Rapid identification of the invasive Small hive beetle (*Aethina tumida*) using LAMP

Running title: Molecular detection of SHB in honeybee hive debris

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Abstract

Background: Small hive beetle (SHB), *Aethina tumida* is an invasive pest of managed honey bees and has invaded Europe from Africa. The main risk point identified for incursions of this pest into the UK is imports of package bees and queens. Surveillance of this pest, carried out by National Bee Unit, involves monitoring imports of live bees and routine screening of sentinel apiaries around the UK through collection of hive debris for visual inspection of pests. Currently, no molecular methods are used for screening hive debris for this pest in the UK. A new invasion of this pest would likely occur at low levels and might present with partial specimens broken up in the hive or eggs from the beetle, difficult to identify by visual inspection. Therefore, we sought to develop a fast and sensitive method for detecting small hive beetle in hive debris by developing a Loop-mediated isothermal amplification (LAMP) assay.

Result: The LAMP assay was able to detect small hive beetle in 19 to 25.37 minutes and did not cross-react with any non-target species tested. The assay was sensitive and could detect 12pg of DNA and it was able to detect less than 1mg of tissue in a 30g complex matrix of honey bee hive debris. The assay could successfully amplify from crude extracts of partial tissue specimens.

Conclusion: This tool will allow rapid field screening of suspect beetle specimens and laboratory screening of hive debris for a notifiable exotic pest of honey bees.

Keywords: *Aethina tumida*, honey bee, small hive beetle, LAMP.

Introduction

Originally from Sub-Saharan Africa, the small hive beetle (SHB), *Aethina tumida* (Nitidulidae: Coleoptera), a serious pest of honeybees, was discovered in Italy in 2014[1-2]. It continues to persist in Calabria, southern Italy, where it was first discovered, and represents a risk to European apiculture. The adult small hive beetle lays eggs in cracks within a bee-hive and larvae hatch in up to six days[3]. The emerging larvae damage the honey bee colony as they develop, by consuming hive products such as honey, pollen and bee larvae [1]. This can result in colony collapse in European subspecies of honey bees as they are not accustomed to the pest. After feeding is complete, the larvae move to the soil to pupate, which makes eradication difficult. They represent a significant risk to apiculture in Europe, through both natural expansion of its new range and through movement or importation of bees infested with the pest.

Tools for rapid and reliable identification of invasive species are crucial to mounting an effective and rapid response. Current methods for identification of *Aethina tumida* in the UK rely on morphological identification but detection may be difficult if infestation is in the initial stages or at low levels (as may be the case a novel incursion). In the UK, sentinel apiaries are screened for statutory notifiable, invasive pests such as small hive beetle on a twice-yearly basis. Small hive beetle may be screened visually by an inspection of the hive but at low levels, may not be spotted on a single inspection. Hive debris samples from sentinel apiaries are submitted to the National Bee Unit laboratories for examination. The method currently used for screening hive debris for this pest involves performing an alcohol flotation on the debris, which is filtered through 4 Endecott sieves of decreasing pore size (0.355 mm, 0.71 mm, 1.0 mm and 1.71 mm). The insect chitin should float while other debris falls to the bottom of the basin. Unfortunately, intact specimens can be damaged in transit or may only be present in the debris in small pieces; this can make a visual inspection of the sample challenging. Additionally, decomposed insect remains do not always float and may aggregate or stick to other materials in the sample such as wax moth silk or beeswax.

Molecular methods may present a more definitive way for detection under these circumstances. The aim of this study was to develop a specific, sensitive LAMP assay for the rapid identification of *Aethina tumida* in honey bee hive floor debris in the laboratory, and from crude extracts of specimens or partial specimens to demonstrate potential field-use to assist with the NBU sentinel apiary screening program. We compare our results to an existing real-time PCR, the laboratory gold standard in many diagnostic fields.

Materials and methods

DNA extraction

Aethina tumida specimens were kindly provided by Dr Franco Mutinelli and Dr Anna Granato at the Intituto Zooprofilattico Sperimentale delle Venezie (Italy). Other invertebrate samples were sourced from collections held at the UK National Bee Unit (Sand Hutton, York) and Fera. DNA for RT-PCR analysis and LAMP specificity and sensitivity testing was extracted from whole adult insect specimens using the QIAamp® DNA mini kit (QIAGEN) following the manufacturer's instructions for DNA purification from tissues. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) following manufacturers' protocols.

DNA was extracted without column purification to test the SHB LAMP assay on larval beetles or partial tissue specimens including a leg, a thorax or an abdomen. This 'crude extract' was obtained by homogenising the sample with an 11.5 mm diameter stainless-steel ball bearing in 500µl (1ml for larvae) PEG buffer (60% PEG 200, 20 mM KOH, pH 13.3-13.5). The sample was homogenised by shaking vigorously by hand for 1 minute and diluted 1:50 and 1:100 in molecular grade water prior to testing by LAMP[4].

Hive debris DNA extraction

Samples of different *Aethina tumida* life-stages were obtained from a rearing facility at Fera Science Ltd., and spiked into samples of honey bee hive debris obtained from honey bee colonies at Fera Science Ltd. Debris (30g) were placed in a 500 ml HDPE wide-mouth bottle with two 20mm stainless steel ball bearings and homogenised in a Minimix auto paint shaker (Merris Engineering Ltd., Ireland) for two minutes at top speed. Two hundred ml of molecular grade water was added to the homogenised debris and the samples were mixed at room temperature for 1 hour on a rocker. Each 30g suspension was sub-sampled into four 50ml falcon tubes, which were centrifuged for 5 minutes at 6,500 rpm/5,900 *x g*. One ml of eluate was removed from the middle of each 50ml sample and

incubated for one hour at 65°C with 20µl proteinase K and 180µl buffer ATL from the QIAamp DNA mini kit (QIAGEN). The remainder of the protocol involved following the manufacturer's instructions for DNA purification from tissues, but elution was carried out in a reduced volume of 100µl.

LAMP assay design and testing

A previously described invertebrate control LAMP assay was used to amplify multiple species designed to regions of homology identified manually in the *28S ribosomal RNA* gene[5]. The invertebrate assay was designed to differentiate non-hornet invertebrate DNA from *Vespa velutina* DNA (to be used in conjunction with the Asian hornet LAMP assay) using an alignment from *Apis mellifera*, *Vespula germanica*, *Vespa velutina* and *Vespa crabro*.

The *A.tumida* species-specific assay was designed to polymorphisms of the mitochondrial cytochrome oxidase subunit I (*mtCOI*) gene which were identified manually. Sequences of *mtCOI* from *A.tumida* from different geographical regions (Australia, Italy, USA, Zimbabwe, South Africa) were obtained from GenBank NCBI (National Center for Biotechnology Information) and aligned using Geneious software to ensure the assay was compatible with beetles from as many regions as possible (Accession numbers: KT380626, KT380628, KT380625, KT380632, KT380634, KT380638, KT380641, AF227647, AF227648, AF227649, AF227650, KC966652, KP134137). *mtCOI* sequences were also obtained from the closely related *A. concolor* (KU665399, GU217509) and *A. suturalis* (KJ480786 and KJ480787), and from species with a *mtCOI* sequence most closely related to *A. tumida* (the pollen beetles, *Meligethes arankae* (AM491706 and AM491705) and *Meligethes thalassophilus* (AM491700 and AM491699) and compared to ensure the assay would not cross-react with these species.

Primer characteristics were assessed using Oligo Calc[6] software. Primers were synthesised by Eurofins-MWG-Operon. FIP and BIP primers were HPLC purified and all other primers were HPSF

purified (table 1). SHB LAMP reactions comprised of 15 µl of isothermal master-mix ISO-004 (Optigene), 2 µM each FIP and BIP primer, 1µM BLoop primer and 0.2µM each F3 and B3 primer in a 25 µl reaction. The invertebrate control assay LAMP was performed as above except isothermal master-mix ISO-001 (Optigene) was used. For purified DNA, 1 µl of DNA was added to each reaction; for crude extracts 5 µl of 1:50 or 1:100 dilution was added. A 65°C amplification step was run for 40 minutes followed by a slow anneal from 98°C to 70°C at 0.5°C per second using the OptiGene Genie®II platform. Each run included no template (negative) and *A. tumida* purified DNA (positive) controls. All samples were tested in duplicate, and all assays were replicated, with two independent assay runs by two different operators. Results were interpreted in terms of time to positive (Tp) value and annealing temperature (Ta). A Ta value outside the expected range and without a Tp value was observed in negative reactions, which was indicative of primer dimers and not considered a positive result. Both a Tp and Ta within the stipulated range are required for a sample to be called positive.

Real-time PCR assay

The real-time PCR primers and probes tested were from Silacci *et al*., 2018[7] and Ward *et al*., 2007[8] (table 1). The SHB assay from Ward et al., 2007[8] was used in conjunction with an invertebrate control assay from Silacci *et al*., 2018[7]. Real-time PCR reactions were comprised of 1 µl DNA in 10 µl reactions containing 5 µl iTaq™ Universal probes reaction mix (BioRad), 375 nM of each primer and 125 nM probe for the *A. tumida* assay and 200nM each primer and 50nM probe for the control assay. Reactions were carried out on a ViiA™ 7 real-time PCR system (ThermoFisher Scientific) using the following cycling conditions: 95°C for 1 minute followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each run included no template (negative) and *A. tumida* (positive) controls. All samples were tested in duplicate and replicated with two different operators.

LAMP assay validation

Analytical specificity of the LAMP assay was tested with DNA from invertebrate samples held at Fera Science Ltd. most likely to be present in honey bee floor debris including *Varroa destructor, Braula coeca, Acarapis woodi, Apis mellifera, Vespa crabro, Vespula germanica, Bombus hypnorum*, and *Galleria mellonella* (greater wax moth). The beetle species tested were *Lasiodites pictus, Brassicogethes (Meligethes) aeneus* (pollen beetle) and *Cryptophagus spp.* (mould beetle). We were unable to obtain other Nitulidae samples including samples of *A. concolor* and *A. saturalis* for verification of our assay as they do not occur in the UK.

Analytical sensitivity was tested through a ten-fold serial dilution of purified *A. tumida* DNA in water, starting from neat samples to a dilution of $1{:}10^6$ (a range from 124ng down to 12.4fg DNA per µl) with each dilution tested in triplicate. For reproducibility testing, specificity and sensitivity testing was performed by two different operators on different days.

Results

Specificity of detection of *Aethina tumida*

LAMP assay

In the SHB specific LAMP assay, amplification was observed in all SHB samples with amplification achieved in 19 to 24:15 minutes (tables 2 and 5) whereas no amplification was observed for non-target species (tables S1 and 2).

The invertebrate control assay, which acts to confirm the presence of DNA, successfully amplified DNA from all invertebrate species tested except *Braula coeca* and *Acarapis woodi* (table 2), although it did detect *A. woodi* with second user testing (table S1) suggesting that it is likely that this assay is not fully optimised for sensitive detection of all invertebrate species. In addition, the real-time PCR confirms that the presence of DNA in the *B.coeca* sample is low with C_T values of 34.5 and above (table 2 and S1), which may have affected the ability for detection using the LAMP assay.

The LAMP assay specific annealing temperatures were 80.98 (range 80.62 (table 2) to 81.35 (table 4) for the SHB assay and 88.21 °C for the invertebrate control assay (range 85.55 (table 4) to 90.86°C (table S1).

The SHB LAMP assay was able to successfully amplify DNA from samples diluted to 12.4pg across three technical replicates (table 3) and reproducible by two independent operators (table S2). The invertebrate control assay was less sensitive, only able to detect at 124pg and above.

The assay was able to detect SHB DNA crudely extracted using PEG buffer from partial specimens of beetles with an amplification time ranging from 23.22 minutes to 39.15 minutes (table 4).

Results from the LAMP assays were compared to real-time PCR assays for species-specific detection of *A. tumida* and a control assay for general invertebrate species. The *A. tumida* real-time PCR assay successfully amplified samples of *A. tumida*, and it was confirmed that there was no amplification in the non-targets tested (table 2); reproducible by two independent operators (Table S1). The limit of detection was tested with the SHB real-time PCR assay and reliable detection of *A. tumida* DNA was possible at 1.24pg (table 3); however with a second user and sensitivity was lower in the second reaction and only detectable using 124 pg (table S2). The invertebrate control real-time PCR assay provided reliable detection of DNA at 1.24pg which was reproducible with a second user (tables 3 and S2).

Detection of *Aethina tumida* DNA from hive debris

DNA extraction of 30g hive debris which was spiked with varying amounts of beetle tissue was performed. Debris was spiked with one beetle, one pupa, one pupa casing (hollowed out manually) and one, two, five, and ten eggs. The real-time PCR assay was able to detect SHB DNA in all samples including those spiked with a single egg, while the LAMP assay could reliably detect up to ten eggs, but samples spiked with five and two eggs were only detected in one out of four subsamples (table 5). The debris sample spiked with a single egg was not detected using the LAMP assay. The extraction and the assays were repeated by a second user (table S3) and while ten eggs were detected, five eggs were detectable in two replicates, two eggs were not detected in any replicate and one egg was detectable in a single replicate.

Discussion

Every year, hundreds of honey bee hive debris samples are submitted to the National Bee Unit as part of the Sentinel Apiary Program to screen for exotic pests of honey bees (Flatman I & Marris G http://www.nationalbeeunit.com/index.cfm?pageid=166). In 2018, 527 debris samples were submitted to the National Bee Unit laboratories from sentinel apiaries and from imports of package bees. Samples were screened for pests using the alcohol flotation method whereby debris is filtered through 4 Endecott sieves of decreasing pore size, with each sieve placed into a basin of pure ethanol and suspect specimens are expected to float so that they may be visually screened. The concern with this method is that specimens of invasive insect may be partial (i.e. legs, wing casings) or of hard to find/identify life stages (i.e. eggs). Also, material from the floor debris can include silk from wax moth cocoons, which can become entangled with the material in the sample making identification of small samples difficult. As early identification of invasive pests of honey bees, such as *Tropilaelaps* and SHB, are essential to mount an effective contingency response, we sought to develop a sensitive method for screening.

Honey bee hive debris is a complex matrix for molecular analysis comprising of wax, pollen and dead bees; it may also contain wax moth silk, a variety of dead insects, plastics from hive products and residues of veterinary medicines used for *Varroa* control. This represents a very difficult matrix for sensitive detection of small amounts of DNA. LAMP assays are robust in their ability to amplify in the presence of inhibitors that would otherwise preclude amplification in a standard PCR reaction[9]. The aim of this study was to develop a LAMP assay for detection of SHB as a method to quickly and reliably identify partial specimens or hard to identify specimens such as eggs and larvae, or SHB within hive debris samples; 30g of hive debris was tested as the NBU laboratory is likely to receive samples of this approximate size, or smaller.

The LAMP assay successfully amplified from crude extracts of SHB larvae, and partial specimens of adult beetle including leg, thorax and abdomen with a Tp ranging from 23:22 to 39:15 minutes, with the single leg taking the longest to amplify. Manual screening for SHB in the apiary is labour

intensive, and a novel introduction would be expected to present (initially) at very low levels making screening very difficult. Dead adult beetles may be damaged and broken up in the hive before falling into the floor debris hindering identification and eggs cannot be identified to the species level without molecular diagnostics. The LAMP assay can detect DNA from crude extracts of partial specimens so is useful for field use or where a rapid result is required for a high-risk sample. LAMP assays can be performed using crude extracts tested on small, portable instruments such as the Genie® (Optigene) that can be used in facilities with minimal laboratory equipment such as sites of import or field stations. It can be operated and interpreted by users with basic training. This LAMP assay may prove potentially useful for testing at any sites of suspected outbreak.

Although LAMP assays are known for their ability to amplify in the presence of inhibitors that would inhibit a real-time PCR reaction[9], the real-time PCR assay was more sensitive than the LAMP assay for detecting small amounts of SHB DNA from a complex floor debris sample. The real-time PCR assay for SHB, developed at the National Bee Unit[8], had a limit of detection of 1 adult beetle (17.28 ±2.84mg of SHB tissue) per 30g of hive debris, while we found a limit of less than 1mg per 30g. This may be due to our use of a different DNA extraction technique and a different TaqMan mastermix (Biorad versus Stratagene). Using the real-time PCR assay, we could detect DNA from a single egg (<1mg of tissue) in 30g floor debris across all four sub-samples, while using the LAMP assay, we found that detection was not as sensitive but could reliably detect 10 eggs; one egg was undetectable, and two and five eggs were only positive in one out of the four sub-samples. While the real-time PCR assay is more sensitive for detection of SHB in debris, the assays are equal in their specificity, the LAMP assay is faster and can be used in a field setting on partial specimens or life stages that are less easy to identify taxonomically. Each assay therefore provides a different benefit depending on the situation. The real-time PCR assay is appropriate for large numbers of debris extracts with no time constraints, processed in a laboratory and with access to experienced personnel. The LAMP assay is more appropriate for urgent samples or in field situations as it can be

used close to a high-risk apiary site on a partial specimen (i.e. leg or thorax) or early life stages (i.e. eggs) which are more difficult to identify morphologically.

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Conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1: Sequences of primers and probes used in this study.

	Primer/probe name	Sequence (5'-3')
LAMP assays		
Invertebrate control assay	InvtCtr-FIP	CTAAGGCCAGCTCAGCGAGGAAGTCTCTATGCGTACTGAAC
	InvtCtr-BIP	GACACCTGCGTTATTCTTTGACAGATCGTTTCTTTACTTACTCGGTT
	InvtCtr-F3	CGATAGGCCGTGCTTTC
	InvtCtr-B3	GCCGGTGAAATACCACTACTT
	InvtCtr-FLoop	GCAAAAGCTGGCTTGATCTC
	InvtCtr-BLoop	TCGAATCGGATCACGCGG
***SHB* assay**	SHB-FIP	CAAATCCGGCTATAATAGCAAATACTGCTAGTAGCCCATTTCCATTATGTA
	SHB -BIP	CAATGATTCCCATTAATTACAGGATTAACTTTACCCTAAGAAATGTTGTGGGAAA
	SHB -F3	CTAGCTAACTCATCTATTGATATT
	SHB -B3	CGTCGAGGTATTCCTCTTA
	SHB -BLoop	TTCAATTCTTTGTAATATTTATTGG
Real-time PCR assays (from Silacci *et al*., 2018[7])		
Invertebrate control assay	18S-F	AATCAGCGTGTCTTCCCTGG
	18S-R	CAATTGCAAGCCCCAATCCC
	18S-probe	FAM-GTAACCCGCTGAACCTCCTT-BHQ
Real-time PCR assays (from Ward *et al*., 2007[8])		
SHB assay	SHB207F	TCTAAATACTACTTTCTTCGACCCATCR
	SHB315R	TCCTGGTAGAATTAAAATATAAACTTCTGG
	SHB245T -probe	FAM-ATC CAA TCC TAT ACC AAC ACT TAT TTT GAT TCT TCG GAC TAM

Table 2: Specificity testing for SHB and Invertebrate control real-time PCR and LAMP assays.

Samples used in this study, their origin and results with the *Aethina tumida* (SHB) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. All values are the mean from duplicate reactions, '-' indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C_T: cycle threshold value.

Sample/Species	Origin	LAMP				Real-time PCR	
		SHB Tp (mm:ss)	SHB Ta (°C)	Invt ctr Tp (mm:ss)	Invt ctr Ta (°C)	SHB C_T	18S ctr C_T
Aethina tumida adult beetle	UK culture	19:00	80.62	11.30	89.14	23.9	16.1
Non-target species							
Vespa crabro	UK	-	74.11	17:45	89.30	-	19.8
Vespula germanica	UK	-	74.0	18:30	89.25	-	20.4
Vespa velutina nigrithorax	UK	-	74.0	20:42	90.79	-	18.0
Braula coeca	UK	-	74.35	-	75.16	-	34.5
Bombus hypnorum	UK	-	74.00	18:37	89.90	-	18.0
Apis mellifera	UK	-	72.78	17:15	89.48	-	18.8
Galleria mellonella	UK	-	-	34:00	90.76	-	36.9
Acarapis woodi	UK	-	-	-	74.22	-	10.9
Lasiodites pictus	UK	-	73.80	35:15	88.24	-	23.4
Varroa destructor	UK	-	73.12	23:30	89.24	-	32.1
Brassicogethes aeneus	UK	-	74:27	21:00	87.44	-	23.2
Cryptophagus spp.	UK	-	74.28	22:15	87.44	-	18.3

Table 3: Sensitivity testing for SHB and Invertebrate control real-time PCR and LAMP assays. Sensitivity of the *Aethina tumida* (SHB) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. Each dilution was tested in triplicate, and all values are the mean from all positive reactions, '-' indicates a negative result.

DNA (per reaction)	LAMP						Real-time PCR			
	Positive replicates	SHB Tp (mm:ss)	SHB Ta (°C)	Positive replicates	Invt ctr Tp (mm:ss)	Invt ctr Ta (°C)	Positive replicates	SHB C_T	Positive replicates	Invt ctr C_T
Aethina tumida 124 ng	3	25:00	80.76	3	25:37	88.08	3	23.9	3	16.6
Aethina tumida 12.4ng	3	25:20	80.84	3	28:20	89.11	3	26.1	3	21.5
Aethina tumida 124pg	3	27:05	80.92	3	38:30	88.31	3	29.6	3	23.2
Aethina tumida 12.4pg	3	30:45	81.11	0	-	-	3	33.6	3	26.6
Aethina tumida 1.24pg	0	-	-	0	-	-	3	38.1	3	31.0
Aethina tumida 124fg	0	-	-	0	-	-	0	-	0	-
Aethina tumida 12.4fg	0	-	-	0	-	-	0	-	0	-

Table 4: Testing crude DNA extract of SHB

Results of detection of SHB from DNA extracts (diluted 1 in 50 or 1 in 100 in water). All values are the mean from duplicate reactions, '-' indicates a negative result. Tp: time to positive value and Ta: annealing temperature.

Sample	SHB Tp (mm:ss)	SHB Ta (°C)	Invert Tp (mm:ss)	Invert Ta (°C)
Positive control SHB DNA neat	24:15	81.35	39:15	85.55
Aethina tumida leg (1:50)	34:45	81.35	-	80.72
Aethina tumida leg (1:100)	39:15	81.20	35:45	85.18
Aethina tumida thorax (1:50)	37:45	79.69	39:45	84.83
Aethina tumida thorax (1:100)	38:45	80.65	35:15	85.67
Aethina tumida abdomen (1:50)	37:50	80.20	-	81.52
Aethina tumida abdomen (1:100)	38:15	80.27	-	80.99
Aethina tumida larva (1:50)	23:22	80.35	32:30	87.46
Aethina tumida larva (1:100)	25:30	80.40	35:45	87.77
No template control	-	-	-	-

Table 5: Testing honey bee hive debris.

Samples spiked into hive debris and results of *Aethina tumida* (SHB) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. All values are the mean from duplicate reactions, '-' indicates a negative result.

Sample	Debris volume	LAMP: SHB		LAMP: Invert		Real-time PCR	
		Tp (mm.ss)	Ta (°C)	Tp (mm.ss)	Ta (°C)	SHB	Invert
Positive control	-	14:30	80.85	23:30	89.55	27.1	24.1
no beetle	30g	-	73.98	23:30	89.55	-	26.4
1 beetle (16mg)	30g	34:05	80.72	23:30	88.85	31.3	28.6
1 pupae (25.2mg)	30g	33:52	80.32	22.22	89.47	27.5	29.5
1 pupa casing (11mg)	30g	34:25	80.32	23:08	89.33	30.5	31.2
10 eggs (<1mg)	30g	38:41	80.30	24:51	89.65	31.3	30.3
5 eggs (<1mg)	30g	34:30*	80.46	21:16	89.34	33.5	30.8
2 eggs (<1mg)	30g	39:15*	80.11	22.37	89.28	34.4	29.1
1 egg (<1mg)	30g	-	73.73	21.58	89.33	36.4	31.2
No template control	N/A	-	-	-	-	-	-

* detected in 1/4 samples