6. Tools for mosquito blood meal identification

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Abstract

The identification of the vertebrate blood meal sources of mosquitoes allows insight to better understand the dynamics of vector-borne pathogens. To do so, different approaches have been used, based on the use of the remains of blood present in the abdomen of recently engorged mosquito females. Among others, different authors have used serological techniques to the more recently developed approaches based on host DNA amplification or mass spectrometry. These methods have allowed researchers to identify the vertebrate hosts of mosquitoes accurately to the species level or, even, at the individual level, providing information on the relative importance of different mosquito species in the transmission of particular pathogens. These approaches have been especially relevant to reveal the contact rates between vectors, susceptible and competent hosts, and mosquito-borne pathogens, including zoonotic ones. Additionally, these methods have revealed important asymmetries in the attraction of mosquitoes towards different host species, allowing to identify key vertebrates for the amplification of some pathogens. This chapter reviews those tools most frequently used for the identification of the blood meals of mosquitoes in order to highlight the main advantages and limitations of these methodologies.

Keywords: barcoding, *cytochrome B*, COI gene, DNA, ELISA, Maldi-TOF

Introduction

The blood feeding behaviour of mosquitoes and their competence for the transmission of different pathogens make these insects one of the main vectors of causative agents of infectious diseases worldwide (Becker *et al.* 2010). Different species of mosquitoes of the genera *Aedes*, *Anopheles* and *Culex*, among others, are considered competent vectors of arbovirus including Dengue virus, Yellow fever virus, Zika virus or West Nile virus (Christophers 1960, Gutiérrez-López *et al.* 2019, Kilpatrick *et al.* 2008, Vasilakis *et al.* 2011). In addition, parasites including protozoan and filarial worms are important mosquito-borne pathogens affecting humans, livestock and wildlife (Christensen and Severson 1993, Valkiūnas 2004).

Mosquito females feed on blood to obtain the resources for egg development. Engorged female mosquitoes with a recent blood meal in their abdomen have been routinely used to identify the potential hosts of these insects in particular areas revealing that mosquitoes show different innate feeding preferences, with some species feeding mostly on mammals while others prefer to bite birds. In addition, different species of mosquitoes use ectotherms as the main blood meal sources, with records of species such as *Uranotaenia sapphirina* feeding on amphibians (Cupp *et al*. 2004) and *Culex hortensis* frequently feeding on reptiles (Martínez-de la Puente *et al.* 2015b).

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Table 1. Examples of studies using different molecular markers for the identification of blood meals of mosquitoes and other hematophagous arthropods.

Gene	Used in mosquito	Other insect used	Hosts identified by the studies	Reference
Mitochondrial DNA				
12S rDNA	not	ticks and Triatominae	mammals, birds, reptiles	Collini *et al.* 2015
COI	yes		vertebrates	Alcaide *et al.* 2009
Cytochrome b	yes		vertebrates	Fornadel *et al.* 2008
16S RDNA	not	biting midges and ticks	reptiles and amphibians	Collini *et al.* 2015
NADH dehydrogenase subunit (ND1)	yes		bats, reptiles and amphibians	Toma *et al.* 2014
Nuclear DNA				
Nuclear Ribosomal DNA (rDNA)	yes		annelids	Reeves *et al.* 2018
Prepronociceptin (PNOC)	not	biting midges	mammals	Hadj-Henni *et al.* 2015
Alu transposable elements	not	sand flies	humans and non-human primates	Siripattanapipong *et al.* 2018
Microsatellite	yes		mammals and birds	Martínez de la Puente *et al.* 2018, Yan *et al.* 2018

In addition, Miyake *et al.* (2019) found that *Aedes baisasi* includes fish as a common blood meal source in its diet. In spite of that, strong evidence support that many mosquito species show a relative opportunistic behaviour being able to feed on blood from different species or groups.

Authors have used different methods used for the identification of the vertebrate hosts of mosquitoes that include, among other, serological techniques, molecular approaches based on the amplification and sequencing of host DNA or mass spectrometry. Altogether, these methods have allowed researchers to accurately identify the vertebrate hosts of mosquitoes to the species level or, even, to the individual level (Alcaide *et al.* 2009, Beier *et al.* 1988, Gomes *et al.* 2001, Tandina *et al.* 2018, Yan *et al.* 2018). Thus, the identification of the vertebrate hosts of mosquitoes may be considered as an important step to evaluate the risk of transmission of mosquito-borne diseases (Coulibaly *et al.* 2016, Githeko *et al.* 1994, Ndenga *et al.* 2016). In particular, the identification of blood meal sources of mosquitoes allows researchers to infer the relative importance of different species of mosquitoes and/or vertebrate hosts in the transmission of particular pathogens by tracing the contact rates between vectors, susceptible and competent hosts, and mosquito-borne pathogens, including zoonotic ones (Ferraguti *et al.* 2020, Kilpatrick *et al.* 2006). These approaches may also allow researchers to identify the importance of particular individuals or groups within host populations (e.g. according to sex, infection status or odour) for the amplification of vector-

borne pathogens (Lefèvre *et al.* 2010, Yan *et al.* 2018). These studies add valuable information to the traditional approaches based on the identification and counting of mosquitoes landing on the skin of animals (Balenghien *et al.* 2006, Ndenga *et al.* 2016) or to studies analysing mosquito attraction towards animals that are stalled in traps (Balenghien *et al.* 2006, Cozzarolo *et al.* 2019).

In this chapter, we review the more frequently used tools for the identification of blood meals of mosquitoes in order to highlight the main advantages and limitations of these methodologies.

Methods used to identify the blood meal sources of mosquitoes

Serological techniques

Techniques including the precipitin tests, such as haemoglobin crystallisation (Washino and Else 1972) and passive hemagglutination inhibition (PHI) (Kirsch and Murray 1969, Tempelis and Rodrick 1972, Weitz 1956), as well as enzyme-linked immunosorbent assays (ELISA) (Gentry *et al.* 1967, McKinney *et al.* 1972) have been widely used to identify the hosts of engorged mosquitoes. All of them basically consist in the identification of the host species of mosquitoes by exposing the blood meal to immunoglobulin G (IgG) conjugated against different potential host species (Mwangangi *et al.* 2003). Although this technique was firstly used to define the role of anophelines in the transmission of human malaria (Tempelis 1975), these studies have been extended to multiple species of mosquitoes of the genera *Aedes*, *Culex* or *Coquillettidia* (Gomes *et al.* 2001, Kurucz *et al.* 2006, Lorosa *et al.* 2010). In spite of that, studies using these methodologies have some limitations. For example, the accuracy of the host identification based on these methods may be limited by the availability of specific anti-sera against target species (Martínez-de la Puente *et al.* 2018). In addition, cross-reactions may occur in presence of blood from closely phylogenetic related species (Boakye *et al.* 1999, Hunter and Bayly 1991). This may limit the utility of this technique in studies performed under natural conditions where numerous vertebrate species are available to mosquito bites. For example, studies using serological techniques under natural conditions fail to identify the hosts of 13-20% of the analysed mosquitoes (Beier *et al.* 1988, Lorosa *et al.* 2010). Furthermore, although many studies are able to identify the avian origin of blood meals, the hosts species of mosquitoes are usually not determined (Lorosa *et al.* 2010). This fact together with the differential competence of different species of vertebrates for the development of pathogens, may limit the epidemiological conclusions obtained in these studies. An example of that could be the case of studies focus on WNV transmission, a pathogen which show a differential ability to develop in different species of birds (Linke *et al.* 2007, Llorente *et al.* 2013, Pérez-Ramírez *et al.* 2014). In example, host competence for WNV of passerines use to be higher than of galliformes and columbiformes (Pérez-Ramirez *et al.* 2014). *Culex quinquefasciatus* is an important vector for WNV, and different studies have shown preference to feed also on birds with a high capacity to amplify the virus (i.e. passeriforms) (Niebylski and Meek 1992, Zinser *et al.* 2004). However, it would be highly recommended to use methods allowing the identification of blood meals of mosquitoes beyond the ordinal level (i.e. species of passeriform), because such preferences may result in important differences in pathogen amplification (Kilpatrick *et al.* 2006).

Molecular approaches

The development of molecular techniques such as polymerase chain reaction (PCR) and sequencing has favoured the identification of vertebrate hosts of mosquitoes to species level (Kent 2009). These methods are based on the amplification of fragments of nuclear or mitochondrial genes of the vertebrate hosts using either species-specific primers or universal primers that are able to

amplify the DNA of a wide range of vertebrates. Different molecular markers have been developed for this objective (Table 1; Borland and Kading 2021). However, the utility of these techniques is also limited by factors including the availability of enough well-preserved genetic material in the mosquito blood meal. The success of host identification decreases as the degradation of the blood meal increases as supported by studies on mosquito species including *Aedes aegypti*, *Anopheles atroparvus* or *Cx. quinquefasciatus* (Martínez-de la Puente *et al.* 2013, Oshaghi *et al.* 2006, Santos *et al.* 2019), although positive identifications are possible after 30-36 hours post ingestion (Oshaghi *et al.* 2006), or even, for up to 72 h after blood feeding (Ngo and Kramer 2003). Unfortunately, for the case of mosquitoes trapped under natural conditions, it is not possible to know the time spanned since blood meal ingestion to mosquito capture. In these cases, mosquitoes could be sorted according to their Sella score which consist of a scale from 1, corresponding to mosquitoes with a recent blood meal, to 7, corresponding to mosquitoes without visible blood and eggs fully developed in their abdomen (Detinova and Bertram 1962). This may be especially useful in wild-trapped mosquitoes, as the Sella scoring of mosquitoes' blood meals was negatively correlated with the success of host identification using DNA amplification and sequencing (Martínez-de la Puente *et al.* 2013, Santos *et al.* 2019).

Other potential limitation of molecular methods is the availability of reference libraries with the nuclear and/or mitochondrial gene sequences for the potential vertebrate hosts (Borland and Kading 2021). Mitochondrial DNA (mtDNA) remains a popular and advantageous sequencing target for arthropod blood meal identification (Kent 2009). A wide variety of animals are able to be identified by mtDNA markers including 12S and 16S Mitochondrial Ribosomal DNA (rDNA) (Collini *et al.* 2015), NADH Dehydrogenase Subunit I (ND1) (Toma *et al.* 2014), the cytochrome b (*cyt* b) and the cytochrome c oxidase subunit I (Cox1 or COI) genes, the last being the most frequently used (Alcaide *et al.* 2009, Kent 2009, Kent and Norris 2005). There are different public databases with information on the genetic sequences for many vertebrate species, including the Barcode of Life initiative, which use the COI gene as a barcode to genetically characterise the worldwide diversity of vertebrates (Hebert *et al.* 2003). Nowadays the Barcode Records on BOLD system (https://www.boldsystems.org/index.php) included 723,297 animal sequences corresponding to 45,744 species. Thus, identification of the vertebrate hosts of mosquitoes could be accurately reached by comparing the obtained sequence from the mosquito blood meals with those available in public databases. In those cases where there are not previous characterisations of the COI sequences of species, using these tools it is also possible to identify the hosts of mosquitoes at lower accurately taxonomic levels (e.g. Family). However, contrary to the case of the *cyt* B and COI genes, for other genes as the prepronociceptin gene (Pnoc) (Hadj-Henni *et al.* 2015) or Nuclear ribosomal DNA (rDNA) markers, the reference libraries available are limited (Reeves *et al.* 2018). In addition, the low variability in the Pnoc sequences of closely related vertebrate species, as in the case of horses and donkeys, may limit the accuracy of host identification (Hadj-Henni *et al.* 2015). This limitation should be considered together with the fact that mammal red blood cells are not nucleated, so the DNA available for molecular analyses based on the amplification of nuclear genes may be lower in these vertebrates compared to groups such as birds.

Mosquitoes´ blood meals from a single host are frequently reported, however mosquitoes are able to feed on different host individuals to complete their blood meals. Records of mixed blood meals exists for a number of species such as *Culex pipiens*, *Aedes albopictus*, or *Aedes japonicus* (Cebrián-Camisón *et al.* 2020) which include mosquitoes fed on different host species or on different individuals of the same species. In the case of molecular approaches, the analyses of these mixed blood meals may provide ambiguous DNA sequences with double peaks in the sequencing electropherograms. With the help of a reference database of potential vertebrate hosts and/

or the amplification of host DNA using specific-designed primers would be possible to identify bloodmeals from different hosts species (Alcaide *et al.* 2009, Kent 2009). This method could be also useful if intraspecific variability in the amplified region exists within species, as in the case of the different haplotypes of sheep identified in the blood meal of several hematophagous vectors in Spain (Calvo *et al.* 2012). However, when this is not the case, alternative methods could be used including the amplification and sequencing of highly variable DNA regions (i.e. microsatellites) at least, when information on the genetic characteristics of the potential hosts of mosquitoes is available (Yan *et al.* 2018).

Molecular fingerprinting based on the identification of host microsatellites have been used to accurately track the hosts of mosquitoes below the species level in studies of the transmission of pathogens affecting humans and other animals. For example, Scott *et al.* (2006) identified the differential susceptibility of humans within a population to the bites of human-malaria vectors. Similarly, these approaches have been also used in studies on the host selection of mosquitoes using different animal models including house sparrows (Yan *et al.* 2018) and pigs (Keven *et al.* 2019). Moreover, these approaches have been applied in studies on conservation biology, to track the individuals of endangered species such as the Iberian lynx (*Lynx pardinus*) fed by *An. atroparvus* in southern Spain (Martínez-de la Puente *et al.* 2015a). Because mosquitoes have a limited dispersal range after feeding on blood (for example, *Cx. quinquefasciatus* reported a maximum of 168 meters based on the study developed by Greenberg *et al.* 2012), this method may provide information on the home range of the target vertebrate individuals. Furthermore, the blood meals of mosquitoes can be also used to identify certain characteristics of their hosts. For example, Burkett-Cadena *et al.* (2014) found that *Culiseta melanura*, *Culex erraticus* and *Culex nigripalpus* female mosquitoes feed more frequently from male than female birds. Similarly, the molecular identification of the sex of vertebrate hosts provides information on host selection by mosquitoes which could be used to build epidemiological models of vector-borne diseases (Teltscher *et al.* 2021). This low-cost methodology allows researchers to study the role of sexual differences in the host utilisation by mosquitoes potentially explaining sex related differences in the incidence of vector borne pathogens.

One important challenge for studies on the identification of mosquito blood meals is the development of *omics* platforms to use in the field. For example, genomic technologies with field-adapted protocols and powerful miniaturised laboratories designed specifically for field deployment, such as MinION (Giordano *et al.* 2017, O'Guinn *et al.* 2004). This platform has demonstrated efficacy in *cyt b* sequencing from wildlife samples (Seah *et al.* 2020), thus being potentially useful to trace the hosts of mosquitoes.

Mass spectrometry associated techniques

Different mass spectrometry techniques have been recently used to determine the origin of arthropod blood meals, including mosquitoes. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) allows to identify host by specifically targeting proteins present in blood fed ticks and triatome (Keller *et al.* 2017, Önder *et al.* 2013). The obtained spectrum can be compared to theoretical spectra generated from known protein sequences and publicly available DNA and protein sequences in databases. Haemoglobin proteins are a well-studied and stable molecule showing an important potential for this technique, especially to analyse degraded insect´s blood meals. In addition, quantitative LC-MS/MS may allow researchers to quantify the amounts of haemoglobin and to detect potential mixed blood meals of mosquitoes (Gerber *et al.* 2003, Natarajan *et al.* 2015). However, this technique shows some important limitations including

the availability of reference sequences of potential host species in published databases. In addition, haemoglobin proteins show high variability across vertebrates, showing some species polymorphic haemoglobin peptides which will reduce the resolution of host identification (Borland and Kading 2021).

The matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) has been recently used to assess the feeding patterns of mosquitoes. MALDI-TOF MS is based on a matrix of proteins that are ionised and, based on their mass-charge relationship, a specific spectrum profile of the analysed species is generated, capable of discerning even closely related species of primates or felines (Niare *et al.* 2016, Tandina *et al.* 2018). MALDI-TOF MS is a quick and easy method to analyse mosquito blood meals using currently available software (Niare *et al.* 2016). However, the use of this technique is limited by the absence of public profile databases. Nowadays, most laboratories build their own libraries considering a limited number of mosquito/host species (Niare *et al.* 2016, Tandina *et al.* 2018). This fact may compromise the use of these approaches for the study of mosquito blood meals of insects collected in the field (Niare *et al.* 2016). Furthermore, as previously discussed for the case of molecular approaches, the degradation of blood meals may affect the quality of the results obtained limiting the accuracy of host identification using MALDI-TOF MS (Niare *et al.* 2016). Vertebrate hosts of mosquitoes could be identified up to 24 h post ingestion, although factors including the storage of the samples could affect to the result obtained (Niare *et al.* 2016). Finally, it is important to highlight that MALDI-TOF have been also used for the identification of mixed blood meals of mosquitoes being able to detect the last and mixed blood meals of *Anopheles gambiae* and *Ae. albopictus* mosquitoes, although it was impossible to identify the first blood meal in successive blood meals (Tandina *et al.* 2020).

Additional information obtained from mosquito blood meals

In addition to reveal mosquito-host interactions, engorged mosquitoes can be also used to monitor the pathogens harboured by the hosts of these insect vectors. This approach known as xenosurveillance was defined as a new surveillance technique that utilises the mosquito blood meals to sample host pathogens (Grubaugh *et al.* 2015); that allows researchers to identify the contact rates of potential mosquito vectors and the pathogens which could transmit (Martínez-de la Puente *et al.* 2015b). These studies may be improved by the use of next-generation sequencing technology which is able to detect a high spectrum of pathogens could be used to detect all the potential pathogens harboured by mosquitoes that could be actively circulating in a particular area. On the other hand, mosquito blood meals could be used to obtain physiological information of the vertebrate hosts of mosquitoes. Blood meals of tse tse flies (Habicher *et al.* 2013) or kissing bugs (Voigt *et al.* 2006) have been used for the quantification of host´s hormones allowing, in example, the determination of pregnancy in female mammals. These approaches could be potentially used for the case of mosquitoes, although its application could be limited by the volume of the blood meal.

Concluding remarks

Mosquito blood meals provide a source of host material which could be used to identify the vertebrate hosts of mosquitoes up to the individual level. Different approaches have been developed during the last decades, with the molecular approaches likely being the most commonly used. However, the different techniques used to identify mosquito feeding have their advantages and disadvantages. Undoubtedly, the technique to be used in each case must be

previously evaluated based on the availability of specialised laboratory equipment and resources, the quality and conservation of the samples and the accuracy of the required identifications. Different approaches could be combined to obtain the most valuable results. For example, the precipitation test such as ELISA can be combined with a subsequent PCR for the specific identification of bird species (Apperson *et al.* 2004). Likewise, given the limited number of species included in the databases of the spectrum generated by MALDI-TOF MS, molecular methods can be used to overcome this limitation, allowing greater specificity in the identification of the hosts of mosquitoes. Altogether, the information obtained from mosquito blood meals may provide a general overview of the importance of the different species of mosquitoes for the transmission of mosquito-borne pathogens of public and animal health relevance.

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