted with virus-free frass homogenate through a 10-fold dilution series. Three independent homogenate dilution series replicates were prepared from the same original homogenates. The diluted frass homogenates were extracted as described above, the DNA diluted to 10 ng/uL and each qPCR assay was run in duplicate on each template, using reaction conditions and

annealing temperature-optimized thermos-cycling profiles described above.

2.6. Statistical analyses

To check for differences between assay types, incubation times and to account for assay replicate series generalized linear mixed models (GLMM) were used including the 'lmer' function from the R-package 'lme4' (Bates et al., 2015). For the different proteinase K incubation times the number of genome equivalents were compared, based on an interaction between the assay type (AdNS and AdVP) and the incubation time (as a 3-level categorical variable), while controlling for incubation and extraction repeats as random variables. To output predictions from these mixed models, 1000 simulations were bootstrapped for each incubation time and each assay using the 'ezPredict' function from the R-package 'ez' (Lawrence, 2016). For the limit of detection (LOD) estimates, the number of genome equivalents from the two assays were compared, using the data from the final dilution level that successfully detected virus in all replicates. For this a GLMM was used that included the replicate dilution series as a random variable. Consistency between replicate dilution series for the different assays was examined by using data from all dilutions and comparing replicate series (with series as a fixed effect and dilution level as the random effect in the mixed models).

3. Results

3.1. Real-time quantitative PCR

3.1.1. qPCR performance parameters

Both the AdNS and AdVP assays displayed near-perfect inverse-linear relationships between the Cq-value and log10 [template] across nine orders of magnitude (R2 = 0.999 for both assays), with excellent PCR reaction efficiencies (94.5% and 93.6% respectively for the AdNS and AdVP assays) as calculated from the respective slopes of these relationships (Supplementary Table I).

3.1.2. Annealing temperature optimization

The qPCR annealing temperature optimization experiments showed that the optimal annealing temperature was 62 °C for the AdNS assay and 58 °C for the AdVP assay, these being the highest annealing temperatures to generate PCR product without compromising the assays' qPCR performance parameters (Supplementary Table I).

3.2. DNA extraction optimization

3.2.1. Proteinase K incubation

The extraction tests with proteinase K showed that a 1 h incubation time was appropriate, with no increase in assay detection sensitivity after this period (Supplementary Fig. 1). Although the baseline sensitivity differed between the assays ($t = 14.7$, $P < 0.001$), the lack of increase in detection sensitivity with increasing incubation times was the same for both assays (assay*time (3 h) interaction: $t = 0.54$; $P = 0.58$ & assay*time (5 h): $t = 0.66$; $P = 0.51$; see Supplementary Figure 1).

3.2.2. Homogenate centrifugation step

Including the homogenate centrifugation step in the protocol and analyzing the supernatant instead of the whole homogenate improved the AdDV detection sensitivity by 1.71x to 5.69x (Table 1).

3.3. Limits of detection, LOD

3.3.1. LOD for the qPCR assay

The limit of detection (LOD) for the qPCR assays was at the 1/1000 dilution level from the baseline (Supplementary Table III).

3.3.2. LOD for the entire protocol

The LOD was also determined for the entire detection protocol, based on successively diluting the virus-contaminated homogenate with virus-free homogenate. Through to the 1/10000 dilution level all replicate extractions and assays had a 100% detection rate, with an estimated 3.37 ± 1.13 AdDV genome equivalents detected by the AdVP assay, and 1.82 ± 1.07 AdDNA genome equivalents by the AdNS assay. There was no statistically significant difference between the replicate homogenate dilution series, for either of the qPCR assays (AdVP: $F2, 40 = 1.15$, $P = 0.33$; & AdNS: $F2, 40 = 1.07$, $P = 0.35$). At all dilution levels, the AdVP assay consistently detected higher levels of AdDV than the AdNS assay ($n = 30$ for each assay; $t = 3.8$, $P < 0.001$; Supplementary Table IV).

4. Discussion

For general health screening of animal populations, it is essential to have a reliable, fast and preferably non-destructive screening and assaying protocol. This is particularly important for intensively reared animals in production facilities where there is a high risk of damaging disease outbreaks. A reliable screening protocol is moreover essential for good animal husbandry and hygiene, so as to minimize production losses, improve animal welfare, protect susceptible wild animal populations from disease and to minimize the risk of contaminating human food or animal feed products (Berggren et al., 2018).

With this study a basic protocol has been established for the analysis of faecal material from the domestic cricket (*A.domesticus*) as a suitable sample type for the detection of AdDV, a lethal virus disease of crickets that is particularly prevalent and damaging in highly intensive cricket rearing facilities. Previous protocols have been developed for the analysis and screening of body parts of crickets (Weissman and Gray, 2012). The main purpose of this study was to establish and optimize principal parameters for a non-invasive diagnostic protocol for AdDV detection based on cricket faeces. The resulting protocol has been developed from a previously published protocol for the qualitative PCR-based detection of AdDV in whole crickets (Szelei et al., 2011). The principal improvements are: the upgrading of the PCR protocol to real-time quantitative detection (qPCR) using the EvaGreen dye-based detection system, which results in much lower limits of detection (LOD) for both the individual qPCR assays and the entire diagnostic protocol than those published previously (Szelei et al., 2011; Weissman and Gray, 2012); the inclusion of a low-speed centrifugation step after frass homogenization to clarify the extract, which facilitates sample management and improves detection sensitivity, and the reduction of the proteinase-K incubation step to one hour without loss of sensitivity. Minor optimizations were made to the annealing temperatures of the qPCR assays themselves, which were optimized at 62oC for the AdNS assay and 58oC for the AdVP assay. There was no significant difference in detection sensitivity or assay performance between the two qPCR assays employed, which are based on different regions of the AdDV genome. The increased sensitivity of the protocol through the inclusion of a clarification step shows that the virus is mostly contained within the soluble fraction for the frass. The improved detection sensitivity is mostly likely through the enrichment of the extracted DNA with AdDV DNA, by pelleting extraneous faecal material.

It has previously been suggested that AdDV is extremely resistant to proteinase-K digestion (Weissman and Gray, 2012; Tijssen et al., 1977). No difference was found in AdDV detection sensitivity between the 1, 3 and 5 h proteinase-K incubation periods. There was no difference between the two qPCR assays throughout this experiment, implying that the two genomic regions where the assays are located were equally affected by the proteinase-K treatments. Either the proteinase-K digestion was ineffective throughout (Weissman and Gray, 2012; Tijssen et al., 1977), in which case only non-packaged AdDV DNA was detected, or highly effective, such that all relevant proteinase digestion was completed after 1 h.

Table 1
Starting quantity (SQ), the variation in SQ-values (± s.d.) and the proportional difference (SQSup/SQHom) between assays based on whole frass homogenate (Homogenate) or the post-centrifugation supernatant of the frass homogenate (Supernatant). All values have been divided by 10^6 for ease of presentation.

	AdNS			AdVP		
Sample	SQ ± s.d Homogenate	SQ ± s.d Supernatant	SQ^{Sup}/SQ^{Hom}	SQ ± s.d Homogenate	SQ ± s.d Supernatant	SQ^{Sup}/SQ^{Hom}
R1	61.90 ± 0.43	112.00 ± 19.4	1.81x	73.00 ± 6.07	125.00 ± 39.6	1.71x
R2	3.20 ± 0.77	9.50 ± 3.84	2.88x	4.13 ± 0.884	8.63 ± 3.63	2.09x
R1 + W1	3.00 ± 3.00	17.10 ± 0.51	5.69x	5.03 ± 0.055	18.40 ± 1.74	3.65x
W1	0	0	0	0	0	0

This study shows that it is possible to use *A. domesticus* frass samples to determine if (reared) cricket populations are infected with *Acheta domesticus* densovirus (AdDV). The protocol can contribute to minimizing the risk and effects of densovirus outbreaks in cricket rearing facilities, improve animal welfare (Gjerris et al., 2016) both through improved disease management and non-destructive sampling, and can be a valuable tool for improved management of commercial cricket rearing facilities. The protocol can be developed further and areas for improvement will arise with its use, such as for example the relationship between bulk frass-based AdDV detection rates and levels and the proportion of infected individuals this represents. The answer to this is partly due to the interaction between the virus and cricket and the resulting behavioral and physiological responses, but further development of the method will bring light to this important area.

Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. Original data is available on request to the corresponding author.

Author contributions

ES, EF & JM conceived and designed experiments; ES performed experiments; ES, ML & ÅB analysed the data; AJ, ML & ÅB contributed reagents/materials/analysis tools; ES, EF, JM, ML & ÅB wrote the paper. All authors read and approved the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.003.

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