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Development of an antigen-based rapid diagnostic test for the identification of blowfly (Calliphoridae) species of forensic significance☆

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ABSTRACT

In this study we examine the limitations of currently used sequence-based approaches to blowfly (Calliphoridae) identification and evaluate the utility of an immunological approach to discriminate between blowfly species of forensic importance. By investigating antigenic similarity and dissimilarity between the first instar larval stages of four forensically important blowfly species, we have been able to identify immunoreactive proteins of potential use in the development of species-specific immunodiagnostic tests. Here we outline our protein-based approach to species determination, and describe how it may be adapted to develop rapid diagnostic assays for the 'on-site' identification of blowfly species.

1. Introduction

Modern forensic science has seen the importance of entomological evidence becoming well established worldwide, particularly when facing crime scenes involving a suspected homicide or manslaughter. One of the defining characteristics of the dipteran group Calliphoridae (blowflies) is the necessity to lay eggs on a proteinaceous substrate, usually the living or necrotic tissue of an animal host; larvae then develop by feeding on this protein-rich matter. Within forensic entomology, blowflies are recognised as some of the most important and robust indicator species, as they are usually among the first insects to colonise a body after death, often within hours [1]. Typically, the immature stages of blowflies, particularly larvae, are collected at a suspected crime scene and are used to establish the minimum post-mortem interval (PMI), using larval age as a form of 'biological clock'. However, the immature stages, particularly early stage larvae, of several forensically important blowfly genera, e.g. *Lucilia* and *Calliphora*, are notoriously difficult to identify on the basis of morphology; the lack of species-specific larval morphological characters can make taxonomic keys very difficult to use, particularly for non-specialist entomologists [2]. Accurate species determination is essential to PMI calculations, as growth and development rates can be highly species-specific; consequently, forensic entomologists are increasingly favouring molecular methods of identification [2–10].

At present, DNA sequence-based analysis, as first suggested by [11], is the only routinely used molecular-based species identification tool [12]. Several loci have been advocated for species identification through phylogenetic inference, whereby the position of unknown specimens within a phylogenetic tree reveals the species taxonomy [7,9,10]. The most commonly used markers include: regions of the mitochondrial cytochrome oxidase subunits I (COI) and II (COII) [2,6,10,13–17] and the nuclear internal transcribed spacers (ITS) [2,18]. Mitochondrial DNA (mtDNA), in particular, is favoured due to its high copy number, ease of isolation, and conserved sequence across taxa with supposed high mutation regions making discrimination between species, and even sub-species, possible [18,19].

Current practice typically employs single gene phylogenies for the identification of forensically important blowflies. However, limitations in the reliability of single locus phylogenies have been reported [20–22]. Such phylogenies only infer evolutionary relationships for the particular gene used, and as such may not represent the true species phylogeny, for example, due to horizontal gene transfer or incomplete lineage sorting at the locus in question. Despite mitochondrial DNA being expected to reach reciprocal monophyly before nuclear genes [19], phylogenetic inference from mtDNA genes has been widely reported to show paraphyly within Calliphoridae, as well as other organisms [20,22]. In particular, blowfly phylogenies based on mitochondrial and nuclear genes have exposed conflicting evolutionary relationships [12,22], with, for example, a COI genealogy rejecting reciprocal monophyly for *Lucilia cuprina* when compared with a 28S rRNA phylogeny [12,18,22]. Such findings imply incomplete lineage sorting for some recently diverged forensically important

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blowflies, and so may prove problematic when relying on phylogenetic methods of diagnosis which themselves assume reciprocal monophyly. See Wells and Stevens [12] for an up to date review of this subject.

While some loci are more routinely used than others, no single gene is approved for use in forensic entomology and, in view of the problems outlined, it appears unlikely that any single gene will be capable of unambiguously identifying all calliphorid species of forensic importance. Consequently, due to the legal implications of forensic evidence, recognition is needed that if the use of molecular methods of species identification is to be continued, a switch to multi-gene approaches, for example using ESTs to screen multiple independent sites across blowfly genomes, is urgently needed. To date, few studies have used multi-gene phylogenies to determine the identity of forensically important flies [22].

The accuracy of phylogenetic inference, particularly when used for species determination of unidentified specimens, is also highly dependant on the experience of the analyst [12]. An important factor commonly misunderstood is the influence of taxonomic sampling, as both the number and choice of taxa represented can have a significant impact on the phylogenetic relationships inferred. In cases where many closely related species are compared, insufficient sampling may lead to intra- and inter-specific ranges of genetic variation overlapping, so affecting the ability to determine the identity of unknown samples [18,23]. A second and potentially immense source of error is through the use of uncritical BLAST searching of databases that rely heavily on individual investigators being responsible for the accuracy and taxonomy of submitted sequence data. At present, the importance of such sources of error remain largely unknown, however, as several recently documented cases illustrate, even some commonly cited forensically important sequence records appear to have been mislabelled, leaving the reliability of reference sequence data in dispute [12]. Consequently, though it is generally accepted that such DNA-based identification of forensically important blowflies is expected to increase in popularity in the near future, emphasis is now also being placed on the need to investigate alternative approaches [12]. In this paper we present our preliminary findings on the development of such an alternative: an antigen-based rapid diagnostic test for the identification of blowfly species of forensic significance.

Due to the presence of several different styles of parasitism within Calliphoridae (saprophage, obligate and facultative), blowfly speciation is likely to have been accompanied by the evolution of different larval proteins relating to their differing life-history strategies [21,24]. For example, obligate and facultative ectoparasitic species (i.e. those capable of feeding on living animal hosts) must have developed defences against both specific and non-specific host immune responses. By investigating similarity and dissimilarity between antigenic (capacity of a substance to induce an immune response) blowfly larval proteins, and the discriminatory power of such a technique between species, we have been able to identify antigenic markers capable of being adapted for use in the development of a protein-based species-specific diagnostic test.

2. Materials and methods

2.1. Blowflies

Four forensically important blowfly species were initially chosen for comparison. These were a single *Calliphora* species, *Calliphora vicina*, and three *Lucilia* species, *L. sericata*, *L. cuprina* and *L. caesar*.

C. vicina is a saprophagic species and larvae feed exclusively on dead or decaying matter. While morphologically very distinct from the other blowfly species investigated, it is an important taxon in forensic entomology and is used extensively in the UK and elsewhere to assess PMI.

The three *Lucilia* species (*L. sericata*, *L. cuprina*, *L. caesar*) are all facultative parasites, meaning they can feed on both necrotic and living tissue, and so were expected to diverge greatly from *Calliphora* species in terms of the proteins expressed by their larvae. As stated, however, the primary objective of this study was to investigate whether reproducible antigenic differences, capable of reliably discriminating between these morphologically similar *Lucilia* species, could be found. *L. sericata* and *L. cuprina*, in particular, are notoriously difficult to discriminate, being almost identical even as adult flies.

2.2. Larval protein preparation

Crude first instar larval protein preparations for *C. vicina*, *L. sericata*, *L. cuprina* and *L. caesar* were prepared by homogenizing whole larvae with deionised water (dH_2O) or with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.2]) and protein concentrations determined spectrophotometrically at 280 nm (Nanodrop; Agilent Technologies Limited, Berkshire).

2.3. Rabbit polyclonal (PAb) antibody production

In order to compare the antigenic properties of larval proteins, rabbit polyclonal antiserum was raised against PBS protein extracts from whole larvae of *L. sericata*. Protein extracts prepared as described were used as immunogen to immunise New Zealand male rabbits using standard immunization procedures [25].

2.4. Gel electrophoresis and Western blotting

Polyacrylamide gel electrophoresis (PAGE) was carried out using the system of Laemmli with 4–20% (w/v) gradient polyacrylamide gels (Bio-Rad Laboratories Limited, Hemel Hempsted, United Kingdom) under denaturing conditions. Larval protein extracts were mixed with Laemmli buffer and denatured by heating at 95 °C for 10 min in the presence of β-mercaptoethanol, before centrifugation and loading onto gels.

The proteins were separated for 1.5 h at 23 °C (165 V). Prestained, broad-range markers (Bio-Rad) were used for molecular mass determinations. For Western blotting, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad) at 4 °C. The membranes were blocked for 16 h at 4 °C with PBS containing 1% (w/v) bovine serum albumin (BSA). The blocked membranes were then incubated with rabbit antiserum diluted 1 in 1000 in PBS containing 0.5% (w/v) BSA (PBSA) for 2 h at 23 °C. After the membranes were washed three times with PBS, they were incubated for 1 h with goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) diluted 1 in 15,000 in PBSA. The membranes were washed twice with PBS and once with PBST (PBS containing 0.05% (v/v) Tween 20), and bound antibody visualised by incubation in substrate solution [25]. The reactions were stopped by immersion in dH_2O and air dried between sheets of Whatman filter paper.

3. Results and discussion

3.1. Western blotting and protein characterisation

First instar larval proteins were resolved by SDS-PAGE and then transferred to PVDF membranes. Membranes were then probed with rabbit antiserum raised against *L. sericata* larval extracts, which reacted with a series of bands for each of the four blowfly

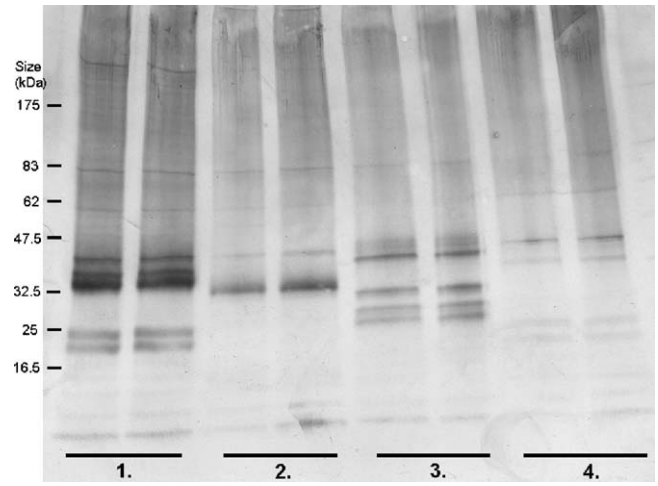

Fig. 1. Western blot analysis of SDS-PAGE resolved first instar larval proteins with antiserum from a *L. sericata* immunised rabbit. *L. sericata* (1), *L. cuprina* (2), *L. caesar* (3), and *C. vicina* (4). Each well was loaded with 10 μg protein.

species tested, Fig. 1. While many of the protein bands were common among all four taxa, a number also appeared to be unique to the each of four taxa.

While initially investigating antigenic similarity and dissimilarity between first instar larval stages of four forensically important blowfly species, our findings have now revealed the potential species discriminatory power of such a technique. Several species-specific diagnostic proteins markers showing potential for use in species determination have been located, and we are currently in the process of characterising these proteins. For example, a particularly striking protein band (Fig. 2; band II, 35.8 kDa) appearing to be specific to *L. sericata* has been located and is currently undergoing characterisation.

A systematic proteomic approach is being used to purify and identify diagnostic immunodominant bands. N-terminal peptide sequencing and MALDI-MS of diagnostic protein markers will be used to perform searches for statistically similar protein sequences in core databases.

Two-dimensional SDS-PAGE gel electrophoresis, which achieves resolution in two dimensions separating proteins first by pH and then by molecular mass, is also planned for each of the four taxa. The major advantage of this technique is the ability to distinguish between different isoforms of a protein with similar

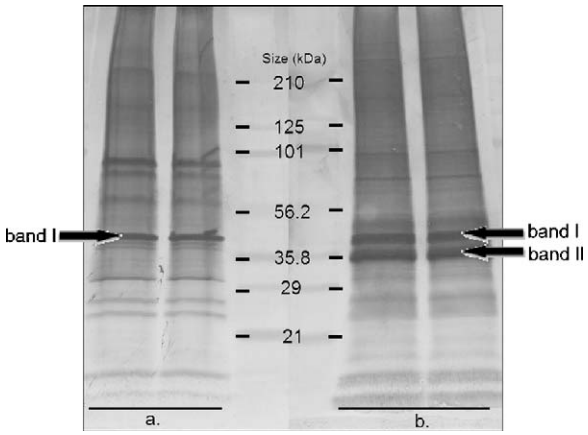

Fig. 2. Western blot analysis of SDS-PAGE resolved *C. vicina* (a) and *L. sericata* (b) first instar larval proteins with antiserum from a *L. sericata* immunised rabbit, illustrating diagnostic immunoreactive bands. Each well was loaded with 10 μg protein.

molecular weights, for example a protein that has been phosphorylated (by addition of a negatively charged group), so helping to identify further potential diagnostic markers.

3.2. Species-specific monoclonal antibodies

The Western blotting experiments carried out in this study utilised rabbit PAb. Our efforts are now focusing towards the production of species-specific murine monoclonal antibodies (MAbs) using the diagnostic proteins identified with the rabbit PAb as immunogens. Raising species-specific MAbs will then allow the development of a rapid diagnostic tool exploiting lateral flow technology, an innovative application to the identification of forensically important blowflies.

3.3. Lateral flow technology

Lateral flow diagnostic tests are a format of semi-quantitative immunoassay for detecting the presence (or absence) of a target antigen (the identified species-specific antigenic protein) in a complex sample of proteins (e.g. whole blood, serum, urine). The most publicised example of the use of this technology is home pregnancy test first introduced by Unipath in 1988. Such technology is now used extensively for the rapid 'on-site' diagnosis of a wide range of human diseases including visceral leishmaniasis [26], HIV/AIDS [27], malaria [28], SARS [29] and invasive aspergillosis [30].

Lateral flow devices work as follows. A species-specific MAb is immobilised to a defined capture zone on a porous nitrocellulose membrane, while the same MAb conjugated to colloidal gold particles serves as the detection reagent. Samples of solubilised antigens are added to a release pad containing the antibody–gold conjugate. The antibody–gold conjugate binds to the target antigen, passes along the porous membrane by capillary action, and binds to the MAb immobilised in the capture zone. Once an antigen extract is prepared and applied to the LFD, the test result is recorded within 10–15 min. Bound antigen–antibody–gold complex is seen as a red line with an intensity that is proportional to the antigen concentration. Anti-mouse immunoglobulin immobilised to the membrane in a separate zone acts as an internal control. In the absence of the target antigen, no complex is formed in the zone containing the solid-phase antibody, and a single control line is seen. In the presence of the target antigen, two lines are clearly visible. One of the key advantages of this technology is the simplicity of the test, typically requiring little or no sample or reagent preparation.

While we believe existing single loci DNA-based methods of species determination to be problematic, we recognise that an extensive global study would be required to provide identification kits for all forensically important blowfly species. We anticipate that our research could develop 'on-site' rapid diagnostic kits for several of the more common blowflies. By reducing the work load of blowfly samples needing to be sent for sequencing analysis, such test kits could prove an innovative supporting role to existing DNA sequence-based methods, allowing forensic entomologists to focus on the more challenging cases of blowfly identification.

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