USING MOSQUITO EXCRETA TO ENHANCE MOSQUITO-BORNE DISEASE SURVEILLANCE

by

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

August 2019

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ABSTRACT

Mosquito-borne pathogens, such as arthropod-borne viruses (arboviruses), parasites and bacteria infect millions of people and animals annually, causing a significant burden to populations living in tropical and subtropical regions of the world. At the same time, changes in land use, climate and biodiversity, together with rapid modes of transportation, have facilitated the spread of these pathogens to areas outside their previously known distribution ranges. With few exceptions, there are no vaccines available against many of these pathogens. Because of this, mosquito control is the primary tool for preventing and reducing the spread of diseases caused by these pathogens. Within this framework, mosquito-borne disease surveillance is fundamental to act ideally as an early warning system or to guide the implementation of control measures and field-based research. Different approaches, summarised in Chapter 2 of this thesis, can be utilised for mosquito-borne disease surveillance, from reporting of human and animal cases, to the use of sentinel animals, and the collection of mosquitoes and subsequent testing for the presence of pathogens. Regarding the latter, field and laboratory studies of mosquito-borne pathogens that require analysis of mosquito samples usually utilise individual or pooled mosquitoes, a body component such as legs and wings, or secretion such as saliva.

Mosquito excreta has been proposed as an alternative sample type for xenomonitoring of parasitic diseases caused by infection with *Brugia malayi* and *Plasmodium vivax*. Recently, it has been demonstrated that *Aedes aegypti* with a disseminated infection excrete dengue virus RNA, which could be used to enhance the sensitivity of mosquito-borne disease surveillance methods. This thesis examines the application of mosquito excreta as a sample type for the detection of mosquito-borne pathogens, such as flaviviruses, alphaviruses and *Plasmodium* in laboratory and field studies. Components include 1) the applicability of mosquito excreta as a sample type for detection of two important Australian arboviruses; 2) the evaluation of the stability of viral RNA in excreta deposited on different substrates under tropical conditions; 3) the concurrent detection of *Plasmodium falciparum* in mosquito excreta, saliva and mosquito salivary glands; and 4) the feasibility of using mosquito excreta for environmental virome sampling by next-generation sequencing (NGS)-based metagenomics.

To assess the applicability of mosquito excreta for the detection of two arboviruses of importance in the Australian context, mosquitoes were exposed to bloodmeals containing either Ross River (RRV) or West Nile virus (WNV). Excreta was collected daily from groups of mosquitoes and analysed by real-time reverse-transcription polymerase chain reaction (RT-rtPCR) or cell culture immunoassay. Similarly, excreta from individual mosquitoes was collected at different time-points and the mosquito's infection status was assessed. For both viruses, viral RNA was detected in excreta from groups of mosquitoes continuously from day 2 to day 15 post-exposure. Viral RNA was also detected from individual mosquitoes at all sampled time-points, and it was correlated with viral dissemination in the mosquito. When comparing detection of viral RNA in excreta versus saliva, the proportion of positive samples was higher for excreta, suggesting that mosquito excreta offers an attractive sample for analysis in laboratory or field situations. Finally, only low levels of infectious virus were detected by cell culture, suggesting a relatively low risk of exposure to personnel handling mosquito excreta.

With the aim of incorporating the collection of mosquito excreta in mosquito traps used for mosquito-borne disease surveillance, the stability of viral RNA in mosquito excreta deposited on different substrates under tropical conditions was evaluated. For this, mosquitoes were exposed to bloodmeals containing WNV and after bloodmeal digestion finalised, mosquitoes were allowed to excrete for 24 hours on either a polycarbonate substrate or a Flinders Associate Technologies (FTA®) nucleic acid preservation card. The mosquitoes were removed, and the samples were either collected (to determine a baseline) or stored in an environmental growth cabinet simulating tropical conditions for 7 and 14 days. Once collected, samples were analysed by RT-rtPCR. No difference was observed in the relative quantity of viral RNA detected on either substrate after 24 hours. However, after 7 and 14 days, there was a significant reduction in the amount of viral RNA detected on polycarbonate. For integration in surveillance programmes, these results suggest that polycarbonate substrates can be used for collecting excreta in traps deployed overnight, whilst FTA® cards are a better alternative in traps that are serviced weekly or fortnightly.

In the context of malaria surveillance strategies, the concurrent detection of *Plasmodium falciparum* in mosquito excreta and saliva by RT-rtPCR was evaluated. For this, mosquitoes were exposed to bloodmeals containing cultured gametocytes and their excreta was collected daily. Additionally, saliva was collected on honey-soaked filter paper cards, and mosquito salivary glands were dissected and examined under the microscope for the presence of sporozoites. Similar to the arboviruses, *P.*

falciparum RNA was detected in excreta as early as four days post-exposure and once the development of sporozoites occurred, it was detected concurrently in both excreta and saliva samples, with a positive association between molecular detection of the parasite in both samples and the proportion of mosquitoes with visible sporozoites in their salivary glands from each container.

To evaluate the feasibility of using mosquito excreta for environmental virome sampling by NGS-based metagenomics, excreta from both experimentally-infected and field-collected mosquitoes from north and southeast Queensland was collected. Total RNA was extracted and reverse transcribed to complementary DNA. Libraries were sequenced using the Illumina NextSeq 500 platform. Bioinformatic analyses demonstrated that mosquito excreta provides sufficient template for NGS, allowing assembly of near-full length virus genomes. Furthermore, this method allowed the identification of seven potentially novel viruses indicating that metagenomic analysis of mosquito excreta has potential for virus discovery and, in the future, unbiased arbovirus surveillance.

The outcomes of this thesis demonstrate that the excretion of pathogens is a general phenomenon that can be exploited in field and laboratory studies of mosquito-borne diseases, with applications ranging from vector competence experiments to enhancing arbovirus surveillance systems. The use of mosquito excreta for mosquito-borne disease surveillance has several advantages since it allows earlier detection of a circulating pathogen, presents a relatively low risk to personnel handling the samples, and viral RNA in excreta is stable in tropical conditions. Finally, mosquito excreta can be used as a sample type for NGS-based metagenomics allowing for detection of arboviruses and virus discovery. Mosquito excreta is the latest addition to the array of sample types available to study and ultimately prevent the spread of vector-borne diseases.

PUBLICATIONS

Please Note: The format of the manuscripts incorporated into the thesis is consistent with that required by the relevant journal.

Ramírez AL, van den Hurk AF, Meyer DB, Ritchie SA. 2018. Searching for the proverbial needle in a haystack: advances in mosquito-borne arbovirus surveillance. Parasit. Vectors 11:320

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Ramírez AL, Hall-Mendelin S, Hewitson GR, McMahon JL, Staunton KM, Ritchie SA, van den Hurk AF. 2019. Stability of West Nile virus RNA in mosquito excreta. J. Med. Entomol. 56: 1135-1138

Ramírez AL, van den Hurk AF, Mackay IM, Yang ASP, Hewitson GR, McMahon JL, Boddey JA, Ritchie SA, Erickson SM. 2019. Malaria surveillance from both ends: concurrent detection of *Plasmodium falciparum* in saliva and excreta harvested from *Anopheles* mosquitoes. Parasit. Vectors 12: e0006771

Ramírez AL, Colmant AMG, Warrilow D, Huang B, Pyke AT, McMahon JL, Meyer DB, Graham RMA, Jennison AV, Ritchie SA, van den Hurk AF. Metagenomic analysis of mosquito excreta for environmental virome sampling. *In preparation*

OTHER PUBLICATIONS

Meyer DB, **Ramírez AL**, van den Hurk AF, Kurucz N, Ritchie SA. 2019. Development and field evaluation of a system to collect mosquito excreta for the detection of arboviruses. J. Med. Entomol. 56: 1116-1121

STATEMENT OF CONTRIBUTION OF OTHERS

My supervisors Scott Ritchie, Andrew van den Hurk, Dagmar Meyer and Susan Laurance assisted with intellectual support throughout my candidature. Unless mentioned otherwise, all the experiments described in this thesis were performed at Public Health Virology, Forensic and Scientific Services, Queensland Health, where I held a student placement under the supervision of Andrew van den Hurk. The mosquito exposures and sample collection described in Chapter 5 were conducted at the Walter and Eliza Hall Institute of Medical Research under the supervision of Sara Erickson. I received financial support in the form of an Australian Institute of Tropical Health and Medicine PhD scholarship. James Cook University waived the tuition fees and provided an internal research account. I received additional funding in the form of an HDRES grant and a publication grant provided by the College of Public Health, Medical and Veterinary Sciences. My research was funded by grants awarded to Scott Ritchie and Andrew van den Hurk including a National Health and Medical Research Council-funded 'Improving Health Outcomes in the Tropical North: A Multidisciplinary Collaboration (HOT NORTH)' grant (number 1131932), an internal Forensic and Scientific Services Research and Development Project grant (number RSS17-031) and a Queensland Government funded Australian Institute of Tropical Health and Medicine Capacity Building Grant.

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Chapter 3 published as: **Ramírez AL, Hall-Mendelin S, Doggett SL, Hewitson GR, McMahon JL, Ritchie SA, van den Hurk AF. 2018. Mosquito excreta: A sample type with many potential applications for the investigation of Ross River virus and West Nile virus ecology. PLoS Negl. Trop. Dis. 12: e0006771**
The study was conceptualised by Ana Ramírez, Scott Ritchie and Andrew van den Hurk. Stephen Doggett provided *Aedes vigilax* eggs and Scott Ritchie provided field collected mosquitoes used in this experiment. Ana Ramírez completed the laboratory infections, sample collection, vector competence experiments and cell culture assays with advice provided by Andrew van den Hurk and Sonja Hall-Mendelin.

Glen Hewitson and Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Data curation, formal analysis, visualisation and writing of the original manuscript was done by Ana Ramírez. Sonja Hall-Mendelin, Stephen Doggett, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Scott Ritchie and Andrew van den Hurk acquired funding for this experiment.

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The study was conceptualised by Ana Ramírez, Scott Ritchie and Andrew van den Hurk. Ana Ramírez and Andrew van den Hurk collected the mosquitoes used in this experiment. Ana Ramírez completed the laboratory infections and sample collection with advice provided by Andrew van den Hurk and Sonja Hall-Mendelin. Glen Hewitson and Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Ana Ramírez analysed the samples using RT-rtPCR and cell culture assays. Kyran Staunton assisted Ana Ramírez with data analysis. Data curation, formal analysis, visualisation and writing of the original manuscript was done by Ana Ramírez. Sonja Hall-Mendelin, Kyran Staunton, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Scott Ritchie and Andrew van den Hurk acquired funding for this study.

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The study was conceptualised by Ana Ramírez, Andrew van den Hurk, Scott Ritchie and Sara Erickson. Sara Erickson exposed the mosquitoes to gametocyte cultures. Ana Ramírez and Sara Erickson performed the experiments and collected the samples. Ian Mackay developed the molecular assay used in this study. Glen Hewitson and Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Data curation, formal analysis, visualisation and writing of the original manuscript was done by Ana Ramírez. All authors read and approved the final manuscript. Andrew van den Hurk, Justin Boddey, Annie Yang and Sara Erickson provided the study materials. Scott Ritchie, Justin Boddey and Andrew van den Hurk acquired funding for the research.

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The study was conceptualised by Ana Ramírez, Andrew van den Hurk and Scott Ritchie. Ana Ramírez and Andrew van den Hurk completed the laboratory mosquito exposures, conducted the field work and collected the samples. Dagmar Meyer assisted Ana Ramírez in different aspects of sample collection in north Queensland. Ben Huang and Alyssa Pyke assisted Ana Ramírez with library preparation and sequencing. Rikki Graham and Amy Jennison provided advice and equipment used for sequencing. Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Ana Ramírez completed the bioinformatic analyses with advice provided by Agathe Colmant, David Warrilow and Alyssa Pyke. Data curation, formal analysis, visualisation and writing of the original manuscript was done by Ana Ramírez. Agathe Colmant, David Warrillow, Ben Huang, Alyssa Pyke, Amy Jennison, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Andrew van den Hurk and Scott Ritchie acquired funding for this study.

A signed statement of contribution of others is provided in Appendix A. Every reasonable effort has been made to gain permission and acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

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I want to thank Scott for his incredible support and encouragement through this adventure. From teaching me the differences between *Culex annulirostris* and *Culex sitiens*, to his valuable lessons on the important skill of cat herding, Scott provided me with the expertise and network necessary to ensure that my research was successful. Scott is an infinite source of mosquito knowledge and I'll be forever grateful for being trained by the one and only. His enthusiasm and creativity are contagious, and he is truly an inspiration. Hopefully one day we will be able to answer the most important question of all: is it mosquito poo or mosquito pee?

There are no words to express my gratitude to Andrew, from whom I have learned so much over the past years. Andrew always believed in my potential and set the bar high, teaching me to always go the extra mile (it is always worth it!). He has been the best mentor one can ask for. Andrew taught me not only the obscure arts of mosquito laboratory and field-work, but also how to play the game. He made this experience so much fun; I will never forget the hundreds of legs and wings removed, MacGyvering traps, getting covered in bat excreta and our close encounter with a very big eastern brown snake. Stay metal.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

ACT – Artemisinin-based combination therapy
BFV – Barmah Forest Virus
CC-EIA – Cell culture enzyme immunoassay
CDC – Centers for Disease Control and Prevention
CO_2 – Carbon dioxide
C_t – Cycle threshold
DENV – Dengue virus
DIAMOND – Double index alignment of next-generation sequencing data
DNA – Deoxyribonucleic acid
EEEV – Eastern equine encephalitis virus
EIP – Extrinsic incubation period

ELISA – Enzyme-linked immunosorbent assay
FP – Filter paper
EVS – Encephalitis vector survey
GAT – Gravid *Aedes* trap
GM – Growth media
IgG – Immunoglobulin G
ISF – Insect-specific flaviviruses
JEV – Japanese encephalitis virus
CHIKV – Chikungunya virus
FBS – Foetal bovine serum
FP – Filter paper
FTA – Flinders Technologies Associates
malERA – Malaria Eradication Research Agenda
MEGAN –Metagenome Analyzer
MVEV – Murray Valley encephalitis virus
NNDSS – National Notifiable Disease Surveillance System
NGS – Next-generation sequencing
PCR – Polymerase chain reaction
PBS – Phosphate buffer saline
PBT – Passive box trap
PE – Post-exposure
qRT-PCR – Real time quantitative RT-PCR
RDT – Rapid diagnostic test
RH – Relative humidity
RNA– Ribonucleic acid
RRV – Ross River Virus
RT-PCR – Reverse transcription polymerase chain reaction
RT-rtPCR – Real time RT-PCR
SLEV – St. Louis encephalitis virus
$TCID_{50}$ – Tissue culture infectious $dose_{50}$
SMACK – Sentinel mosquito arbovirus capture kit
UAV – Unmanned aerial vehicle
WEEV – Western equine encephalitis virus
WNV – West Nile Virus
WNV_{KUN} – West Nile Virus Kunjin subtype
YFV – Yellow fever virus
ZIKV – Zika virus

1

INTRODUCTION

Vector-borne pathogens are responsible for almost a fifth of the total global burden of infectious diseases, causing more than 700,000 deaths annually, with more than 80% of the population living in areas of risk. These pathogens exact their greatest toll in tropical and subtropical regions, where they affect the more disadvantaged populations (WHO 2017). However, in recent years, these pathogens have started to occur in areas outside of their previously known distributions, as exemplified by the spread of West Nile (Gubler 2007), Japanese encephalitis (van den Hurk et al. 2001), chikungunya (Petersen and Powers 2016) and Zika viruses (Baud et al. 2017). Although difficult to forecast, it is likely that anthropogenic changes in climate, land use and biodiversity will have an effect on the spread of mosquito vectors, host-pathogen interactions and the emergence or re-emergence of zoonotic diseases. (Rizzoli et al. 2019). Additionally, rapid modes of transportation such as air travel have facilitated the movement of viraemic/parasitaemic people and vectors across the globe resulting in the emergence of vector-borne pathogens in novel areas and vectors (Kilpatrick and Randolph 2012).

There are around 3,000 mosquito species in the world, but only about 100 of these species are epidemiologically important in their role in transmission of pathogens to humans (Rozendaal 1997). Nevertheless, mosquitoes are the best-known vectors of vector-borne diseases (Bartlow et al. 2019) and are considered the deadliest animals in the world (Kamerow 2014). Mosquitoes transmit arthropod-borne viruses (arboviruses), parasites and bacteria that infect millions of people and animals. The dengue viruses are responsible for $\sim$ 96 million clinical cases per year (Bhatt et al. 2013), with mortality rates ranging between $<$ 1% to 30% for severe cases if left untreated (Edelman 2005). Malaria, the vector-borne disease with the highest death toll, caused millions of cases and more than 400,000 deaths in 2017 alone (WHO 2018).

With the exception of yellow fever and Japanese encephalitis viruses (Frierson 2010, Hegde and Gore 2017), there are no vaccines currently available against most of mosquito-borne pathogens, so mosquito management is often the only option for prevention and control of these diseases. In this context, mosquito-borne disease surveillance is crucial for detecting elevated pathogen activity and act as an

early warning system to guide the implementation of control measures (Ramírez et al. 2018). Over the last decade, novel technologies have been developed to detect evidence of pathogen circulation in wild mosquito populations. Chapter 2 of this thesis provides a review of the literature on traditional and novel methods used for arbovirus surveillance. From the use of sentinel animals, reporting of human cases and detection of viruses in mosquitoes to the development of sugar-based surveillance systems and next-generation sequencing, the review of the literature identifies opportunities to enhance mosquito-borne disease surveillance and sets the context for this thesis.

Field and laboratory investigation of mosquito-borne pathogens requires analysis of mosquito samples, either individually, in pools, or a body component, or secretion such as saliva. It has been recently demonstrated that dengue virus RNA can be detected in the excreta of mosquitoes with a disseminated dengue infection (Fontaine et al. 2016) which can potentially be exploited to enhance mosquito-borne disease surveillance. Similarly, mosquito excreta has been proposed as a sample type for surveillance or xenosurveillance of *Plasmodium* parasites (Pilotte et al. 2016, Cook et al. 2017). However, it is not known if the excretion of nucleic acid applies to other pathogens, and, in the case of *Plasmodium*, how soon after a blood meal and for how long it can be detected and its correlation with sporozoite detection in salivary glands.

The overarching objective of the experiments presented in this thesis was to evaluate the application of mosquito excreta as a sample type for the detection mosquito-borne pathogens, such as flaviviruses, alphaviruses and *Plasmodium* in laboratory and field studies. This thesis is divided into seven chapters: a review of the literature on methods for arbovirus surveillance, five empirical chapters and a final chapter integrating and discussing the outcomes of all chapters.

In Chapter 3, I assessed the applicability of mosquito excreta as a sample type for detection of the arboviruses, Ross River virus, (Togaviridae: Alphavirus) and West Nile virus (Flaviviridae: Flavivirus) from groups and individual mosquitoes sampled during the extrinsic incubation period of the virus. I tested excreta samples by reverse-transcription real-time polymerase chain reaction (RT-rtPCR), and the detection of viral RNA in excreta was correlated with the infection status of the mosquito. I compared the results obtained from excreta samples with results obtained from saliva samples obtained during sugar feeding (van den Hurk et al. 2007). Finally, I tested whether the virus excreted by mosquitoes was infectious to determine if mosquito excreta could pose a workplace health and safety hazard

for personnel handling excreta samples. The results from this chapter provided me with outcomes that led me to further assess the utility of detecting viruses in mosquito excreta in the field (Chapter 4).

Indeed, the results obtained from Chapter 3 indicate that mosquito excreta has potential to be used as a sample type for detecting arboviruses in the field. With the aim of incorporating the collection of mosquito excreta in mosquito traps, in Chapter 4, I evaluated the stability of West Nile virus RNA in excreta deposited on different substrates held at tropical conditions for up to two weeks. Based on the results from this experiment, I was able to provide recommendations on methodology to enhance collection of mosquito excreta for downstream processing and detection of arboviruses. Excitingly, it has been recently demonstrated that arboviruses can be detected in field-collected mosquito excreta samples using this approach (Meyer et al. 2019).

In Chapter 5 of this thesis, I collected the excreta and saliva over the duration of the period of sporogony from mosquitoes that were previously exposed to blood-meals containing gametocytes of *P. falciparum*. Excreta samples were analysed by RT-rtPCR and the results compared with the presence of sporozoites in the salivary glands as assessed by dissection and light microscopy. The findings presented in this chapter are the first report of concurrent detection of *P. falciparum* nucleic acid in mosquito excreta and saliva from the same mosquito cohorts.

Based on the results obtained in the previous chapters, I evaluated the applicability of mosquito excreta for environmental virome sampling by next-generation sequencing (NGS)-based metagenomics (Chapter 6). Samples from experimentally infected mosquitoes were sequenced and analysed for the presence of arboviruses, indicating that it is possible to assemble near-full length arbovirus genomes from excreta samples. Based on these results, field-collected samples from north and south east Queensland were sequenced and analysed for the presence of RNA viruses, showing the rich virome of mosquito excreta, including seven potentially novel viruses and suggesting that mosquito excreta can be used for virus discovery. This is the first reported study to investigate mosquito excreta as samples for NGS-based metagenomics.

Finally, Chapter 7 integrates the findings from all chapters of this thesis, and discusses the opportunities and limitations of using mosquito excreta for mosquito-borne disease surveillance in the field or in laboratory studies.

2

SEARCHING FOR THE PROVERBIAL NEEDLE IN A HAYSTACK: ADVANCES IN MOSQUITO-BORNE ARBOVIRUS SURVEILLANCE

The work presented in this chapter, entitled "Searching for the proverbial needle in a haystack: advances in mosquito-borne arbovirus surveillance" was published in Parasites and Vectors, May 2018. doi: 10.1186/s13071-018-2901-x

2.1 CONTEXTUAL LINKAGE

Arbovirus surveillance is critical for the prevention and control of mosquito-borne arboviruses. In this chapter, I review the literature on traditional and novel methods used for arbovirus surveillance and describe the strengths and limitations of using different approaches.

Ramírez et al. Parasites & Vectors (2018) 11:320
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Parasites & Vectors

REVIEW Open Access

Searching for the proverbial needle in a haystack: advances in mosquito-borne arbovirus surveillance

Ana L. Ramírez[1*], Andrew F. van den Hurk[3], Dagmar B. Meyer[1,2] and Scott A. Ritchie[1,2]

Abstract

Surveillance is critical for the prevention and control of mosquito-borne arboviruses. Detection of elevated or emergent virus activity serves as a warning system to implement appropriate actions to reduce outbreaks. Traditionally, surveillance of arboviruses has relied on the detection of specific antibodies in sentinel animals and/or detection of viruses in pools of mosquitoes collected using a variety of sampling methods. These methods, although immensely useful, have limitations, including the need for a cold chain for sample transport, cross-reactivity between related viruses in serological assays, the requirement for specialized equipment or infrastructure, and overall expense. Advances have recently been made on developing new strategies for arbovirus surveillance. These strategies include sugar-based surveillance, whereby mosquitoes are collected in purpose-built traps and allowed to expectorate on nucleic acid preservation cards which are submitted for virus detection. New diagnostic approaches, such as next-generation sequencing, have the potential to expand the genetic information obtained from samples and aid in virus discovery. Here, we review the advancement of arbovirus surveillance systems over the past decade. Some of the novel approaches presented here have already been validated and are currently being integrated into surveillance programs. Other strategies are still at the experimental stage, and their feasibility in the field is yet to be evaluated.

Keywords: Arboviruses, Surveillance, Mosquito, Sentinel animals, Honey-based surveillance, Next-generation sequencing

Background

Arthropod-borne viruses (arboviruses) transmitted by mosquitoes are of public health and veterinary importance globally causing disease syndromes including encephalitis, viral haemorrhagic disease and arthritis. Dengue viruses (DENVs) alone cause an estimated 96 million clinical cases a year, especially in the tropics and sub-tropics [1]. The flaviviruses, Japanese encephalitis virus (JEV) and West Nile virus (WNV), are major causes of viral encephalitis throughout their geographical range. Recently, the expansion of chikungunya (CHIKV) [2] and Zika (ZIKV) [3] viruses in the Western Hemisphere, and the yellow fever (YFV) outbreaks in Africa [4] and Brazil [5] have highlighted the continuing threat emerging and re-emerging arboviruses pose.

With the exception of YFV [6] and JEV [7], there are currently few vaccines or antiviral drugs available against most of these viruses. Thus, prevention and control of most arboviruses is almost solely reliant on effective mosquito management. This can be enhanced by surveillance, where detection of elevated or emergent virus activity serves as a warning system to implement appropriate actions to reduce the severity and duration of outbreaks. However, designing an appropriate arbovirus surveillance system is challenging. Arboviruses have complex transmission cycles with dual-host tropism: they replicate in vertebrate hosts (such as birds or mammals) and arthropod hematophagous vectors (such as mosquitoes or ticks) [8]. This complexity needs to be accounted for, and an ideal surveillance system should rely on different sources of information (Fig. 1), and can include

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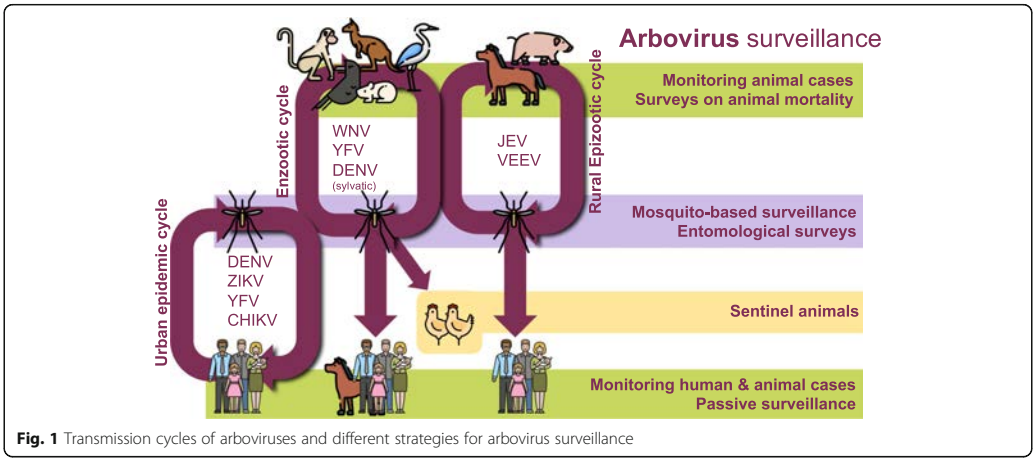

Fig. 1 Transmission cycles of arboviruses and different strategies for arbovirus surveillance

meteorological data, evidence of virus infection in vertebrate hosts, entomological surveys, virus detection in vectors, and reports of human or animal disease. The scale of surveillance can vary regionally [9] and is particularly challenging in remote locations, or in areas with limited resources and infrastructure.

Given the broadness of this subject, few attempts have been made to provide a synthesis of arbovirus surveillance methods. The objective of this review is to describe the development and implementation of mosquito-borne arbovirus surveillance strategies. First, we evaluate traditional methods that have been commonly used where arboviruses are a public health threat, then outline and assess recently developed methodologies, before identifying future research needs.

Methods for arbovirus surveillance

Monitoring human and animal disease

Human or animal case surveillance relies on hospitals, laboratories and health practitioners notifying public health authorities of confirmed or suspected cases of arbovirus infection that occur in the population. Almost every state in the United States conducts surveillance of human WNV cases as a part of the national arbovirus surveillance system, ArboNET [10], whilst in Australia, human arbovirus disease notifications are monitored using the National Notifiable Disease Surveillance System (NNDSS) [11]. These surveillance systems require strict case definitions and laboratory diagnostic testing criteria, as well as demographic, clinical, laboratory and epidemiological information [12]. In the summer and autumn of 1999, reports of dead crows played a critical role in identifying the outbreak of WNV in New York [13]. With bird cases often preceding human cases by up to 3 months, it served as an ideal early warning system for WNV [14]. In Argentina [15] and Brazil [16], dead howler monkeys acted as an early warning for sylvatic transmission of YFV and prompted vaccination campaigns in the human population in 2008 and 2017, respectively.

A major limitation of monitoring human and animal cases is that confirmatory laboratory testing is not available in many limited resource countries, so arboviral disease is diagnosed on clinical symptoms. However, symptoms can overlap between arboviruses, as well as with non-arbovirus pathogens, complicating their clinical diagnosis. Furthermore, most arbovirus infections are mild, or sub-clinical, which may lead to them being under-reported. Ultimately, using human and animal case data is not ideal, since it indicates that active transmission is already occurring.

Vertebrate host arbovirus surveillance: sentinel animals

Sentinel animals provide evidence of virus activity and increased risk to the target animal or human population [17]. For this, immunologically naïve animals are deployed in a specific location, bled on a defined schedule, and tested for the presence of virus-specific antibodies as an indication of exposure. Virus isolation or molecular detection on pre-seroconversion blood samples can provide an isolate and/or a sequence for genotypic analysis of circulating virus strains [18]. A suitable sentinel animal should: (i) be susceptible to the monitored virus; (ii) develop an antibody response that can be detected in serological assays; (iii) have low morbidity and mortality; (iv) be attractive to the vector; (v) be easy to handle; and (vi) allow for multiple sampling [12].

Different vertebrate species are used as sentinels (Table 1) and choice of animal is dependent on the target virus. In terms of WNV surveillance, some studies

Table 1 Animal species that have been used as sentinels for arbovirus surveillance

Animal	Virus	Example location	References
Chickens	WNV	USA, UK	[119, 120]
	SLEV	USA	[121]
	MVEV, WNV_{KUN}	Australia	[122]
Pheasants	WNV, SLEV, EEEV	USA	[123, 124]
Pigs	JEV	Japan, Australia, Thailand	[125–127]
Dogs	WNV	USA, Africa	[128, 129]
	JEV	Japan, Thailand	[130, 131]
Sheep and goats	RVFV	Africa, Saudi Arabia	[132–134]
Cattle	BTV, Akabane	Australia, Papua New Guinea, Japan	[135–137]
Horses	EEEV, WEEV	Argentina	[138]
	WNV, SLE	Colombia	[139]
Hamsters	EEEV, VEEV	USA, Central and South America	[140–143]
Non-human primates	YFV	Brazil, Argentina	[144–147]

Abbreviations: WNV, West Nile virus; SLEV, St. Louis encephalitis virus; MVEV, Murray Valley encephalitis virus; WNV_{KUN}, West Nile virus (Kunjin subtype); EEEV, eastern equine encephalitis virus; JEV, Japanese encephalitis virus; RVFV, Rift Valley fever virus; BTV, bluetongue virus; WEEV, western equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus; YFV, yellow fever virus

have suggested that the use of sentinel chickens is the most sensitive indicator of virus activity, when compared with other methods, such as detection of seroconversion in wild birds and virus isolation from mosquito pools [19]. Whilst they can undoubtedly serve as an early warning system, in some areas of the USA, sentinel chickens to monitor WNV have proven unsuccessful, since seroconversions were detected only after the onset of human cases [20, 21].

Even though sentinel animal surveillance enables the timely detection of circulating arboviruses, it also comes with limitations. In many cases, the locations of enzootic arbovirus foci are unknown or difficult to access. Thus, animals are placed near towns, which may be too far from virus foci to detect elevated activity [22]. Furthermore, some animals serve as amplifying hosts (i.e. pigs for JEV) increasing the risk of transmission to humans [23]. Additionally, the cost of rearing and replacing sentinel animals, especially in remote locations, can be prohibitive [24, 25], and bleeding large animals presents a workplace health and safety hazard [26]. There are also ethical considerations associated with the use of sentinel animals [27]. Finally, closely related viruses (i.e. JEV, WNV and Murray Valley encephalitis virus (MVEV)) can cross-react in some serological assays, requiring confirmation by other methods to obtain unequivocal results [28].

Another approach to vertebrate host surveillance relies on monitoring wild vertebrates or livestock, which are captured, sampled and released [12]. However, one of the biggest issues with surveillance of these animals is the cross-reaction between antibodies and the interpretation of the results. Given that many of these animals are mobile, it is difficult to determine exactly when and where an animal acquired the infection, especially since IgG antibodies are present for the life of the animal.

Mosquito-based arbovirus surveillance

Mosquito-based arbovirus surveillance monitors vector populations and virus infection prevalence within them. Mosquitoes are collected, identified, pooled by species or other taxonomic grouping, and sent to the laboratory where they are tested for virus infection status. There are different strategies for mosquito collection. In areas with low-level mosquito infections or early in the transmission season, efforts should be directed towards performing targeted surveillance at "hotspots" where a high likelihood of arbovirus presence is suspected; as vector populations increase later in the season, the number of sampling sites should be expanded for broader monitoring [29]. There are a variety of commercial traps designed to collect mosquitoes, the design of which and application have been comprehensively reviewed elsewhere [30, 31]. It is essential that the selection of the collection method takes into consideration the physiological and behavioural characteristics of the studied vector [32] (Table 2).

A variety of methods have been utilized for detection of arboviruses in captured mosquitoes. Historically, arbovirus isolations were conducted in animals, such as suckling mice and chickens. With the development and establishment of cell lines, virus isolation in cell culture became the gold standard for arbovirus detection from pools of mosquitoes. This method can only detect viable viruses, so a cold chain keeping samples at ultralow temperatures during transport needs to be maintained to

Table 2 Collection methods commonly used for mosquito-based arbovirus surveillance

Mosquito behaviour	Collection method	Advantages	Disadvantages	References
Host seeking	Human-landing catches[a]	Larger collections than resting or oviposition collections. Collections can be increased by using CO_2 or chemical lures	Most traps require batteries or AC power to operate. Depending on environmental conditions, the fan components are prone to malfunction. Require CO_2 as the primary attractant	[148]
	BG Sentinel			[149]
	CDC-light trap			[150]
	EVS-trap			[151]
	Mosquito Magnet™			[152]
	Animal baited traps			[153–155]
Resting	CDC-backpack aspirator	More blood fed mosquitoes collected, ideal for blood meal analysis	Labour intensive and inefficient mosquito capture	[156]
	Prokopack			[157]
	Resting boxes			[158–160]
Oviposition	Sticky ovitraps	Mosquitoes have bloodfed and thus a higher probability of detecting positive mosquitoes. Targets *Aedes*-borne viruses such as DENV and CHIKV	Smaller collections than other methods, thus all mosquitoes can be easily processed	[161–163]
	Gravid *Aedes* trap (GAT)			[66]
	CDC-gravid trap			[164]

[a]Although this method has been used for arbovirus studies in the past, it has considerable drawbacks, including the risk of infection to the collector, which is considered unethical even illegal in some countries

Abbreviations: CDC, Centers for Disease Control and Prevention; EVS, Encephalitis virus surveillance

preserve virus infectivity [33]. Maintenance of a cold chain requires the use of dry ice or liquid nitrogen shippers in the field, which can be logistically challenging. Virus isolation is time consuming and obtaining definitive results can take weeks, which defeats the purpose of using it for early warning. Some viruses do not replicate on common cell lines used in the laboratory. This can be the case for previously unrecognized or unknown viruses, such as insect-specific flaviviruses (ISF) that do not grow in vertebrate cells [34]. Virus isolation can be expensive and requires special infrastructure and trained personnel. However, even with these limitations, virus isolation is still an important method for arbovirus diagnostics, as it increases viral titer, which allows for full genome sequencing and provides viruses for phenotypic characterization.

Nucleic acid detection using RT-PCR has become one of the most popular methods of virus detection and has potentially displaced virus isolation as the new gold standard. Real time quantitative RT-PCR (qRT-PCR) platforms, such as TaqMan®, are ideal for routine testing of mosquitoes, since they reduce processing time significantly (sometimes to less than an hour), allowing for high throughput screening [35, 36]. Since these assays detect both infectious virus and RNA, they have comparable or better sensitivity than virus isolation [37]. Depending on the protocol or application, these techniques enable the detection of one infected individual from a pool of up to 5000 non-infected mosquitoes [38, 39]. Additionally, although a cold chain is still recommended, it has been possible to detect viral RNA from dead mosquitoes kept for several weeks in hot and humid conditions by qRT-PCR [33, 39, 40]. Currently, a variety of qRT-PCR assays exist for the detection of almost every arbovirus of human (and veterinary) importance, with some even available in multiplex format [41]. In spite of this, it is important to note that RT-PCR and qRT-PCR will only pick up RNA from viruses that the primers and probes were designed to detect [42]. Historically, one of the main drawbacks of this method has been its high installation and reagent costs, limiting its use in low-resource settings. However, recently, costs associated with qRT-PCR have dropped considerably making it an accessible alternative for routine screening.

Rapid antigen detection assays were initially developed to test clinical samples but have proven to be a useful tool to test mosquito pools in the field [43]. These assays allow for qualitative detection of arboviruses, and have the advantage of being rapid, without the need for specialized equipment. Currently, there are tests commercially available for a variety of viruses including CHIKV [44], DENV [45] and WNV [43], among others. In Singapore [46] and Malaysia [47], a dengue NS1 rapid test has been used to detect infected mosquitoes as part of a routine surveillance programme. These tests have shown high specificity for the target virus, although some assays have reduced sensitivity when compared with molecular methods [48, 49]. However, although they may provide an underestimate of infection rate, they provide a first screen and have applicability in regions without access to more resource intensive or expensive diagnostic capacity.

Traditional mosquito-based surveillance systems that target processing of pools of mosquitoes come with inherent limitations. Mosquito populations often have very low carriage rates, whereby only one in 1000 mosquitoes is actually infected [50]. To increase the probability of detection, large numbers of mosquitoes are required,

resulting in numerous mosquitoes to identify, pool and test, increasing laboratory costs and turnaround time. Additionally, many traps require attractants, such as CO_2, to increase collections. This comes in the form of dry ice or pressurized cylinders, which may not be readily available, or only allow overnight deployment of the trap. A cold chain of storage at < -50 °C is required to preserve the integrity of the virus for detection, which can be a challenge in remote locations. Finally, specialized laboratory equipment and infrastructure is required for diagnostics, which might not be available in developing countries.

Novel methods for arbovirus surveillance

The majority of mosquito species feed on carbohydrates (i.e. flower nectar, honeydew or rotting fruit) which are the primary energy source of their diet [51]. One exception is *Ae. aegypti*, which appears to obtain enough energy from blood and rarely feeds on sugar in domestic environments [52]. The ingestion of carbohydrates is important for the survival of the mosquito, and plays an indirect role in disease transmission, allowing an infected female to live long enough to become infective [53]. It was hypothesized by Doggett et al. [54] and confirmed by van den Hurk et al. [55], that infected mosquitoes expectorate virus while sugar feeding, which can be detected using molecular assays. This finding led to the development of novel sugar-based approaches for the detection of arboviruses in mosquitoes in the field. This system integrates purpose-built CO_2-baited box traps, which house nucleic acid preservation cards (Flinders Technology Associates, FTA® cards) soaked in honey and on which mosquitoes feed and expectorate onto [56]. The FTA® cards inactivate any expectorated viruses and preserve the liberated RNA. The cards are then sent to the laboratory in the post without requirement of a cold-chain, where they are screened for viruses using molecular assays.

Commonly used traps employed to collect mosquitoes (i.e. CDC-light trap and Encephalitis Virus Surveillance, EVS, trap) require batteries to operate which can be logistically challenging. To circumvent this limitation, a non-powered CO_2-baited passive box trap (PBT) was developed by Ritchie et al. [57] to collect and house mosquitoes. A variation of the PBT, the sentinel mosquito arbovirus capture kit (SMACK) was developed to increase mosquito survivorship and consequently increase the probability of infected mosquitoes feeding on the FTA® card [58]. Although designed for weekly or fortnightly servicing, the SMACK has demonstrated similar trap efficacy to the CDC-light trap and EVS trap in overnight collections, making it an alternative to traps that require batteries to operate.

Free-standing sugar bait stations have the potential to be used instead of CO_2-baited traps [59]. These stations consist of a dental wick soaked in sucrose solution and a floral lure, such as phenyl acetaldehyde. Mosquitoes lured to the station feed on the wick, which is tested for expectorated viral RNA. The sugar bait stations do not require CO_2 or electricity, so a number of stations can be deployed simultaneously, thus increasing geographical coverage. In a proof of concept, the sugar bait stations detected WNV before sentinel animals seroconverted in California. However, this method appears more efficacious in arid habitats, probably because of lack of competition with other sucrose sources, such as floral nectars. As sugar bait stations facilitate increased geographical coverage, they may have higher costs associated with analysing an increased number of samples, although this would be offset by savings by not having to use CO_2 baited light traps.

Sugar-based surveillance has several advantages over traditional methods. When mosquito populations are elevated, sorting becomes time consuming, and a high number of pools can overwhelm laboratory capacity. When combined, these issues can reduce the ability to provide results in a timely manner. Sugar-based methods potentially overcome these issues, since only 1-2 FTA® cards per trap are tested, compared to a variable number of mosquito pools. As only transmitting mosquitoes will yield positive results, the presence of virus in saliva expectorate is a better estimate of transmission risk. FTA® cards can preserve viral RNA for up to 28 days [56], making this an ideal alternative for surveillance in remote or difficult to access locations, where regular servicing of traps is not feasible. Results suggest that sugar-based surveillance is a more sensitive indicator of arbovirus activity than sentinel animals. In northern Australia, it has been possible to detect WNV_{KUN} before sentinel animal seroconversions [60]. However, a comparison of the sugar-based surveillance system with existing strategies still needs to be thoroughly evaluated. Sugar-based surveillance, using either SMACK or EVS traps, has been successfully incorporated into existing surveillance programs in Australia, with multiple detections of MVEV, WNV_{KUNV}, RRV, BFV, Edge Hill virus and Stratford virus [61–64].

Honey-soaked FTA® cards have the potential to be integrated into surveillance of *Ae. aegypti*-borne arboviruses. The cards have been used in Biogents sentinel traps (BGS traps) and modified double sticky ovitraps for the detection of CHIKV in French Guiana [65]. The approach appeared time consuming with only one CHIKV positive FTA® card out of 234 analysed. Traps that are more efficient at collecting *Ae. aegypti* may be able to increase trap collections, thus increasing the likelihood of detecting virus. For instance, the Gravid *Aedes*

Trap (GAT) [66] collects 2.4 times more *Ae. aegypti* and significantly more gravid females than double sticky ovitraps [67], which could increase the chances of finding positive mosquitoes. However, *Ae. aegypti* collections are usually small, and in many cases, it would be easier to pool the mosquitoes (or alternatively, squash them into FTA® cards [68]) and process them by molecular methods.

Like any system, sugar-based surveillance has some limitations. Perhaps its main limitation is that the cycle threshold (C_t) values obtained by real time RT-PCR are high (> 30 cycles), reflecting the relatively small amount of saliva expectorated by mosquitoes [69]. Additionally, this method will only detect positive mosquitoes after the extrinsic incubation period which, depending on the virus, can last from two to 14 days. Thus, the proportion of mosquitoes in a population that survive to transmit the virus can be quite low. In order to increase mosquitoes feeding on the FTA® cards, trapped mosquitoes must be kept alive in the trap for as long as possible. The SMACK was developed to include a water reservoir in the trap to increase humidity, the lack of which can be a problem in remote and arid locations. To save on reagent costs, some agencies will wait until they have sufficient samples to batch together, which can extend the turnaround time. Finally, sugar-based surveillance does not provide data on the mosquito species that expectorated the virus. Instead, detection of virus on a FTA® removed from a trap could be used to trigger intensive trapping to collect mosquitoes for pooling and processing to provide information on potential vectors at a given time point or location.

A potential way to increase sensitivity of sugar-based surveillance systems is through the collection and analysis of mosquito excreta. When mosquitoes feed on a sucrose solution it takes approximately 30 min for it to reach the midgut, after which excreta is ejected from the anus [70]. In terms of pathogen detection, the focus has mainly been on the detection of filarial nematodes, such as *Brugia malayi* [71] and *Plasmodium vivax* [72]. In the late 1920s, de Beaurepaire Aragão and da Costa Lima performed a series of experiments in which they infected rhesus macaques with the excreta collected from YFV infected *Ae. aegypti* [73–75]. Laboratory-based experiments have recently demonstrated that *Ae. aegypti* with a disseminated infection excrete DENV RNA, which can be detected through qRT-PCR [76]. The rate of detection was higher in excreta samples, 89%, compared with 33% for saliva samples. This suggests that collection of excreta from trapped mosquitoes could enhance the sensitivity of current sugar-based surveillance systems. This is not surprising, given that mosquitoes excrete considerably more fluid than they salivate (~1.5 μl [77] *vs* 4.7 nl [69]). Integration of excreta collection into current surveillance systems would require modification of current trap designs to selectively capture mosquito excreta.

Advances in arbovirus detection, characterization and data interpretation

Next-generation sequencing for the detection of arboviruses

Traditionally, diagnostic assays utilised in arbovirus surveillance programs only screen for characterised endemic and enzootic viruses. Because virus specific primers and probes are used for molecular diagnostics, it is likely that many other viruses, whether pathogenic or not, remain undetected. Metagenomic analysis using next-generation sequencing (NGS), allows for the simultaneous identification of viruses, mosquito species, and endosymbionts, such as *Wolbachia*, from a single mosquito in a single reaction [78] without prior sequence knowledge. This approach relies on bioinformatics tools to analyse the millions of sequence reads [79–81] and the availability of high-quality sequence databases to analyse the large and complex datasets generated. In Australia, viral metagenomics has been used for the identification of multiple arboviruses, including novel rhabdoviruses, bunyaviruses [82] and mesoniviruses [83] from field collected mosquitoes.

At this stage, NGS methods have some disadvantages compared with other molecular methods of virus detection. NGS is less sensitive than qRT-PCR for the detection of samples with low virus titres [84]. At present, the costs associated with NGS are higher than the cost of qRT-PCR, and its associated equipment has a relatively large laboratory footprint. It also requires intimate bioinformatics knowledge and reference sequence databases to analyse the data produced. Over the past years, there has been advancement in the hardware used for NGS, with equipment getting smaller and cheaper. The first hand-held portable sequencer (MinION) is already available on the market. This platform reduces processing time significantly (e.g. < 6 hours for detection of CHIKV from blood samples [85]). Even with operational challenges, the MinION's high portability and low energy requirements have enabled its use in extreme field conditions [86] and it has been used to investigate outbreaks of Ebola [87] and *Salmonella* [88]. It has recently been demonstrated that the MinION can be used for metagenomic arbovirus detection from infected mosquitoes [89], so it could be used during arbovirus outbreaks. Although the MinION still has limitations, such as high error rates and requirement for an internet connection for base calling, technologies like this, together with lower reagent costs, will be crucial in making sequencing accessible in the field in the near future.

Xenosurveillance

Mosquitoes have the potential to act as environmental samplers ("biological syringes") that feed on the blood of a variety of vertebrate hosts. Xenosurveillance offers an alternative to directly sampling hosts, a process that is time consuming and requires individual informed consent in the case of humans or animal ethics approval, in the case of veterinary pathogens. Mosquitoes can be used as a proxy for syringe sampling of small animals for virus titer determination [90]. This approach has mainly been used to study vector-borne pathogens, such as filarial parasites [91] or apicomplexans [92]. For example, in Sri Lanka, xenosurveillance has been successfully used to map areas with persistent *Wuchereria bancrofti* after mass drug administration programmes [93]. Furthermore, it has been possible to detect DENV from (non-competent) *Anopheles stephensi* mosquitoes 24 h after ingestion [94]. In addition to viruses that actively replicate in them, engorged mosquitoes potentially possess viruses or other pathogens that do not replicate in them but might be present in hosts they feed upon [95]. Xenosurveillance monitors these potential non-vector borne human and animal pathogens [96] by performing nucleic acid detection or vector enabled metagenomics [97] on mosquito samples. Mosquitoes have been successfully used to monitor non-mosquito borne pathogens such H5N1 influenza virus [98], Epstein-Barr virus, canine distemper virus [96], human herpesvirus, human papillomaviruses, anelloviruses and circoviruses, among others [95].

One of the main limitations of xenosurveillance is the difficulty in collecting sufficient blood engorged mosquitoes for analysis. Some of the methods to collect engorged mosquitoes (i.e. use of an aspirator) are labour intensive and can be intrusive, especially when sampling inside houses and villages [99]. To circumvent this issue, mosquito excreta could be used to provide the template for xenosurveillance. Indeed, hepatitis B virus, which does not replicate in the vector, has been detected in mosquito excreta by RT-PCR and Southern Blot up to 7 days after the ingestion of an infectious blood meal [100].

Emerging technology

Integration of data acquisition, storage and sharing methodologies, such as cloud networks and geographic information systems, will form an integral component of surveillance and control programmes. An example of this is the Intelligent Dengue Monitoring technology (MI-Dengue) developed in Brazil [101]. MI-Dengue consists of an array of tools to collect gravid *Ae. aegypti* females, collect field data, detect virus and create georeferenced infestation maps that are available in real time, providing information to optimize vector control. This system has been successful at reducing dengue in the municipalities that have adopted it.

In the age of mobile phones, social media and internet, citizen science will undoubtedly play an important role in disease surveillance in general. In Spain, Mosquito Alert was implemented as a system to collect reports of invasive *Ae. albopictus*. To date, it has more than 30,000 registered participants [102]. As a part of the GLOBE project sponsored by NASA, Mosquito Habitat Mapper merges data generated by citizens with satellite-based research [103]. Interestingly, with minimal training, the data generated by programmes like these is considered as reliable as data collected by experts [104]. Mobile phones, even low-end ones, can also be used as acoustic sensors to identify mosquito species [105]. All these initiatives will allow large-scale data acquisition, which is critical for adequate mosquito control.

Over the past 20 years, single device detection platforms for clinical and environmental analyses have been rapidly evolving. A promising technique for integration into surveillance programmes is the use of microfluidic devices [106] and biosensors [107] which are designed to process very small volumes of liquid, requiring minimal amount of sample and reagents to yield results in minutes [108, 109]. Some applications of these devices include diagnosis of infections caused by DENV [110–112] and CHIKV [113] from clinical samples, detection of DENV NS1 antigen from pools of mosquitoes [114] and genotyping of closely related *Anopheles* species [115].

Conclusions

Over the past decade, there have been key scientific advances in arbovirus surveillance, particularly with regard to sample collection, virus detection and data analysis. Table 3 summarises the relative advantages and disadvantages of current and emerging surveillance methodologies. Alternative samples for virus detection, such as mosquito excreta, may enable more sensitive detection of arboviruses than existing methodologies. It has been proposed that we are on the cusp of a revolution in genomic epidemiology [116]. With NGS technologies becoming more accessible in the near future, they will enable the collection of real-time in-depth genetic information on circulating arboviruses before or during an outbreak. There is still room for improvement of surveillance systems used in remote locations where surveillance coverage is limited by cost and limited access to sites. Use of other sources of CO_2 in mosquito traps (such as fermentation using yeast) [117] or CO_2-free systems could provide an alternative in areas where dry ice or pressurized gas cylinders are not available. Deployment of in-field portable molecular laboratories or point of care assays could provide same-day assessment of arbovirus circulation and rapid response in these locations [118]. In the future, other technologies,

Table 3 Summary of traditional and novel arbovirus surveillance methods

Method	Advantages	Disadvantages	Application
Monitoring human and animal disease	Uses data that is already being collected by hospitals, health practitioners, and animal health personnel	Overlap of clinical symptoms within arboviruses and other pathogens. Not ideal for early warning since active transmission will be already occurring	National disease surveillance databases
Sentinel animals	Can act as an early warning system	Animals can be amplifying hosts. High costs associated with animal rearing. Cross reactivity between closely related arboviruses when using serological assays	Routine surveillance, inform control strategies
Virus isolation from pools of mosquitoes	Increases virus titer allowing for genotypic and phenotypic characterization	Time consuming. Requires special infrastructure (biological containment). Requires a cold chain	Routine surveillance, virus identification, inform control strategies
Virus detection in pools of mosquitoes using molecular assays	Allows high throughput screening. High sensitivity	Will only detect RNA from viruses that the assays were designed to detect. Requires special infrastructure	Routine surveillance, research, inform control strategies
Virus detection in pools of mosquitoes using rapid antigen detection assays	Rapid. Does not require specialized equipment. Lower cost	Lower sensitivity than molecular methods	Routine surveillance in low resource settings
Sugar-based surveillance	Does not require a cold chain. Only 1-2 samples per trap are tested potentially compared with 1000s of mosquitoes using other methods of surveillance. Better estimation of transmission risk	Relies on a nanoliter amounts of expectorate. Mosquitoes need to be kept alive for as long as possible to increase feeding on cards. Cannot be used to incriminate mosquito species as vectors. Requires special infrastructure	Routine surveillance, ideal for remote locations
Next-generation sequencing of mosquito samples	Does not require prior information (will detect any arbovirus present in the sample)	High cost. Requires bioinformatics knowledge. Requires special infrastructure	Research, virus discovery
Xenosurveillance	Mosquito acts as an environmental sampler. Allows detection of viruses that do not replicate in the mosquito	Blood engorged mosquitoes are difficult to collect	Research and surveillance of arboviruses and other pathogens

such as unmanned aerial vehicles (UAVs) could be used to automate sample collection in difficult to access locations increasing the coverage of surveillance. Regardless of the surveillance system, there are always going to be issues and limitations, which can vary between jurisdictions. Currently, the extent of arbovirus surveillance varies between countries and even states with many jurisdictions lacking any form of monitoring. There is a need for sharing of arbovirus surveillance intelligence between public health agencies at regional level as a means to apply better control measures. Moreover, the implementation issues that might arise from new approaches cannot be underestimated. Agencies that are familiar with set methodologies may be reluctant to adopt new technologies or not have the capacity to implement change. Because of this, when designing new arbovirus surveillance methodologies, there should be a clear understanding of the needs and limitations of field, laboratory and public health personnel.

Abbreviations

DENV: Dengue virus; JEV: Japanese encephalitis virus; WNV: West Nile virus; CHIKV: Chikungunya virus; ZIKV: Zika virus; YFV: Yellow fever virus; SLEV: St. Louis encephalitis virus; RRV: Ross River virus; BFV: Barmah Forest virus; MVEV: Murray Valley encephalitis virus; BTV: Blue tongue virus; RVFV: Rift Valley fever virus; EEEV: Eastern equine encephalitis virus; WEEV: Western equine encephalitis virus; ISF: insect-specific flaviviruses; IgG: immunoglobulin G; PCR: polymerase chain reaction; RT-PCR: reverse-transcription polymerase chain reaction; RNA: ribonucleic acid; FTA®: Flinders Associate Technologies; PBT: passive box trap; SMACK: sentinel mosquito arbovirus capture kit; C_t: cycle threshold; GAT: gravid *Aedes* trap; NGS: next-generation sequencing; UAV: unmanned aerial vehicle

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3

MOSQUITO EXCRETA: A SAMPLE TYPE WITH MANY POTENTIAL APPLICATIONS FOR THE INVESTIGATION OF ROSS RIVER VIRUS AND WEST NILE VIRUS ECOLOGY

The work presented in this chapter, entitled "Mosquito excreta: A sample type with many potential applications for the investigation of Ross River virus and West Nile virus ecology" was published in PLoS Neglected Tropical Diseases, August 2018. doi: 10.1371/journal.pntd.0006771

3.1 CONTEXTUAL LINKAGE

As described in the literature review (Chapter 2), it has been previously demonstrated that mosquitoes excrete dengue virus RNA, suggesting that there is potential to use mosquito excreta as a biological sample. In the context of my thesis, the first step was to evaluate if the excretion of arboviruses was a general phenomenon. For this, I designed a laboratory study where I experimentally infected mosquitoes with two arboviruses of importance in the Australian context: Ross River virus and West Nile virus. Then, I collected mosquito excreta and tested it for the presence of viral RNA using RT-rtPCR assays. Through this experiment, I was able to determine the time-frame of excretion and its relation with the infectious status of the mosquito, two key pieces of information for the use of mosquito excreta in laboratory- or field-based applications. With the aim of establishing if excreta could pose a workplace health and safety hazard for people handling the samples, I then evaluated if the excreted virus was infectious. The protocols for housing mosquitoes and collecting excreta samples I developed in this experiment were subsequently used for the laboratory studies described in Chapter 4, Chapter 5 and Chapter 6.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Mosquito excreta: A sample type with many potential applications for the investigation of Ross River virus and West Nile virus ecology

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Abstract

Background

Emerging and re-emerging arthropod-borne viruses (arboviruses) cause human and animal disease globally. Field and laboratory investigation of mosquito-borne arboviruses requires analysis of mosquito samples, either individually, in pools, or a body component, or secretion such as saliva. We assessed the applicability of mosquito excreta as a sample type that could be utilized during studies of Ross River and West Nile viruses, which could be applied to the study of other arboviruses.

Methodology/Principal findings

Mosquitoes were fed separate blood meals spiked with Ross River virus and West Nile virus. Excreta was collected daily by swabbing the bottom of containers containing batches and individual mosquitoes at different time points. The samples were analyzed by real-time RT-PCR or cell culture enzyme immunoassay. Viral RNA in excreta from batches of mosquitoes was detected continuously from day 2 to day 15 post feeding. Viral RNA was detected in excreta from at least one individual mosquito at all timepoints, with 64% and 27% of samples positive for RRV and WNV, respectively. Excretion of viral RNA was correlated with viral dissemination in the mosquito. The proportion of positive excreta samples was higher than the proportion of positive saliva samples, suggesting that excreta offers an attractive sample for analysis and could be used as an indicator of potential transmission. Importantly, only low levels of infectious virus were detected by cell culture, suggesting a relatively low risk to personnel handling mosquito excreta.

Conclusions/Significance

Mosquito excreta is easily collected and provides a simple and efficient method for assessing viral dissemination, with applications ranging from vector competence experiments to

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complementing sugar-based arbovirus surveillance in the field, or potentially as a sample system for virus discovery.

Author summary

Testing for the presence of arboviruses in mosquitoes used in laboratory experiments or surveillance usually involves collecting samples, from pools of hundreds of mosquitoes to the legs and wings of an individual mosquito and testing them by different methods. These methods can be labour intensive and costly and require sacrificing the mosquitoes. Arbovirus detection can be made from mosquito saliva; however, the amount of saliva mosquitoes expel is very small, making detection difficult. Here we demonstrate that mosquitoes excrete Ross River and West Nile viruses at levels sufficient to be detected by molecular assays as early as 2 days after they have fed on an infected blood meal. The amount of live (infectious) virus in excreta is low, suggesting that mosquito excreta poses a relatively low risk to people handling the samples. Mosquito excreta is easily collected in the laboratory and has a range of applications including experiments designed to incriminate mosquito species as vectors (i.e. vector competence experiments), arbovirus surveillance in the field, and discovery of previously unknown viruses.

Introduction

It has been estimated that vector-borne diseases account for almost 20% of the global burden of infectious diseases, with more than 80% of the world's population living in areas at risk [1]. Mosquitoes are the most important vectors of arthropod-borne viruses (arboviruses) globally. In recent years, many arboviruses have emerged or re-emerged due to several factors. High viral mutation frequency, widespread urbanization, and changes in land use, together with globalization and the growth of air travel, facilitate vector population increase and dispersal, and enable rapid transit of viremic humans [2, 3, 4]. Since few vaccines and antiviral therapies are available, critical work to understand and prevent arbovirus outbreaks must be undertaken both in the laboratory, by performing vector competence experiments to incriminate candidate species, and in the field by undertaking studies of virus ecology, as well as routine surveillance to identify periods of elevated virus activity.

Vector competence refers to the ability of a mosquito or other hematophagous arthropod to acquire, replicate, and successfully transmit a pathogen [5]. This is a key parameter to estimate vectorial capacity, namely the potential of a mosquito population to transmit an infectious agent to a susceptible host population [6]. Vector competence is determined by intrinsic factors that regulate virus infection of the midgut, escape from the midgut into the hemocel and associated tissues (dissemination), and finally infection of the salivary glands [7]. In the laboratory, vector competence is evaluated usually by feeding mosquitoes an infectious bloodmeal or allowing them to feed on an infected vertebrate. After a period of time, their ability to transmit the pathogen is evaluated. Several methods are used to assess transmission in the laboratory. Historically, transmission was evaluated by allowing mosquitoes to feed on susceptible vertebrate hosts (such as suckling mice) and then assessing infection (e.g. via clinical changes in the mice) [8, 9]. However, many arboviruses lack an appropriate model vertebrate host that will produce sufficient viremia or antibodies after exposure to be detected using standard laboratory assays [10]. Additionally, not all laboratories have the required biological security to

allow handling vertebrate hosts in the same space as mosquitoes. Transmission can also be assessed *in vitro*, by forcing mosquitoes to salivate into capillary tubes [11] and then testing the expectorate for virus by inoculation in cell culture or by molecular assays. This method is relatively simple and removes ethical and logistical issues with working with live vertebrates. However, it can be an insensitive system to demonstrate transmission for some arboviruses, such as dengue viruses (DENVs) and chikungunya (CHIKV) [12,13]. Although not ideal, an alternative to estimate transmission potential is to test mosquito legs, wings, and/or heads, and use dissemination as a proxy for transmission [14]. This method fails to take into account possible salivary gland barriers to transmission [7, 15] and may overestimate the true transmission rate. The main limitation of *in vitro* methods is that since the mosquitoes must be sacrificed, they provide an end-point measurement preventing longitudinal measurements from the same individual.

In the field, routine arbovirus surveillance is carried out to detect elevated viral activity in order to implement disease control measures. Different strategies can be used for arbovirus surveillance [16] and one of the most widespread methods is the collection, identification, pooling and testing of wild mosquitoes by molecular assays or virus isolation. However, mosquito-based surveillance is time consuming and requires a continuous cold-chain to preserve virus viability for downstream processing. To overcome these limitations, a mosquito-free surveillance system based on the detection of arboviruses in saliva of infected mosquitoes has recently been developed [17, 18]. Saliva is collected on honey-baited nucleic acid preservation cards (Flinders Associate Technologies, FTA), which inactivate the virus and preserve viral RNA. Viral RNA is then eluted from the cards and detected using standard molecular assays. Importantly, the RNA preserved on the FTA cards serves as a template for nucleotide sequencing allowing strain identification and genotyping. This system has been successfully incorporated into routine surveillance programmes in Australia and is generally effective, as evidenced by numerous detections of arboviruses from multiple locations [19, 20, 21, 22]. Similar approaches using honey-baited cards or sugar-baited wicks have been evaluated in Florida [23] and California [24, 25]. Like any novel or emerging technology, there is always an opportunity to enhance the sugar-based arbovirus surveillance system. Since only a limited number of virions are passed during salivation [26, 27], the amount of virus on the FTA cards is generally of low concentration, indicating that the diagnostic assays are operating at their limits of detection [22]. This may lead to false negatives or insufficient template for downstream nucleotide sequencing. Additionally, this method will only detect mosquitoes after the extrinsic incubation period (EIP) which can take up to 14 days for some arboviruses. Finally, infection rates and vector species identification cannot be determined from honey-baited cards [28].

An exciting new application involves the collection of a previously overlooked sample. It was recently demonstrated by Fontaine et al. [29] that DENV RNA can be detected in excreta from *Aedes aegypti* mosquitoes with a disseminated infection. Since collection of excreta does not require sacrificing the mosquito, it allows for [a]time-to-event[o] estimation of the time for dissemination, and consequently, an estimation of the EIP when used as a proxy for transmission potential, in individual mosquitoes. Detection of viral RNA in mosquito excreta can also be used to select mosquitoes based on extreme phenotypes (viral refractory or susceptible) for experiments exploring the genetic basis of a complex trait. Mosquito excreta can potentially be used to complement sugar-based surveillance. Indeed, it appears that viral RNA detection in excreta is more sensitive than detection in saliva (89% vs 33% for DENV) [29]. Detection of arboviruses from excreta of infected mosquitoes could enable more sensitive detection of arboviruses than existing honey-baited FTA cards relying on collection of mosquito saliva alone.

The main objective of the current study was to determine whether mosquitoes excrete the Australian endemic arboviruses Ross River virus (RRV; family *Togaviridae*, genus *Alphavirus*,)

and West Nile virus (Kunjin strain, WNV_{KUN}; family *Flaviviridae*, genus *Flavivirus*) at levels sufficient to be detected by real-time reverse transcription polymerase chain reaction (RT-PCR) molecular assays. Building upon the Fontaine et al. [29] findings, we also determined if the association between virus dissemination and excretion extends to other arboviruses. Then, as a way to potentially enhance the sensitivity of the sugar-based surveillance system, we compared the detection of RRV and WNV_{KUN} in mosquito excreta with virus detected in saliva via filter paper cards. Importantly, in the context of workplace health and safety regulations affiliated with arbovirus surveillance systems, we evaluated whether excreted virus was infectious.

Materials and methods

Viruses

RRV was isolated from a pool of *Verrallina carmenti* collected from the Cairns suburb of Yorkeys Knob, Queensland, Australia in 2007 [30]. The virus had been previously passaged three times in African green monkey kidney (Vero) cells (ATCC, CCL-81). WNV_{KUN} was isolated from a pool of *Culex annulirostris* collected in the Gulf Plains region of Queensland, Australia in 2002 [31]. The virus had been previously passaged twice in porcine-stable equine kidney (PSEK) cells [32] before a final passage in *Aedes albopictus* (C6/36) cells (ATCC, CRL-1660).

Mosquitoes

Aedes vigilax was selected based on its status as the coastal vector of RRV in Australia [33]. Eggs from colonized *Ae. vigilax* were obtained from NSW Health Pathology-ICPMR, Westmead Hospital, Westmead, Australia. The colony was originally established at the Malaria Research Unit at Ingleburn in 1986 from material collected near Townsville, Queensland. Eggs were hatched in 2L of 33% seawater containing ~45 mg of brain-heart infusion powder. Larvae were reared at 26ÊC12:12 L:D and fed fish flakes (Tropical Flakes, Aqua One®, Ingleburn, Australia). Pupae were placed in 150 mL containers inside a 30 x 30 x 30 cm insect rearing cage. Emerged adults were held at 26ÊC,75% RH and 12:12 L:D, and maintained on 15% honey solution *ad libitum*.

Culex annulirostris was selected based on its status as the primary WNV_{KUN} vector in Australia [34]. Adult mosquitoes were collected in February 2017 using passive box traps [35] baited with CO_2 (1kg dry ice) and operated for 14 h (1700±0700) in a mixed *Melaleuca* and mangrove swamp near Cairns, Australia (−16.826613Ê,145.707065Ê).These field mosquitoes were transported to the laboratory where they were briefly anesthetized and female *Cx. annulirostris* were sorted and maintained on 15% honey solution *ad libitum* at 26ÊC,75% RH and 12:12 L:D. Since there is no evidence that WNV_{KUN} circulates in the Cairns region [30], it is unlikely that the mosquitoes had acquired the virus in the field.

Virus exposure

Mosquitoes were starved for 24 h before oral infection with virus. Five to 7 day-old female *Ae. vigilax* were offered RRV diluted in washed defibrinated sheep blood (Institute of Medical and Veterinary Science, Adelaide, Australia) at 37ÊCusing a Hemotek membrane feeding system (Discovery Workshops, Accrington, Lancashire, UK) with pig intestine as a membrane. *Cx. annulirostris* were exposed to WNV_{KUN} diluted in washed defibrinated sheep blood via the hanging drop method [36]. To determine the virus titer of the blood at the time of feeding and to assess if there was any reduction in titer, a 100 μL sample of the blood/virus mixture was taken before and after feeding, diluted in 900 μL of growth media (GM; Opti-MEM (Gibco,

Invitrogen Corporation, Grand Island, NY) containing 3% foetal bovine serum (FBS; *In Vitro* Technologies, Australian origin), antibiotics and antimycotics), and stored at -80ÊC. After feeding, mosquitoes were briefly anesthetized with CO_2 gas, and blood-engorged females sorted and placed in modified containers (see below) or in 900 mL containers covered with 100% polyester gauze (Spotlight Pty Ltd, Australia). All mosquitoes were maintained at 28ÊC, 75% RH and 12:12 L:D within an environmental growth cabinet for 15 days.

Collection of excreta from mosquito batches

For each virus, 20 batches of 5 mosquitoes were placed in modified 200 mL polypropylene containers for excreta collection. The gauze-covered containers had a false floor made of fiberglass insect screen that allowed excreta to pass through onto a parafilm M (Bemis NA, Neenah, WI) disc situated about 5 mm below the screen to avoid cross contamination. Mosquitoes were fed on cotton balls soaked in 15% honey dyed with blue food colouring to allow for excreta visualisation and were replaced daily. Excreta was collected daily from day 2 to day 15 post-exposure (PE) using a cotton swab (Livingstone International, Rosebery, Australia) moistened with GM + 3% FBS. Each swab was placed in a 2 mL tube containing 1 mL GM + 3% FBS and stored at -80ÊC. Parafilm discs were replaced daily to avoid cross contamination. Mosquito mortality was also recorded daily. To compare the sensitivity of detection of viral RNA in excreta with the sensitivity of detection in saliva expectorates, on day 14 PE, mosquitoes were allowed to feed on a 4 cm^2 filter paper card (FP; low chamber filter paper, Bio-Rad Laboratories, California) soaked in 100% honey dyed with red food colouring. After 24 h, the FP cards were removed, placed in a 2mL tube containing 1 mL GM + 3% FBS and stored at -80ÊC.

Collection of excreta from individual mosquitoes

At three different timepoints (RRV: 7, 10, 14 days PE; WNV_{KUN}: 6, 11, and 14 days PE), 20 individual mosquitoes were placed into 70 mL containers modified with the same design as described above. A 1 cm^2 FP card soaked in 100% blue honey was offered as a sugar source. The mosquitoes were allowed to feed on the cards for 18±24 h, after which the excreta and the cards were collected as described above.

Assessment of infection, dissemination and transmission rates from mosquito cohorts

Because the mosquitoes used for the batches and individual analyses were derived from a cohort exposed to the same infectious blood meal, we assessed the infection, dissemination and transmission rates only from the experiments that used individual mosquitoes. Saliva was collected using the *in vitro* capillary tube method described by Aitken [11] from mosquitoes described above. Bodies and legs+wings were stored separately in a 2mL tube containing 1 mL GM + 3% FBS with a single 5 mm stainless steel bead to assess for infection and dissemination, respectively. Saliva expectorates were expelled into a 2mL tube containing 500 μL of GM + 3% FBS. All samples were stored at -80ÊC.

Virus assays

The blood/virus mixtures were titrated as 10-fold dilutions in 96-well microtiter plates containing confluent C6/36 cell monolayers. Bodies and legs+wings were homogenized using a QIAGEN Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at 26 hz and centrifuged briefly at 14,000 g. Mosquito homogenates (bodies, legs+wings) and saliva expectorates collected using capillary tubes were filtered using a 0.2 μm membrane filter (Pall Corporation, Ann Arbor, MI).

Filtered mosquito homogenates were inoculated in duplicate and filtered saliva expectorates were inoculated in quadruplicate onto confluent C6/36 monolayers in 96-well microtiter plates. To assess the viability of virus in excreta, 50 excreta samples collected from mosquito batches (10 samples, 5 time points) were homogenized and filtered as described above, and inoculated as neat (not diluted) and as 10-fold dilutions onto confluent C6/36 monolayers in 96-well microtiter plates. Plates were incubated at 28ÊCfor 7 days before being fixed in PBS/20% acetone with 0.2% BSA and stored at -20ÊC. Virus infection in cells was assessed using a cell culture enzyme immunoassay (CC-EIA) using monoclonal antibodies: B10 for RRV and 4G2 for WNV_{KUN} [37] (provided by Roy Hall, University of Queensland, Australia).

Thawed excreta samples were homogenized in the Tissue Lyser II as describe above. Thawed FP cards were maintained on ice and briefly vortexed every 5 min for 20 min [17]. Viral RNA was extracted from the excreta supernatant and eluted FP cards with a QIAxtractor (Qiagen, Hilden, Germany) using the QIAmp One-For-All nucleic acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral RNA was detected using real-time TaqMan RT-PCR assays specific for RRV [38] and WNV [22] in a Rotor-Gene 6000 real-time PCR cycler (Qiagen, Australia). With each run, positive controls included an extraction control (bovine viral diarrhoeal virus, BVDV) and a positive virus control extracted from a virus stock with known titer. Negative controls included at least one negative extraction control and a no-template control (molecular grade water). For each sample, the threshold cycle number (C_t) was determined; lower C_t values correspond to a greater amount of viral template. Any sample with a C_t value $\geq$40 was considered negative [39].

Analysis

For all the samples titrated in the CC-EIA, 50% endpoints (tissue culture infectious $dose_{50}$, $TCID_{50}$) were calculated using the method of Reed-Muench [40] and expressed as $TCID_{50}$/mL. The Mann-Whitney U test was used to determine if there was a difference between the C_t values observed for excreta samples from batches and individuals, and between excreta samples and saliva expectorates on FP cards. Fisher's exact test was used to compare the difference in between detection of viral RNA in excreta and detection of virus by CC-EIA in legs+wings, as an indication of virus dissemination. Scatter plots, heat maps and all statistical analyses were performed using GraphPad Prism version 7.0c (GraphPad Software, La Jolla CA, www.graphpad.com).

Results

Infection, dissemination and transmission rates in mosquito cohorts

For RRV with *Ae. vigilax*, the mean (± SD) virus titer at the time of feeding was $10^{8.1 \pm 0.1}TCID_{50}$/mL and the overall infection rate was 82% (Table 1). For WNV_{KUN} with *Cx. annulirostris*, the mean (± SD) virus titer at the time of feeding was $10^{7.3 \pm 0.3}$ $TCID_{50}$/mL and the overall infection rate was 42% (Table 2). All *Ae. vigilax* with confirmed RRV midgut infection developed a disseminated infection. Transmission of RRV was first observed on day 8 PE when 9/19 mosquitoes expectorated the virus. Only 76% (19/25) of *Cx. annulirostris* with confirmed WNV_{KUN} midgut infection developed a disseminated infection. Transmission of WNV_{KUN} was first observed on day 12 PE when 3/20 mosquitoes expectorated the virus.

Detection of viral RNA in excreta from batches of mosquitoes

RRV and WNV_{KUN} viral RNA was excreted every day from day 2 PE onward in both *Ae. vigilax* and *Cx. annulirostris*, respectively, at levels sufficient to be detected by real-time RT-PCR.

Table 1. Infection, dissemination and transmission rates in *Ae. vigilax* exposed to $10^{8.1\pm0.1}$TCID$_{50}$/mL of RRV tested at different days post exposure (PE).

Day PE	Infection[a]			Dissemination[b]			Dissemination/ Infection[c]			Transmission[d]			Transmission/ Dissemination[e]		
	n	%	95%CI	n	%	95%CI	n	%	95%CI	n	%	95%CI	n	%	95%CI
8	19	79	56±92	19	79	56±92	15	100	76±100	19	47	27±68	15	60	36±80
11	19	89	67±98	19	89	67±98	17	100	78±100	19	32	15±54	17	35	17±59
15	17	76	52±91	17	76	52±91	13	100	73±100	17	29	13±53	13	38	18±65
Total	55	82	69±90	55	82	69±90	45	100	91±100	55	36	25±50	45	44	31±59

[a]Number of mosquitoes tested, percentage of mosquitoes containing virus in their bodies, 95% confidence intervals

[b]Number of mosquitoes tested, percentage of mosquitoes containing virus in their legs+wing, 95% confidence intervals

[c]Number of infected mosquitoes, percentage of infected mosquitoes containing virus in their legs+wings, 95%CI, percentage, 95% confidence intervals

[d]Number of mosquitoes tested, percentage of mosquitoes containing virus in their expectorate collected in capillary tubes, 95% confidence intervals

[e]Number of mosquitoes with disseminated infection, percentage of mosquitoes with disseminated infection containing virus in their expectorate collected in capillary tubes, 95% confidence intervals

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With the exception of one batch of *Ae. vigilax* and one batch of *Cx. annulirostris*, viral RNA was detected in excreta from all the batches of mosquitoes on at least one day (Fig 1). For RRV positive samples, C_t values ranged from 24.6 to 38.8. For WNV_{KUN} positive samples, C_t values ranged from 26.6 to 39.2.

Detection of viral RNA in excreta from individual mosquitoes

It was possible to detect RRV RNA in excreta from individual *Ae. vigilax* on all days tested PE (Fig 2). Sixty-four percent (35/55) of samples were positive, with C_t values ranging from 25.1 to 37.6. No significant difference ($P>0.05$) was observed between the median C_t values from

Table 2. Infection, dissemination and transmission rates in *Cx. annulirostris* exposed to $10^{7.3\ \pm0.3}$TCID$_{50}$/mL of WNV_{KUN} tested at different days post exposure (PE).

Day PE	Infection[a]			Dissemination[b]			Dissemination/ Infection[c]			Transmission[d]			Transmission/ Dissemination[e]		
	n	%	95%CI	n	%	95%CI	n	%	95%CI	n	%	95%CI	n	%	95%CI
7	20	40	22±61	20	15	4±37	8	38	13±70	20	0	0±19	3	0	0±62
12	20	45	26±66	20	40	22±61	9	89	54±100	20	15	4±37	8	38	13±70
15	19	42	23±64	19	42	23±64	8	100	63±100	19	26	11±49	8	63	30±87
Total	59	42	31±55	59	32	22±45	25	76	56±89	59	14	7±25	19	42	23±64

[a]Number of mosquitoes tested, percentage of mosquitoes containing virus in their bodies, 95% confidence intervals

[b]Number of mosquitoes tested, percentage of mosquitoes containing virus in their legs+wing, 95% confidence intervals

[c]Number of infected mosquitoes, percentage of infected mosquitoes containing virus in their legs+wings, 95%CI, percentage, 95% confidence intervals

[d]Number of mosquitoes tested, percentage of mosquitoes containing virus in their expectorate collected in capillary tubes, 95% confidence intervals

[e]Number of mosquitoes with disseminated infection, percentage of mosquitoes with disseminated infection containing virus in their expectorate collected in capillary tubes, 95% confidence intervals

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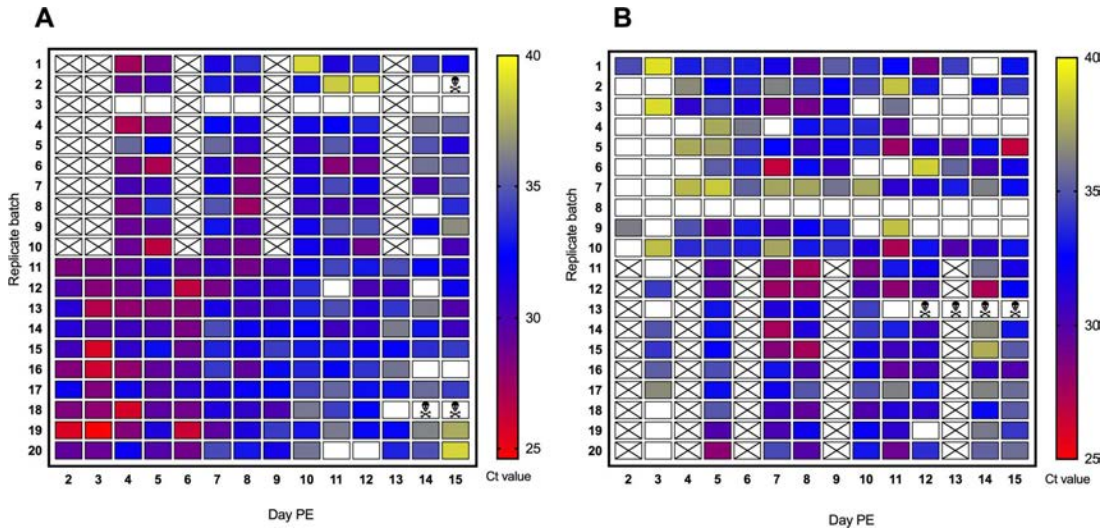

Fig 1. Real-time RT-PCR detection of arboviruses in excreta from 20 batches of 5 mosquitoes. (A) Detection of RRV RNA from *Ae. vigilax* excreta collected daily from day 2 to day 15 post exposure (PE) (B) Detection of WNV_{KUN} RNA from *Cx. annulirostris* excreta collected daily from day 2 to day 15 post exposure (PE). Lower C_t values correspond to a greater amount of viral template; a blank square indicates that viral RNA was not detected. A skull indicates that the container was removed from the experiment due to mortality of all 5 mosquitoes. X = not tested.

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excreta collected from batches of mosquitoes and from individual mosquitoes, with the exception of day 8 PE where the median C_t value for batches was higher (30.8 vs 27.5; $P = 0.0001$, S1 Fig).

WNV_{KUN} RNA was detected in excreta samples from individual *Cx. annulirostris* tested on all days PE (Fig 3). Twenty-seven percent (16/59) of samples were positive, with C_t values ranging from 28.9 to 39.2. No significant difference ($P>0.05$) was observed between the median C_t values from excreta collected from batches of mosquitoes and from individual mosquitoes (S2 Fig).

Association between disseminated infection and excretion of arboviruses

From 55 *Ae. vigilax* individuals tested, 45 (82%) mosquitoes had disseminated RRV infection. We detected RRV RNA in the excreta of 35 (78%) mosquitoes with a disseminated infection. None of the mosquitoes without a disseminated infection had positive excreta. From 59 *Cx. annulirostris* individuals tested, 19 (32%) had disseminated WNV_{KUN} infection. Thirteen (68%) mosquitoes with a disseminated infection had excreta positive for WNV_{KUN} RNA. Only 3 (8%) mosquitoes without disseminated infection had positive excreta. For both RRV and WNV_{KUN}, there was a significant ($P<0.0001$) association between disseminated infection and excretion of viral RNA.

Comparison of detection of arboviruses in excreta and saliva

Saliva deposited on FP cards from batches of mosquitoes on day 15 PE was tested for viral RNA. For *Ae. vigilax*, the proportion of RRV positive excreta samples was higher than the proportion of RRV positive FP cards (89% (16/18) vs 22% (4/18); $P<0.0001$). For *Cx. annulirostris*,

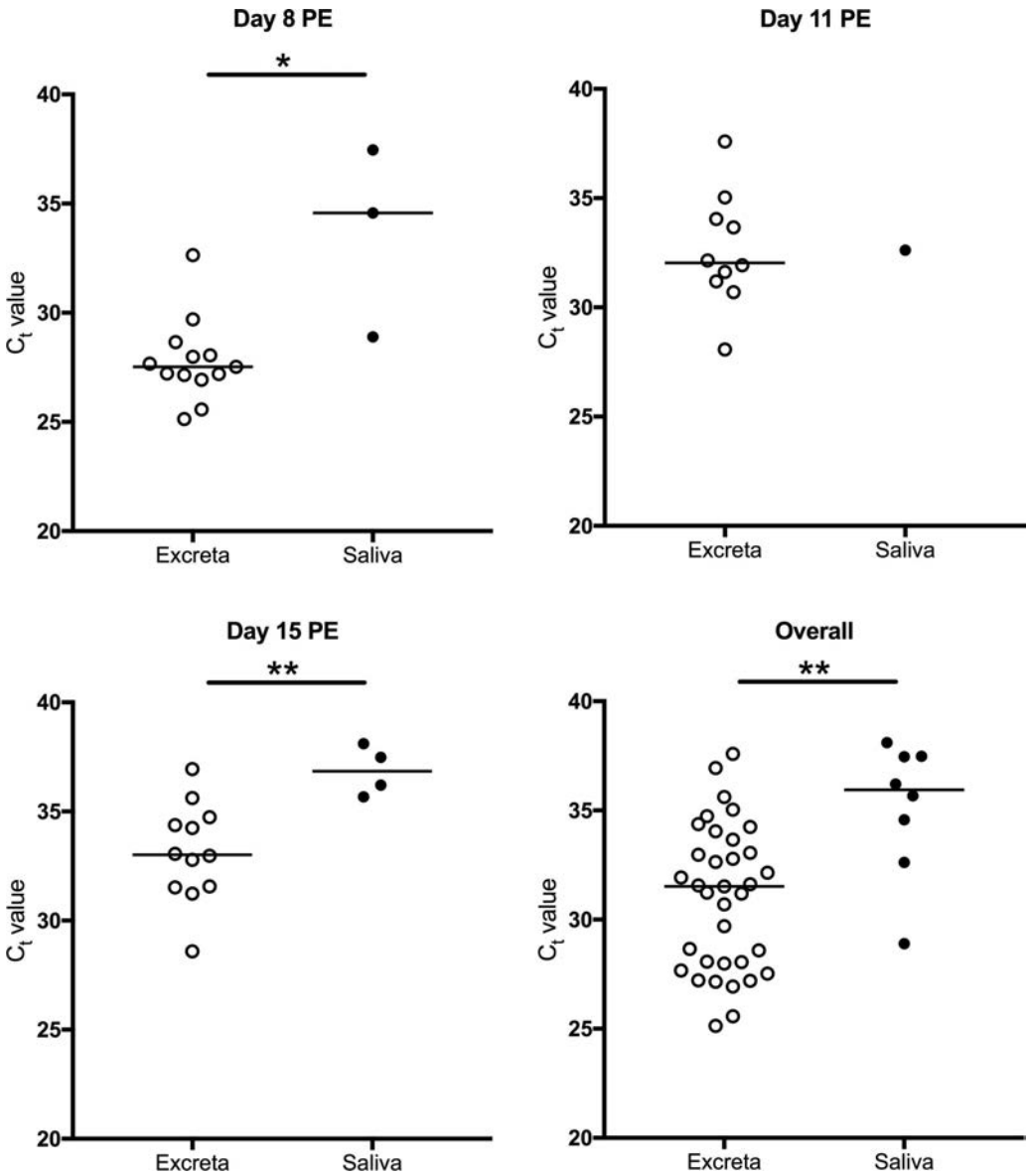

Fig 2. Detection of RRV RNA by real time RT-PCR in excreta swabs and saliva expectorates (filter paper cards). Samples collected over 18±24 h from individual *Ae. vigilax* sampled at different timepoints post exposure (PE). Bars denote medians. $P<0.05$ (*), $P<0.001$ (**), $P<0.0001$(***). Each point represents an individual mosquito.

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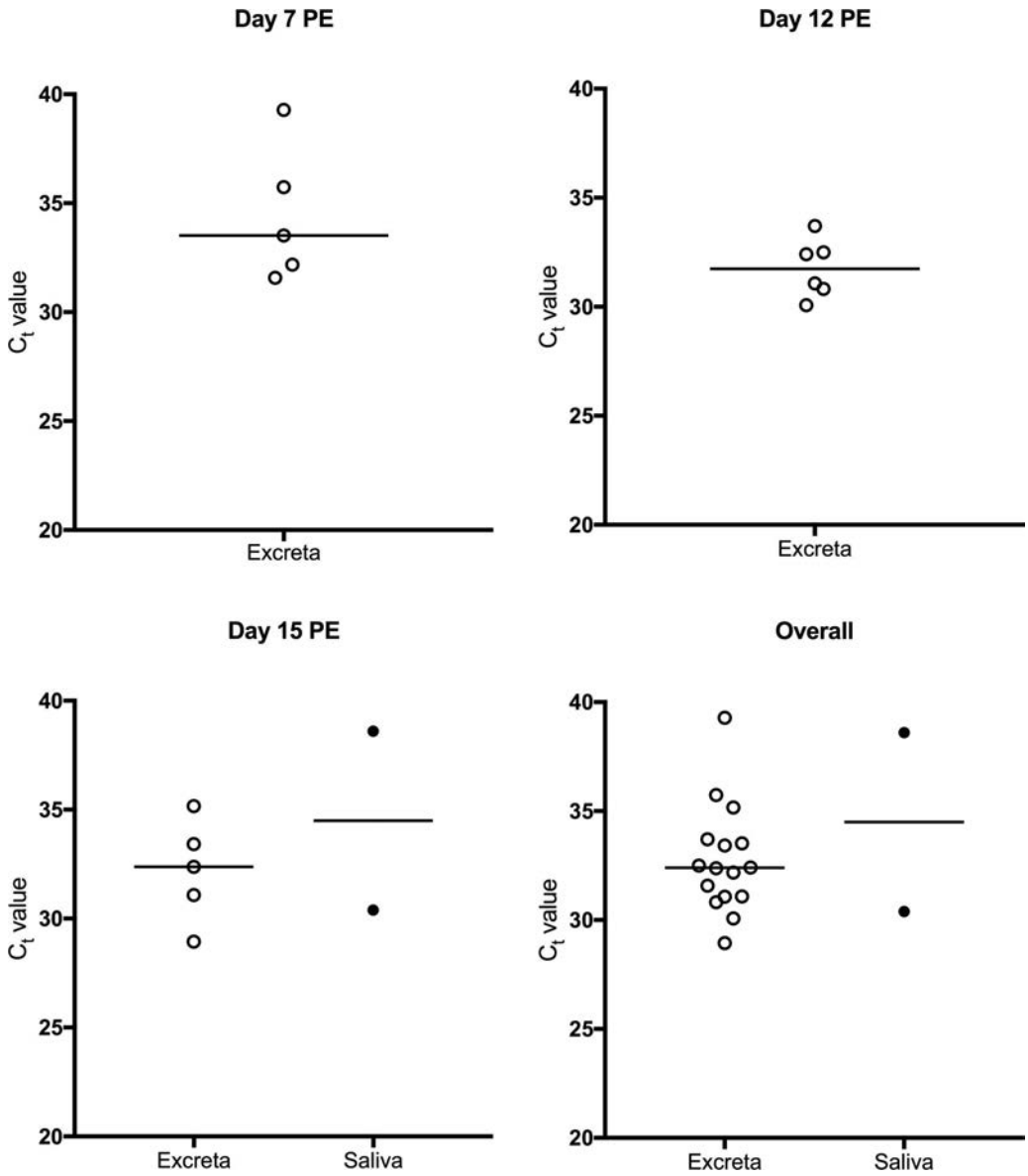

Fig 3. Detection of WNV_{KUN} RNA by real time RT-PCR in excreta swabs and saliva expectorates (filter paper cards). Samples collected over 18±24 h from individual *Cx. annulirostris* sampled at different timepoints post exposure. Bars denote medians. $P<0.05$ (*), $P<0.001$ (**), $P<0.0001$(***). Each point represents an individual mosquito. No mosquitoes expectorated virus onto filter paper cards on days 7 and 12 PE.

https://doi.org/10.1371/journal.pntd.0006771.g003

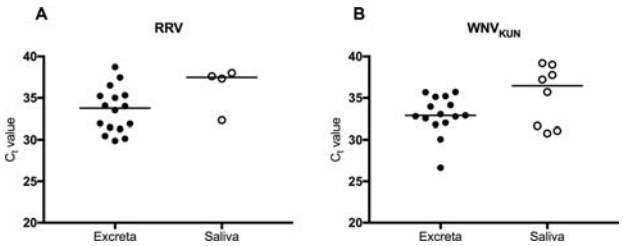

Fig 4. Detection of viral RNA in excreta and saliva expectorates (filter paper cards) from mosquito batches on day 15 post-exposure. (A) Detection of RRV RNA by real time RT-PCR in excreta and filter paper cards collected over 18± 24 h from batches of 5 *Ae. vigilax*. (B) Detection of WNV_{KUN} RNA by RT-PCR in excreta and filter paper cards collected over 18±24 h from batches of 5 *Cx. annulirostris*.

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the proportion of WNV_{KUN} positive excreta samples was higher than the proportion of WNV_{KUN} positive FP cards (79% (15/19) vs 42% (8/19); $P = 0.0448$). For both viruses, no significant difference ($P>0.05$) was observed between the median C_t values obtained from positive excreta and saliva expectorates on FP cards (Fig 4)

There was a significant difference ($P < 0.05$) between the proportions of RRV positive excreta and RRV positive FP cards obtained from individual *Ae. vigilax* at each time point (Table 3). With the exception of day 11 PE, where only one FP card was positive, median C_t values were significantly different between excreta and FP cards (day 8 PE: $P<0.05$; day 15 PE: $P<0.01$; overall: $P<0.01$; Fig 2).

For WNV_{KUN} only 2 FP cards were positive on day 15 (Fig 3). With the exception of day 15 PE, there was a significant difference ($P < 0.05$) between the proportions of WNV_{KUN} positive excreta and FP cards obtained from *Cx. annulirostris* at different time points (Table 3). There was no significant difference ($P>0.05$) between median C_t values obtained from excreta and FP samples (Fig 3).

Specificity and sensitivity of viral RNA detection in excreta and FP cards as a proxy for viral dissemination were calculated as described by [29]. Mosquitoes with a confirmed disseminated infection (assessed by CC-EIA) and a positive RT-PCR result were considered true positives

Table 3. Proportion of excreta and saliva (filter paper cards) from individual mosquitoes positive for viral RNA by real-time RT-PCR tested at different days post exposure (PE).

Mosquito	Virus	Day PE	n	Excreta[a]		Saliva[b]	
				%	95%CI	%	95%CI
Ae. vigilax	RRV	8	19	68	46±85	16*	5±38
		11	19	53	32±73	5*	0±26
		15	17	71	47±87	24*	9±48
		Total	55	64	50±75	14*	7±26
Cx. annulirostris	WNV_{KUN}	7	20	25	11±47	0*	0±19
		12	20	30	14±52	0*	0±19
		15	19	26	11±49	11	2±33
		Total	59	27	17±40	3*	3±12

[a]Percentage of positive excreta samples, 95% confidence intervals

[b]Percentage of positive saliva samples (filter paper cards), 95% confidence intervals

*Fisher's exact test two-tailed *P*-value <0.05 for comparison with excreta

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(TP) and those with a disseminated infection but a negative RT-PCR result were considered false negatives (FN). Mosquitoes without a disseminated infection and negative RT-PCR result were considered true negatives (TN) and those without a disseminated infection and positive RT-PCR result were considered false positives (FP). Using excreta as a proxy for viral dissemination, detection of RRV in excreta is highly specific (100%) and moderately sensitive (78%, 95%CI: 66±90). In contrast, detection of RRV in FP cards is highly specific (100%) but only slightly sensitive (18%, 95%CI: 7±29). For WNV_{KUN}, detection in excreta also is highly specific (93%, 95%CI: 84±100) and moderately sensitive (68%, 95%CI: 48±90) while detection in FP cards is highly specific (100%) but slightly sensitive (11%, 95%CI: 0±24).

Viability of arboviruses in excreta

To evaluate whether the excreted virus was infectious, 50 samples collected from batches of mosquitoes from each experiment (10 batches from 5 time points, RRV: day 2, 3, 6, 9 and 13 PE; WNV_{KUN}: day 2, 4, 6, 9 and 13 PE) were inoculated onto C6/36 cells and virus infection confirmed using the CC-EIA. Only 3 samples (6%) from different batches on different days had sufficient material to quantify the amount of RRV (day 2PE: $10^{3.06}$ $TCID_{50}$/mL; day 3PE: $10^{1.30}$ $TCID_{50}$/mL; day 9PE: $10^{1.80}TCID_{50}$/mL). Trace amounts of viable RRV were found on 8% (4/50) of the samples. In these samples CC-EIA indicated the presence of the virus in at least one well, but it was below the calculation cut-off value. Only one sample from day 9 PE showed trace amounts of viable WNV_{KUN} (2%, 1/50).

Discussion

Our results confirm that mosquitoes exposed to RRV or WNV_{KUN} excrete viral RNA at levels sufficient to be detected by molecular assays. Our findings, together with previous observations on the excretion of DENV RNA by *Ae. aegypti* [29] support the hypothesis that the excretion of arboviruses by mosquitoes is a general phenomenon. Interestingly, even when the infection rate of WNV_{KUN} in *Cx. annulirostris* (42%) was lower than the infection rate of RRV in *Ae. vigilax* (82%), we were able to detect viral RNA in excreta from batches of mosquitoes continually from day 2 to day 15 PE. This indicates that the detection of viral RNA in excreta is not a result of a high mosquito infection rate under laboratory conditions. Blood meal digestion times vary between mosquito species, but generally 72 hours after feeding it has finalized [41]. Similar to the results of Fontaine et al., we observed brown excreta spots from digested blood meals in samples from day 2 and 3 PE, hence it is possible that viral RNA from those samples came directly from the blood meal. From day 4 onward, no dark excreta spots were visible, indicating that blood meal digestion was completed. The excreta from individual mosquitoes also provided sufficient material for detection of viral RNA at all timepoints indicating that the method is sensitive enough regardless of the volume of excreta collected. Indeed, we were able to detect viral RNA from containers with as little as one visible blue excreta spot.

We observed a correlation between viral dissemination and excretion of viral RNA. RRV RNA was not detected in excreta from any individual *Ae. vigilax* tested without a disseminated infection. Only 3 excreta samples from *Cx. annulirostris* without disseminated infection but with confirmed midgut infection were positive for WNV_{KUN} RNA. However, it is important to note that viral dissemination was assessed by cell culture, which is less sensitive than RT-PCR [42] and may have failed to detect low titer disseminated infection. RRV disseminates quickly in *Ae. vigilax*; 2 days after ingesting an infectious bloodmeal [33] with transmission occurring from day 3±4 PE [43]. Similarly, dissemination of WNV_{KUN} in *Cx. annulirostris* is detectable as early as day 3, with initial transmission observed on day 5 and increasing from day 10 to day 14 PE [44]. We detected RRV and WNV_{KUN} RNA in 90% and 70% excreta

samples from batches of *Ae. vigilax* and *Cx. annulirostris*, respectively, collected on day 4 PE, when viral dissemination has already occurred for both viruses. Our results from individuals and batches of mosquitoes support the idea that testing mosquito excreta could be used in vector competence experiments as an indicator of viral dissemination or as a proxy for virus transmission potential for arboviruses that do not have a suitable transmission model, such as the DENVs, without having to sacrifice the insects. A limitation of this method is that it is impossible to distinguish viral RNA resulting from blood meal digestion from that being excreted because of viral dissemination. In order to avoid false positives, excreta samples should be collected after blood meal digestion has finalized.

For both batches and individual mosquitoes (overall), the proportion of positive excreta samples was higher than the proportion of positive saliva samples, suggesting that excreta offers an attractive sample for analysis of mosquitoes with disseminated infection in the laboratory and potentially in the field. Although specificity of detection of viral RNA when used as a proxy for viral dissemination in both excreta and saliva is high, sensitivity is at least 4 times higher for excreta compared to saliva (RRV: 78% vs 18%: WNV_{KUN}: 68% vs 11%). Indeed, for WNV_{KUN} only 2 saliva samples were positive for viral RNA. These differences in sensitivity are expected, since detection of viral RNA in excreta and saliva result from different processes: dissemination and transmission. Not all mosquitoes with a disseminated infection transmit the virus, and the existence of a salivary gland infection barrier, where the virus is unable to enter or establish infection of the salivary glands prior to transmission has been documented. [7, 15]. In this experiment, only 44% and 42% of the mosquitoes with a disseminated infection transmitted RRV and WNV_{KUN}, respectively, as measured by the capillary tube method. The median C_t values obtained from positive saliva expectorates were significantly higher than those from positive excreta samples obtained from individual mosquitoes. This is not surprising, since the volume of fluid excreted by mosquitoes is higher than what they expectorate (~1.5 μl [45] vs 4.7 nl [41]). This difference was not observed in batches of mosquitoes, possibly because there was more than one mosquito expectorating onto each filter paper card, potentially increasing the amount of viral RNA.

There is potential for mosquito excreta to be applied to enhance arbovirus surveillance. Honey-based surveillance provides a better estimate of transmission risk than testing pools of mosquitoes, since only transmitting mosquitoes will yield positive results [17, 46]. However, the proportion of mosquitoes in a population that survive the extrinsic incubation period can be low. Given that arboviruses can be detected in excreta as early as 2 days after the ingestion of an infectious blood meal, mosquito excreta could be used to obtain evidence of arbovirus circulation earlier. These results could be used to prompt intensive mosquito trapping for pooling and processing by traditional methods. Since mosquitoes expel only small quantities of saliva, the amount of virus on FTA cards is generally of low concentration which may lead to false negatives [22]. In this study, we observed that detection of arboviruses in excreta is more sensitive than detection in saliva. Further experiments will be required to establish if large amounts of excreta from non-infected mosquitoes would reduce the ability to detect viral RNA from the excreta of a single mosquito and to evaluate its performance under field conditions. Additionally, a methodology would need to be developed to collect and preserve the viral RNA from excreta in light traps and passive mosquito traps [18, 35] in a way that is convenient for routine surveillance. Recently, a method was described to collect mosquito excreta for xenomonitoring of filarial parasites, malaria, and trypanosomes, using super hydrophobic cones to concentrate excreta either into tubes or FTA cards, enabling detection of parasite DNA from the samples [47]. Finally, mosquito excreta could be used as an exploratory sample for virus discovery or metagenomic analysis by providing a template for next generation

sequencing, greatly reducing associated costs (one sample vs several pools of mosquitoes per trap).

Only low or trace amounts of viable virus were found in excreta samples. It has been proposed that arbovirus virions in the midgut are inactivated by digestive proteases that affect the integrity of their envelope, rendering the virion non-infectious [7]. The sample with the highest titer (RRV, $10^{3.06}$ $TCID_{50}$/mL) was obtained on day 2 PE and it is possible that this [a]higher[o] viral titer resulted from the digestion of the recently acquired infectious blood meal. It is unlikely that mosquito excreta has a role as an alternative route of transmission under field conditions. Firstly, arboviruses are labile in the environment; in fact, viability of arboviruses in infected mosquitoes decreases rapidly after their death in hot and humid conditions [48]. Mosquito excreta also contains digestive enzymes [49] which could continue to inactivate remaining virions once they have been excreted. Secondly, arbovirus infection via aerosol has only been observed under circumstances of high virus concentration [50]. Studies to test Japanese encephalitis virus (JEV) vaccines using *Rhesus* macaques exposed intranasally to JEV required at least 6.6 x 10^6 infectious units per animal to achieve infection [51, 52]. Our results obtained from batches of 5 mosquitoes with a high infection rate showed only low or trace amounts of viable virus. In the field, where only 1±2 mosquitoes out of thousands in a trap might be infected, the amount of viable virus in excreta would be even lower. Finally, it is well documented that mosquito saliva plays an important role in facilitating arbovirus transmission [53] and excreta lacks salivary proteins responsible for generating favourable replication conditions in the vertebrate host.

There are some factors that influence the outcome of experiments that rely on experimental infection of mosquitoes. A limitation of our study was the use of field collected *Cx. annulirostris*. It has been documented that the source of the vector population plays a role in the outcome of vector competence studies [54]. Unknown factors such as age, previous exposure to other pathogens, temperature and vector microbiome can affect vector competence and the reproducibility of the experiment [55, 56]. Differences in blood meal titers could also influence rates of excreta detection. Midgut infection and escape barriers are dose dependent [57]. Females exposed to higher viral doses tend to develop a disseminated infection quicker. In contrast, females ingesting lower viral doses have lower infection rates and take longer to amplify the virus [58]. In our study, both mosquitoes were exposed to high viral titers, which could explain the early detection of viral RNA in excreta resulting from viral dissemination. While excreted viral RNA is detected earlier from mosquitoes exposed to higher titers, Fontaine et al. did not observe a difference in the amount of DENV RNA excreted between low and high titers. Further experiments will be required to determine if this applies to other arboviruses.

Important work to understand and prevent arbovirus outbreaks is undertaken in the laboratory and in the field analysing different mosquito samples. Mosquito excreta is an easily collected sample and provides a simple and efficient method for assessing virus dissemination in vector competence experiments. Although the use of mosquito excreta to enhance sugar-based arbovirus surveillance is still at experimental stage, our results suggest that excreta offers an attractive sample for analysis that could enable earlier and more sensitive detection of circulating arboviruses, and potentially be used for virus discovery.

Supporting information

S1 Fig. Detection of RRV RNA by real time RT-PCR in excreta from batches and individual mosquitoes. Samples collected over 18±24 h from batches and individual *Ae. vigilax* sampled at different timepoints post exposure (PE). Bars denote medians. $P<0.05$ (*), $P<0.001$

(**), $P<0.0001$(***). Each point represents either a batch of 5 or an individual mosquito.
(TIFF)

S2 Fig. Detection of WNVKUN RNA by real time RT-PCR in excreta from batches and individual mosquitoes. Samples collected over 18±24 h from batches and individual *Cx. annulirostris* sampled at different timepoints post exposure (PE). Bars denote medians. $P<0.05$ (*), $P<0.001$ (**), $P<0.0001$(***). Each point represents either a batch of 5 or an individual mosquito.
(TIFF)

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4

STABILITY OF WEST NILE VIRUS (FLAVIVIRIDAE: FLAVIVIRUS) RNA IN MOSQUITO EXCRETA

The work presented in this chapter, entitled "Stability of West Nile Virus (Flaviviridae: Flavivirus) RNA in mosquito excreta" was published in the Journal of Medical Entomology, June 2019. doi: 10.1093/jme/tjz044

4.1 CONTEXTUAL LINKAGE

The results I have presented in Chapter 3 demonstrate that it is possible to detect viral RNA in the excreta from mosquitoes with a disseminated arbovirus infection. In order to develop protocols to collect excreta in the field, it is necessary to determine if viral RNA in excreta is stable under tropical conditions over the periods of time in which mosquito traps are deployed. Additionally, the substrate on which the excreta is deposited could also play a role in preserving RNA stability in the field. For this, I designed an experiment where I infected mosquitoes with West Nile virus, allowed them to excrete on two different substrates and stored the substrates for up to two weeks in an incubator simulating tropical conditions. The results and recommendations presented in this chapter can be used as a guide for the development of field methodologies to collect mosquito excreta utilising different traps and trapping schedules.

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Research

Stability of West Nile Virus (Flaviviridae: Flavivirus) RNA in Mosquito Excreta

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Abstract

Arbovirus surveillance is crucial for the implementation of vector-borne disease control measures. Recently, it has been demonstrated that mosquitoes with a disseminated arbovirus infection excrete viral RNA, which can be detected by molecular methods. Thereby, mosquito excreta has been proposed as a sample type that could be utilized for arbovirus surveillance. In this study, we evaluated if West Nile virus (Kunjin strain, WNV_{KUN}) RNA in *Culex annulirostris* Skuse (Diptera: Culicidae) excreta deposited on different substrates could be detected after storage for up to 2 wk at tropical conditions of high heat and humidity. No significant drop in relative quantity of WNV_{KUN} RNA (determined by comparison of C*t* values) in excreta deposited on Flinders Associate Technologies (FTA) cards was observed over 14 d, suggesting that RNA was stable for that time. There was no significant difference in relative quantity of WNV_{KUN} RNA in excreta deposited on FTA cards or polycarbonate substrates after 24 h. However, after 7 and 14 d, there was a significant decline in the relative quantity of viral RNA in the excreta stored on polycarbonate substrates. For incorporation in arbovirus surveillance programs, we recommend the use of polycarbonate substrates for excreta collection in mosquito traps deployed overnight, and the integration of FTA cards in traps serviced weekly or fortnightly. Polycarbonate substrates facilitate the collection of the majority of excreta from a trap, and while FTA cards offer limited area coverage, they enable preservation of viral RNA in tropical conditions for extended periods of time.

Key words: mosquito, arbovirus, excreta, viral RNA stability, surveillance

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5

MALARIA SURVEILLANCE FROM BOTH ENDS: CONCURRENT DETECTION OF *PLASMODIUM FALCIPARUM* IN SALIVA AND EXCRETA HARVESTED FROM *ANOPHELES* MOSQUITOES

The work presented in this chapter, entitled "Malaria surveillance from both ends: concurrent detection of *Plasmodium falciparum* in saliva and excreta harvested from *Anopheles* mosquitoes" was published in the Parasites and Vectors, July 2019. doi: 10.1186/s13071-019-3610-9

5.1 CONTEXTUAL LINKAGE

Based on the results described in Chapter 3, in the context of malaria surveillance, I set to investigate if I could also detect parasites in mosquito excreta. It has been demonstrated that it is possible to detect *Plasmodium falciparum* in mosquito excreta by molecular methods, however, a study to determine the time-frame of detection in excreta and a comparison with detection in saliva had not been conducted. For this, I designed two experiments where I collected excreta and saliva from *Anopheles stephensi* mosquitoes that had been previously exposed to different cultures of *P. falciparum* gametocytes. I collected, homogenised and extracted the samples using the protocols developed in Chapter 3, before testing them by RT-rtPCR. The findings presented in this chapter are the first report of concurrent detection of *P. falciparum* RNA in mosquito excreta and saliva from the same mosquito cohorts.

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Parasites & Vectors

RESEARCH Open Access

Malaria surveillance from both ends: concurrent detection of *Plasmodium falciparum* in saliva and excreta harvested from *Anopheles* mosquitoes

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Abstract

Background: Malaria is the most important vector-borne disease in the world. Epidemiological and ecological studies of malaria traditionally utilize detection of *Plasmodium* sporozoites in whole mosquitoes or salivary glands by microscopy or serological or molecular assays. However, these methods are labor-intensive, and can over- or underestimate mosquito transmission potential. To overcome these limitations, alternative sample types have been evaluated for the study of malaria. It was recently shown that *Plasmodium* could be detected in saliva expectorated on honey-soaked cards by *Anopheles stephensi*, providing a better estimate of transmission risk. We evaluated whether excretion of *Plasmodium falciparum* nucleic acid by *An. stephensi* correlates with expectoration of parasites in saliva, thus providing an additional sample type for estimating transmission potential. Mosquitoes were exposed to infectious blood meals containing cultured gametocytes, and excreta collected at different time points post-exposure. Saliva was collected on honey-soaked filter paper cards, and salivary glands were dissected and examined microscopically for sporozoites. Excreta and saliva samples were tested by real time polymerase chain reaction (RT-rtPCR).

Results: *Plasmodium falciparum* RNA was detected in mosquito excreta as early as four days after ingesting a bloodmeal containing gametocytes. Once sporogony (the development of sporozoites) occurred, *P. falciparum* RNA was detected concurrently in both excreta and saliva samples. In the majority of cases, no difference was observed between the C_t values obtained from matched excreta and saliva samples, suggesting that both samples provide equally sensitive results. A positive association was observed between the molecular detection of the parasites in both samples and the proportion of mosquitoes with sporozoites in their salivary glands from each container. No distinguishable parasites were observed when excreta samples were stained and microscopically analyzed.

Conclusions: Mosquito saliva and excreta are easily collected and are promising for surveillance of malaria-causing parasites, especially in low transmission settings or in places where arboviruses co-circulate.

Keywords: Malaria, Mosquito, Saliva, Excreta, Sporozoite, *Plasmodium falciparum*, *Anopheles stephensi*

Background

Malaria is the deadliest vector-borne disease, with an estimated 219 million cases and 435,000 deaths in 2017 alone [1]. More than 90% of the cases occur in sub-Saharan Africa, and children under five years are the most vulnerable group. *Plasmodium falciparum* is the most prevalent causative agent of human malaria and has the most severe clinical manifestations [2]. The parasites are transmitted to humans by anopheline mosquitoes. More than 70 *Anopheles* species are competent vectors of malaria and more than half of these are responsible for

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transmitting the majority of human malaria parasites [3, 4].

Malaria control, elimination and ultimately, eradication, are global priorities, with 21 countries committed to eliminate malaria by 2020 [5]. Malaria elimination is achieved through a combination of antimalarial treatments (such as artemisinin-based combination therapy, ACT), vector control and source reduction of mosquito larval habitats. Surveillance is a crucial component of malaria intervention programmes, providing information to guide initiatives and measure their impact [6] and is regarded as one of the three fundamental pillars of the Global Technical Strategy [7]. Malaria surveillance strategies are dependent on the level of transmission, where lower levels of transmission require increased efforts to detect new cases and transmission foci. Generally, malaria surveillance focuses on passive or active case detection, monitoring of anti-malarial drug resistance and entomological surveillance, including detection of insecticide resistance [8].

Detection of *Plasmodium* in mosquitoes is an essential parameter used to estimate metrics of exposure and transmission intensity. The sporozoite, the infectious stage of the parasite in the mosquito is usually the target of these efforts. There are several approaches to detect sporozoites in field-collected mosquitoes. Traditionally, their salivary glands are dissected and observed under a compound microscope for the presence of sporozoites [9, 10]. Alternatively, enzyme-linked immunosorbent assays (ELISAs) have been used to detect sporozoite protein in salivary glands or pools of mosquitoes [11, 12]. Rapid diagnostic tests in dipstick format have also been developed [13, 14], with results comparable to those obtained by ELISA [15]. A variety of polymerase chain reaction (PCR) methods are available, with high sensitivity and versatility [16–18]. However, although certainly useful, these techniques have limitations. Dissection and observation of salivary glands is time-consuming, require skill and expertise, can fail to detect infections with low numbers of sporozoites and is not species specific. Immunoassays can yield false positives that need to be confirmed by molecular methods [10, 19, 20]. Even though PCR assays allow for high-throughput analysis, the identification and sorting of mosquitoes can be labor-intensive, especially for larger collections. They also require specialized facilities, equipment and expertise, which is often not available in low resource settings. Finally, all these methods can overestimate transmission, since not all the sporozoites present in the salivary glands will be ejected by a feeding mosquito [21].

Analysis of mosquito saliva for the presence of pathogens provides a better estimate of transmission risk. Mosquito saliva has been used for the study of other mosquito-borne diseases, particularly arthropod-borne viruses (arboviruses), both in the field and the laboratory [22, 23]. It had been demonstrated that *P. falciparum* could be detected in mosquito saliva collected by forced salivation [9], but it was not until recently that *P. falciparum* sporozoites were detected in saliva expectorated on honey-soaked nucleic acid preservation cards, allowing for detection without killing the mosquito [24]. Alternatively, mosquito excreta has emerged as a promising sample for the study of arboviruses [25–27], filarial parasites and malaria [28]. Mosquito excreta has the added potential to be used for xenomonitoring, where the mosquitoes are used as "flying syringes" to sample vertebrate hosts to monitor human and animal diseases and methodologies are being developed to collect mosquito excreta in the field [29].

The primary objective of the current study was to determine, through proof of concept, if *P. falciparum* could be detected by molecular assays concurrently in excreta and saliva of *Anopheles stephensi* mosquitoes. We also correlated the detection of the parasite in excreta and saliva with salivary gland sporozoite infection in the mosquitoes. Finally, we analyzed excreta samples microscopically for evidence of recognizable parasites.

Methods

Parasite maintenance

The asexual stages of *P. falciparum* NF54 were maintained at 4% hematocrit in human O-positive erythrocytes (Australian Red Cross, Melbourne) in RPMI-HEPES with 10% heat-inactivated human serum (Australian Red Cross, Melbourne) in an atmosphere of 94% N, 5% CO_2, 1% O_2 [30]. Gametocytes were generated as described previously, using the crash method [31]. After 17 days, gametocytes were quantified by Giemsa smears, harvested, and five different blood meals prepared by dilution to 0.3% stage V gametocytemia in human serum for feeding to mosquitoes [30].

Mosquito rearing

Experiments were performed using *Anopheles stephensi* mosquitoes (John Hopkins School of Public Health strain) at the Walter and Eliza Hall Institute of Medical Research. Larvae were fed a 1:1 ratio of TetraMin and Nutrafin Max tropical fish food flakes. After adult emergence, mosquitoes were provided sugar cubes and water in a cotton wick *ad libitum*. Females were offered mouse blood in water-jacketed, glass membrane feeders (Lillie Glassblowers, Inc., Georgia, USA) to stimulate egg production. All mosquitoes were maintained at 26 °C, 80% RH and 12:12 L:D for the duration of the study.

Exposure of mosquitoes to *P. falciparum* gametocyte cultures and analysis of parasite development

Four- to five-day-old mosquitoes were deprived of sugar overnight (10–14 h) prior to being exposed to *P. falciparum* gametocytes. Females were aspirated into 0.946 l paper cartons (Castaway Food Packaging, Australia) secured with mesh lids where they were offered a gametocytemic blood meal through a water-jacketed, glass membrane feeder. Two hours after feeding, mosquitoes were CO_2 anesthetized and sorted on wet ice. Only fully engorged females were maintained, whilst males, non-fed and partially-fed females were discarded. Fully engorged females were immediately placed in a 24.5 cm^3 mesh cage (Bugdorm-42222, Bugdorm, Taichung, Taiwan), with sugar cubes and a water wick, or in modified containers for excreta collection (see below). At day 8 post-exposure (PE), the midguts from 16–23 cold-anesthetized and ethanol-killed mosquitoes from each cohort were dissected and stained with 0.1% mercurochrome (w/v) in water, and oocysts per mosquito enumerated by microscopy. At day 17 PE, 30–32 salivary glands from mosquitoes from each cohort were dissected and pooled before being homogenized in PBS with a pestle to release sporozoites. After filtering through glass wool, sporozoites were counted using a Neubauer hemocytometer, and each cohort sample was counted in triplicate.

Collection of mosquito excreta and saliva

Two experiments were conducted to evaluate the use of mosquito excreta and saliva for *P. falciparum* detection. In the first experiment, groups of mosquitoes were followed over time to establish the time of first detection in excreta. For this, 20 batches of 5 mosquitoes which had been exposed to two different gametocytemic blood meals were placed in modified 150 ml polypropylene containers for excreta collection [26]. The containers had a fiberglass insect screen floor to allow excreta to pass through onto a parafilm disc and the top opening of the containers was covered in mesh. Mosquitoes were maintained on cotton pledgets soaked in 15% honey water dyed with blue food coloring for excreta visualization. Excreta was collected daily from day 4 to 14 PE using a cotton swab moistened with PBS. Swabs were placed in a 1.5 ml tube with 500 µl PBS and stored at − 80 °C. Cotton pledgets and parafilm discs were replaced daily to avoid cross-contamination and mortality was recorded daily.

In the second experiment, from day 15 to 19 PE, excreta and saliva were collected from groups of mosquitoes and the presence of sporozoites in their salivary glands was visually assessed. For this, 3 groups of 5 mosquitoes from cohorts that had fed on 5 different blood meals containing gametocytes were placed in modified containers as described above. For daily saliva collection, mosquitoes were allowed to feed on a 4 cm^2 filter paper card (FP; low chamber filter paper, Bio-Rad Laboratories, California) soaked in 100% honey dyed with blue food coloring. After 24 h, excreta was collected as previously described, whilst the FP cards were removed and placed in a 1.5 ml tube containing 0.5 mL PBS and stored at − 80 °C. The mosquitoes were CO_2 anesthetized, ethanol-killed, and the salivary glands dissected and assessed for the presence of sporozoites using a compound microscope. The sporozoite rate of the container was calculated as the number of mosquitoes with sporozoites in their salivary glands per the number of surviving mosquitoes in the container.

Detection of *Plasmodium* spp. by real-time RT-rtPCR

Thawed excreta samples were agitated using a Qiagen Tissue Lyser II (Qiagen, Hilden, Germany) for 3 min at 26 Hz and centrifuged for 1 min at 14,000×*g* [26]. Thawed FP cards were maintained at 4 °C and briefly vortexed every 5 min for 20 min [22]. RNA was extracted from excreta samples and FP card eluates using a QIAmp One-For-All Nucleic Acid Kit (Qiagen, Hilden, Germany) in a QIAxtractor (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A Taqman real-time RT-PCR (RT-rtPCR) assay (modified from [32]) was used to detect *Plasmodium* spp. The assay amplifies a conserved region of the *18S* rRNA gene. The primers and probe were: forward primer (5′-AGG AAG TTT AAG GCA ACA ACA GGT-3′); reverse primer (5′-GCA ATA ATC TAT CCC CAT CAC GA-3′); and probe (5′-6FAM-TGT CCT TAG ATG AAC TAG GCT GCA CGC G-BHQ-1-3′). Primer and probe oligonucleotides were synthesized by Sigma-Aldrich (Castle Hill, Australia). The reaction mix was prepared using SuperScript III Platinum one-step quantitative RT-PCR system (Invitrogen, Carlsbad, CA) and contained 0.4 µl of SuperScript III/Platinum *Taq* mix, 10 µl of 2× reaction mix, 50 nM of ROX reference dye, primers and probe in a final optimized concentration of 900 nM and 150 nM respectively, 5 µl of extracted RNA and nuclease-free water to produce a final volume of 20 µl. The assays were run in a Rotor-Gene 6000 real-time PCR cycler (Qiagen, Australia) with cycling conditions as follows: (i) one cycle at 50 °C for 5 min; (ii) one cycle at 95 °C for 2 min; and (iii) 50 cycles of 95 °C for 3 s and 60 °C for 30 s. Each run included a positive extraction control (bovine viral diarrheal virus, BVDV) and a positive *P. falciparum* control extracted from sporozoites; a negative extraction control and a no-template control (molecular grade water). The cycle threshold number (C_t) was determined for each sample; any sample with a $C_t > 40$ was considered negative.

To determine the assay's limit of detection, a sample of quantified sporozoites from salivary glands was extracted as described above, and 10-fold dilutions were used to generate a standard curve with undiluted RNA and each dilution (10^{-1} to 10^{-8}) tested in triplicate.

Visualization of *P. falciparum* in mosquito excreta

A total of six aliquots from 10 excreta samples that were positive by RT-rtPCR were air-dried, methanol-fixed and dyed with 11% Giemsa stain diluted in distilled water for 60 min before being washed with water, dried and examined using a compound microscope under 1000× magnification.

Statistical analyses

All data sets were tested for normality using Shapiro-Wilks tests. Differences in salivary gland infection between cohorts were analyzed using a one-way ANOVA followed by Tukey's multiple *post-hoc* comparison test. Differences in oocyst counts between cohorts and C_t values for excreta and expectorate between days and between groups were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Differences in C_t values between excreta and saliva were analyzed using the Wilcoxon matched-pairs signed rank test. Differences between the proportion of positive saliva and excreta samples were analyzed using the Fisher's exact test. Associations between sporozoite rates and C_t values from saliva and excreta were analyzed using Spearman's rank correlation. All figures, Kaplan-Meier survival curves and statistical analyses were performed using GraphPad Prism version 7.0c (GraphPad Software, La Jolla, CA, http://www.graphpad.com).

Results

Parasite development in the mosquito

There was no difference in mosquito survival distributions between cohorts (Log-Rank statistic $\chi^2_{(3)}=4.415$, $P=0.220$; Additional file 1: Figure S1). The overall oocyst rate (prevalence of mosquito infection) in mosquito midguts at day 8 PE was 72.7%, ranging from 55% for cohort C to 91% for cohort B (Fig. 1a). There was a significant difference between the median number of oocysts between groups (Kruskal-Wallis one-way ANOVA, $H_{(4)}=15.67$, $P=0.0035$, Fig. 1b). On day 17 PE, the mean number of sporozoites per mosquito ranged from 2490 in cohort C to 9730 in cohort A (Fig. 1c). There was a significant difference between groups as determined by one-way ANOVA ($F_{(4,\ 15)}=54.11$, $P<0.0001$). However, *post-hoc* analysis showed that there was no significant difference in sporozoite load between some groups. Consequently, for further analyses of the second experiment, the cohorts were grouped as high sporozoite load (AE), mid sporozoite load (B) and low sporozoite load (CD) where applicable.

Plasmodium falciparum sporozoite RT-rtPCR detection threshold

To determine the RT-rtPCR assay's limit of detection, a standard curve was prepared using RNA extracted from quantified sporozoites purified from mosquito salivary glands on day 17 PE. Serial dilutions of parasite RNA resulted in an R^2 of 0.9451 and a slope of −2.92, demonstrating the linear relationship between the logarithm of the number of parasites and C_t value within a 4-$\log_{10}$ dynamic range (Additional file 2: Figure S2). At a C_t value > 40 P. *falciparum* could not be detected.

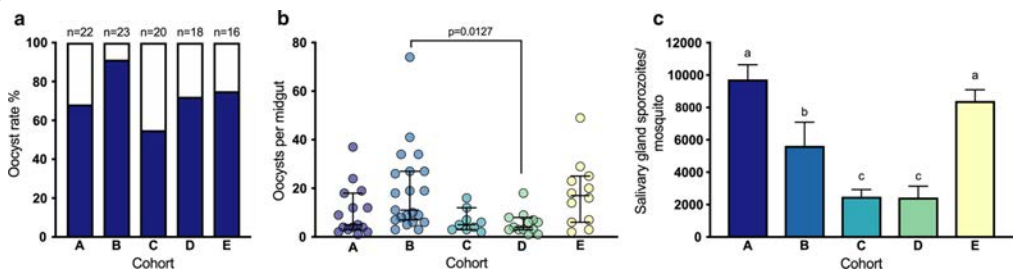

Fig. 1 Parasite development in mosquito cohorts exposed to five different bloodmeals (indicated as cohorts **a**–**e**). **a** Proportion of mosquitoes with oocysts in their midguts at day 8 PE. **b** Oocyst counts per mosquito midgut 8 days PE (median and 95% CI, Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test). Each dot corresponds to one midgut. **c** Salivary gland sporozoite loads per mosquito 17 days PE. Different letters indicate statistically significant differences between groups (mean ± SEM, $n=4$, one-way ANOVA test followed by Tukey's multiple comparison test, $P<0.05$)

Mosquitoes excrete *P. falciparum* material soon after ingesting an infectious blood meal

The excreta from 10 containers from each of 20 original containers from the two cohorts was analyzed over time. *Plasmodium falciparum* was detected in mosquito excreta by RT-rtPCR as early as day 4 PE in both cohorts (Fig. 2). For cohort A (68.2% oocyst rate and 9730 ± 910 sporozoites per mosquito), excreta samples collected from 8 out of 10 containers were positive at least once from day 4 to day 14 PE, with 10% (11/110) samples positive for *P. falciparum* overall. C_t values ranged from 27.5 to 37.9. For cohort B (91.3% oocyst rate and 5630 ± 1460 sporozoites per mosquito), excreta samples collected from 8 out of 10 containers were positive at least once for the duration of the experiment, with 16% (18/110) samples positive for the parasite and C_t values ranging from 25.5 to 39.7. No statistically significant difference was observed between mean C_t values between the cohorts (Two sample t-test, $t_{(2)} = 0.5236$, $P = 0.6048$). For both cohorts, no positive samples were observed on day 10 and day 12 PE.

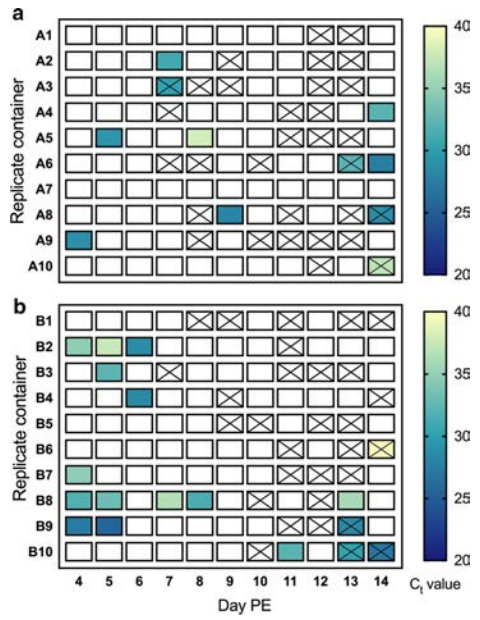

Fig. 2 Time series RT-rtPCR detection of *P. falciparum* in excreta from groups of 5 *An. stephensi* mosquitoes. Excreta was collected daily from day 4 to 14 post-exposure (PE). **a** Mosquitoes with 68.2% oocyst rate and 9730 ± 910 sporozoites per mosquito. **b** Mosquitoes with 91.3% oocyst rate and 5630 ± 1460 sporozoites per mosquito. Lower C_t values correspond to a greater concentration of starting template; a blank cell indicates that *P. falciparum* RNA was not detected. An X indicates containers with no visible excreta spots

Plasmodium falciparum can be detected concurrently in mosquito excreta and saliva after sporogony

Plasmodium falciparum sporozoites were microscopically observed in the salivary glands of at least one mosquito removed from each of the containers analyzed in the second experiment (75/75). *P. falciparum* was detected by RT-rtPCR in 89% (67/75) of saliva samples and 91% (68/75) of excreta samples collected from day 15 to 19 PE, with no significant difference between these proportions (Fisher's exact test, $P > 0.9999$). No significant differences were observed in median C_t values of saliva samples between days within the same cohort (Kruskal–Wallis one-way ANOVA, $P > 0.05$) or from excreta samples between days within the same cohort (Kruskal-Wallis one-way ANOVA, $P > 0.05$). Thus, the samples from different days from the same cohort were analyzed together from this point onward. No statistically significant differences were observed between median C_t values obtained from saliva and excreta, except the mosquitoes with a medium sporozoite load, where the median C_t value was lower in excreta than saliva (Fig. 3a; 27.9 *vs* 30.0, Wilcoxon matched-pairs signed rank test $W_{(14)} = 14$, $P = 0.0134$). When comparing detection between mosquitoes with different sporozoite loads, a statistically significant difference was observed in median C_t values from saliva (Fig. 3b; Kruskal–Wallis one-way ANOVA, $H_{(2)} = 15.61$, $P = 0.0004$). A statistically significant difference was also observed in median C_t values from excreta between these cohorts (Fig. 3c; Kruskal–Wallis one-way ANOVA, $H_{(2)} = 11.39$, $P = 0.0034$).

All containers from which saliva and excreta were harvested from had at least one mosquito with sporozoites in their salivary glands. The overall sporozoite rate for these mosquitoes was 60%, and the sporozoite rates were 66%, 56% and 54% for high, mid and low sporozoite load cohorts, respectively. A negative association was observed between the sporozoite rate of the container and the C_t value in saliva (Fig. 4a; Spearman's rank correlation $\rho_{(65)} = -0.5408$, $P < 0.0001$): the higher the proportion of mosquitoes with sporozoites in their salivary glands, the lower the C_t value (indicating higher amounts of the template). For excreta, this association was lower but still negative (Fig. 4b; Spearman's rank correlation $\rho_{(66)} = -0.3595$, $P = 0.0026$).

Visualization of *P. falciparum* life stages in excreta samples

A subsample (10/68) of the excreta samples that were positive for *P. falciparum* by RT-rtPCR were examined microscopically in sextuplicate. No visual evidence of

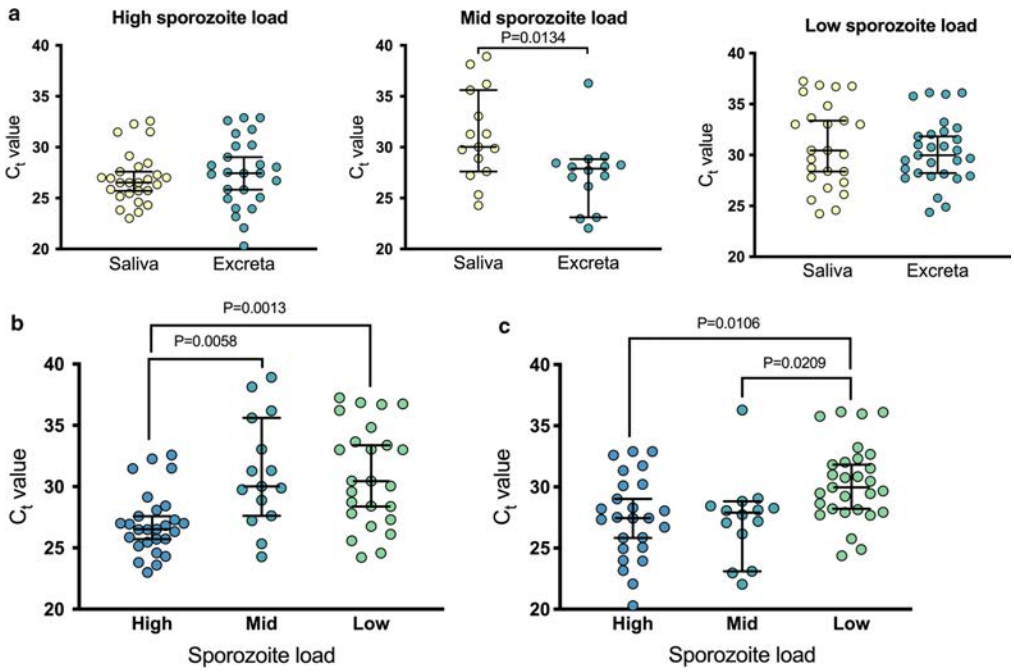

Fig. 3 RT-rtPCR detection of *P. falciparum* in mosquito secretions collected from mosquitoes with high, middle and low sporozoite loads on day 15 to 19 post-exposure. **a** Detection of *P. falciparum* in saliva vs excreta in mosquitoes from the cohorts with different sporozoite loads. Wilcoxon matched-pairs sign ranked test. Detection of *P. falciparum* in saliva (**b**) excreta (**c**) from mosquitoes with different sporozoite loads. Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison test. Data are the median C_t value ± 95% CI. Each dot represents a group of 5 mosquitoes in a container. Lower C_t values correspond to a greater concentration of starting template

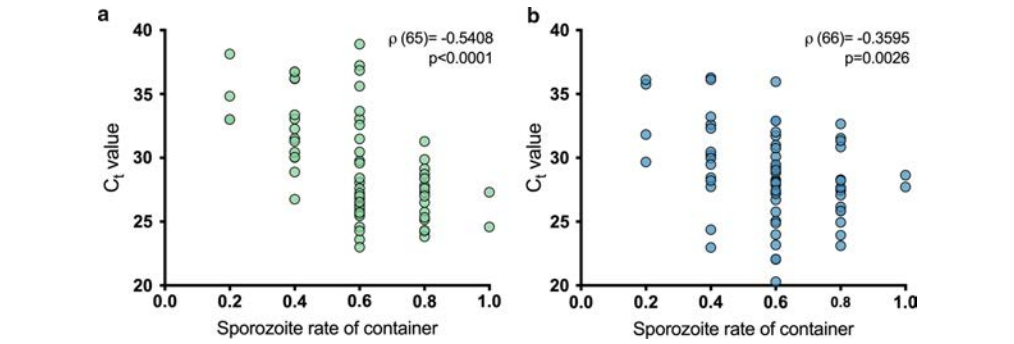

Fig. 4 Spearman's rank correlation between presence of sporozoites in salivary glands and RT-rtPCR detection of *P. falciparum*. Each dot represents a group of 5 mosquitoes in a container, sampled from day 15 to 19 post-exposure. Correlation between sporozoites and C_t values obtained from saliva (**a**) and excreta samples (**b**)

sporozoites or other life stage of the parasite was found in these samples.

Discussion

Given the limitations of traditional methods to study mosquito-borne diseases, there has been concern in finding innovative or alternative samples for analysis. Mosquito saliva expectorated during sugar feeding has been used for research and surveillance of arboviruses [22, 23], and recently mosquito excreta has been proposed as a sample to enhance the sensitivity of saliva detection or for molecular xenomonitoring [25–28]. To our knowledge, this is the first study to evaluate the excretion and expectoration of *P. falciparum* in parallel with parasite development in the mosquito. Our results confirm previous findings that *Plasmodium* can be detected in mosquito excreta [28] and saliva deposited on filter paper cards after sugar feeding [24].

Previous studies have demonstrated that *P. falciparum* DNA can be detected in mosquito excreta on days 2–3 PE [29]. In this study, excreta was not collected until day 4 PE, to allow for blood meal digestion, which takes approximately 72 hours to be completed [33]. Our results indicate that *P. falciparum* nucleic acid in mosquito excreta continues to be detectable after blood meal digestion from day 4 to at least 19 PE. The source of the nucleic acid or the parasite life stage in excreta is unknown, but several hypotheses could explain its presence. Once a mosquito feeds on an infected host, it ingests gametocytes, the sexual stage of the parasite. An hour later, fertilization occurs, and by 24 hours the ookinete enters the midgut were oocysts establish and begin mitosis [34]. It has been suggested that some of the material excreted in the early days could be metabolized merozoites [28], the asexual parasites in the intraerythrocytic cycle, which are present in ingested blood meal in a ratio of about 156 merozoites per gametocyte [35] and which cannot infect, or survive in, the mosquito.

Additionally, early stage parasite development from ookinete to oocyst is closely related with blood-meal digestion; ookinetes that fail to traverse the midgut and transform to oocysts after digestion are destroyed [36]. From day 11 to 16, the oocysts burst producing thousands of sporozoites that migrate through the hemocoel to the salivary glands [37]. This is an inefficient process: some of these oocysts may be unsuccessful in producing sporozoites and the released sporozoites can fail to navigate, invade or survive in the salivary glands, with less than 20% of the sporozoites released by oocysts reaching salivary glands [38]. The remaining sporozoites are degraded in the hemocoel [39], and although the mechanism is unknown, it is possible that the residue finds its way to the Malpighian tubules to be excreted with other unwanted substances of the hemolymph. Although 40% and 91% of the containers sampled from day 11 to 14 and 15 to 19, respectively, were positive for *P. falciparum* by RT-rtPCR, no distinguishable parasites were observed under microscopy following Giemsa staining in any of the analyzed samples. Further studies of the contents of mosquito excreta are required to determine the source of the excreted nucleic acid.

We were able to detect *P. falciparum* sporozoites deposited on filter paper cards after sugar feeding on days 15 to 19 PE. Our results expand the results from Brugman et al., who detected sporozoites on cotton wool pledgets from day 18 to 24 [24]. It is likely that the differences in C_t values in saliva samples between cohorts are due to differences in sporozoite rates and not in sporozoite loads. The high sporozoite load cohort also had the highest sporozoite rate (66%) compared to the other cohorts (56% and 54% for mid and low sporozoite cohorts respectively). This was further demonstrated by the negative association between the C_t values obtained from saliva samples and the sporozoite rate of the container. Studies have suggested that the sporozoite load in the salivary glands is not a predictor for sporozoite transmission [40] because the structure of the salivary glands limits the number of sporozoites that are expectorated [21].

With the exception of the mid sporozoite load cohort, no significant differences were observed between detection of *P. falciparum* in excreta and saliva in samples collected after sporogony. It is interesting to note that although mosquitoes from this cohort had the highest oocyst rate (91%) at day 8 PE, the sporozoite rate from day 15 to 19 was moderate (56%) in the context of this experiment. This could explain the lower C_t values observed in excreta, since many of the sporozoites produced by the oocysts may have failed to reach the salivary glands, and may have been destroyed and possibly voided in excreta.

In this study we did not directly evaluate the detection of the parasite in secretions from individual mosquitoes. However, we were able to detect *P. falciparum* in the saliva and excreta from 80% of containers where just one mosquito had a salivary gland infection, indicating that the method is sensitive enough to detect the parasites from an individual mosquito. Previous studies have demonstrated that trace amounts of *Brugia malayi* DNA are detectable in samples that contain excreta from as many as 500 uninfected mosquitoes [28]. Similarily, it does not appear that the saliva of numerous uninfected mosquitoes affects the detection of arboviruses in mosquito expectorate. It is unlikely that saliva or excreta from many mosquitoes would interfere with the detection of

P. falciparum from a single infected mosquito. However this needs to be further evaluated.

Detection *of Plasmodium* in mosquito excreta and saliva has applications in the laboratory. Observation of oocysts in mosquito midguts can be used as an estimation of mosquito infectivity [41]; however, oocysts are not visible until 6–7 days after ingesting an infectious bloodmeal, making it impossible to determine the infectious status of mosquitoes for a week [36]. Our results suggest that excreta could be monitored after bloodmeal digestion as soon as day 4, allowing for an earlier estimation of the potential of the parasite to establish a midgut infection in a non-destructive manner. An important component of vectorial capacity is the estimation of the period of sporogony, the period from which a mosquito ingests gametocytes to when it can transmit sporozoites to a receptive host [42]. Traditionally, this has relied on the detection of sporozoites in mosquito salivary glands. Monitoring the expectoration of the parasite could be a useful tool for exploring genetic traits and different environmental conditions that influence this period, allowing for a precise measurement of time-to-event in individual mosquitoes [43]. Genetic analyses and drug and vaccine development studies often rely in infected mosquitoes feeding on animal models, such as mice [44, 45] and non-human primates [46, 47]. Since our method allows for non-destructive screening of the parasite in the vector, mosquitoes that are transmitting could be pre-selected to increases the chances of transmission and potentially reducing the number of animals used in an experiment.

The analysis of mosquito saliva and excreta could also be implemented for malaria surveillance in the field. Currently, parasite detection in mosquitoes requires testing thousands of mosquitoes, either individually by microscopy or in pools by ELISA or molecular methods. Indeed, as transmission of a pathogen decreases, larger numbers of mosquitoes are necessary to improve the likelihood of capturing the less frequent occurrence of infection. Honey-based surveillance using nucleic acid preservation cards or wicks to collect mosquito saliva has been successfully incorporated by public health agencies in Australia and USA for routine surveillance of arboviruses [23, 48, 49] with several advantages over traditional methods. First, it reduces the number of samples that need to be processed down to 1–2 samples per trap. Secondly, the cards or wicks do not require a cold chain, making the method a logistically attractive approach. Finally, detection of the pathogen in mosquito saliva gives a better estimate of transmission risk, since only the mosquitoes that are transmitting will yield a positive result. Recently, it has been demonstrated that detection of arboviruses in excreta can be used to enhance the sensitivity of honey-based surveillance since the volume of the sample is larger [25, 26]. In the context of malaria surveillance, honey-based methods could be incorporated in regions with known co-circulation of malaria and arboviruses with the advantage of detecting all the circulating pathogens from one sample. Since it is not possible to determine how many mosquitoes expectorated or excreted in a trap, it is not possible to calculate an entomological metric, such as the sporozoite rate. Additionally, although a positive excreta result would not be sufficient to suggest that the mosquitoes in the trap are transmitting *Plasmodium*, it would indicate that the parasite is circulating. However, together with geolocation and mapping of larval habitats and areas of human activity [50], a positive result can be used to identify potential foci of transmission. This is particularly interesting in low transmission settings or to monitor re-establishment after elimination. In this study we used RT-rtPCR for pathogen detection, but the use of portable and automated rapid diagnostic test (RDT) devices for detection of the parasite in mosquito saliva and excreta samples needs to be assessed. Although the majority of RDTs available for *Plasmodium* focus on diagnosis of human samples [51], a VecTest dipstick assay for detection of sporozoites from mosquitoes has been developed [13, 14]. Dipstick assays have the advantage of providing results within minutes and do not require specialized equipment or infrastructure. Currently, a centrifugal microfluidic multiplex vector-diagnostic platform (LabDisk) to be used with mosquitoes is being evaluated [52]. The sensitivity of these assays is not as good as PCR-based detection, but given that collection of mosquito saliva and excreta is relatively simple, it could be coupled with RDTs or portable devices for use in low-resource settings and remote locations.

Conclusions

The development of methods to estimate malaria transmission in low-transmission settings has been identified as one of the objectives by the malaria Eradication Research Agenda (malERA) [53]. As elimination targets are met, it is evident that novel approaches will be needed to ensure that transmission foci are identified, and re-establishment is prevented. Mosquito saliva and excreta have potential to be added to the array of samples supporting the crusade for malaria elimination and eradication. The samples are relatively easy to collect and can be used by surveillance programmes to detect evidence of malaria transmission, especially in low resource settings since the number of samples that need to be tested is reduced. Finally, as evidenced by studies of other mosquito-borne diseases, it appears that excretion of pathogens by infected mosquitoes is a general phenomenon that can be exploited for research and surveillance applications.

Additional files

Additional file 1: Figure S1. Kaplan–Meier survival curves for cohorts of mosquitoes exposed to five different gametocyte cultures. The survival distribution was not different between cohorts (Log-Rank statistic $\chi^2_{(3)}=4.415$, $P=0.220$).

Additional file 2: Figure S2. RT-rtPCR standard curve. The standard curve was prepared using a suspension of *P. falciparum* sporozoites isolated from mosquito salivary glands. X-axis corresponds to the concentration of triplicate serially diluted template; Y-axis corresponds to RT-rtPCR C_t values.

Abbreviations
ACT: artemisinin-based combination therapy; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; PE: post-exposure; FP: filter paper; RT-rtPCR: real-time reverse transcription polymerase chain reaction; RDT: rapid diagnostic test; malERA: malaria Eradication Research Agenda.

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Authors contributions
ALR and SME performed the experiments. ALR, GRH and JLM analyzed the samples. AFvdH, JAB, ASPY and SME provided the study materials. ALR, AFvdH, SAR and SME conceptualized the study. IMM developed the molecular assay. ALR performed the data analyses. ALR wrote the initial draft of the manuscript. SAR, JAB and AFvdH acquired the funding for the research. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate
Protocols involving mice were conducted in strict accordance with the recommendations in the National Statement on Ethical Conduct in Animal Research of the National Health and Medical Research Council and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2014.030).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

6

METAGENOMIC ANALYSIS OF MOSQUITO EXCRETA FOR ENVIRONMENTAL VIROME SAMPLING

The work presented in this chapter is in preparation for submission as a manuscript and is included in such format.

6.1 CONTEXTUAL LINKAGE

Through my thesis, I have successfully demonstrated that RT-rtPCR can be used to detect arboviral (Chapter 3, Chapter 4) and *Plasmodium* RNA (Chapter 5) from mosquito excreta samples. As mentioned in Chapter 2, advances have been made in next-generation sequencing-based metagenomic technologies which allow the simultaneous identification of viruses, endosymbionts and even mosquito species in a single reaction without prior sequence knowledge. Since this approach has already been used to screen pools of mosquitoes, in this chapter I evaluated the use of next-generation sequencing for unbiased identification of RNA viruses in excreta collected from experimentally-infected and field-collected mosquitoes.

Metagenomic analysis of mosquito excreta for environmental virome sampling

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6.2 ABSTRACT

Traditional testing for arboviruses in mosquitoes requires a priori knowledge and the utilization of appropriate assays for their detection. As a sample type, mosquitoes can potentially be a vast source of additional information, including detection of unexpected or novel arboviruses, non-arboviral pathogens ingested from hosts they feed on and their own genetic material. Next-generation sequencing (NGS) is a rapidly advancing technology that allows us to potentially obtain all this information from a mosquito sample without any prior knowledge of virus, host or vector. Moreover, it has been recently demonstrated that pathogens, including arboviruses and parasites, can be detected in mosquito excreta by molecular methods. In this study, we investigated whether RNA viruses could be detected in mosquito excreta by NGS. For this, excreta samples were collected from mosquitoes experimentally exposed to either Ross River or West Nile viruses, and from field mosquitoes collected across Queensland, Australia. Total RNA was extracted from the excreta samples, reverse transcribed to complementary DNA (cDNA) and sequenced using the Illumina NextSeq 500 platform. Bioinformatic analyses from the generated reads demonstrate that mosquito excreta provides sufficient template for NGS, allowing the assembly of near full-length genomes. By using this approach, we identified Australian Anopheles totivirus, Wuhan insect virus 33 and Hubei odonate virus 5 along with seven potentially novel viruses closely related to members of the order *Picornavirales* (2/7) and to previously described, but unclassified, RNA viruses (5/7). Our results suggest that metagenomic analysis of

mosquito excreta has great potential for virus discovery and for unbiased arbovirus surveillance in the near future.

6.3 BACKGROUND

In effect, female mosquitoes, act as environmental samplers ("biological syringes") that feed on the blood of a variety of vertebrate hosts. Mosquitoes not only harbour arthropod-borne viruses (arboviruses) which are capable of replicating within and being transmitted by mosquito vectors, but also potentially carry other non-arboviruses that do not replicate in mosquitoes but might be present in hosts they feed upon. Traditionally, molecular diagnostic assays widely utilized in arbovirus surveillance programs only screen for characterized endemic and enzootic viruses. It is likely that many other viruses, regardless of pathogenicity, may remain undetected. Metagenomic analysis using next-generation sequencing (NGS), allows the unbiased identification of viruses, mosquito species, and endosymbionts, such as *Wolbachia*, from a single mosquito in a single reaction (Hall-Mendelin et al. 2013). Viral metagenomics has been successfully used in Australia for the identification of multiple arboviruses, including novel rhabdoviruses, bunyaviruses (Coffey et al. 2014) and mesoniviruses (Warrilow et al. 2014) from field-collected mosquitoes.

Vector-enabled metagenomics could also be used as a tool to monitor human and animal diseases (Brinkmann et al. 2016), an application often referred to as xenosurveillance (Grubaugh et al. 2015). Xenosurveillance offers an alternative to directly sampling hosts, a process that is time-consuming and requires individual informed consent in the case of humans, or in the case of veterinary pathogens, animal ethics approval. By using this approach, mosquitoes have been successfully used to detect circulating H5N1 influenza virus (Barbazan et al. 2008), Epstein-Barr virus, canine distemper virus (Grubaugh et al. 2015), human herpesvirus, human papillomaviruses, anelloviruses and circoviruses, among others (Ng et al. 2011). This methodology has also been used to study other pathogens, such as filarial parasites (Bockarie 2007) or apicomplexans (Fernandez de Marco et al. 2016). In Sri Lanka, xenosurveillance has been successfully used to map areas with persistent circulation of *Wuchereria bancrofti* after mass drug administration programmes (Rao et al. 2016).

Metagenomic analysis of fecal samples from vertebrate species has been used to identify viruses (Ge et al. 2012, Moreno et al. 2017), describe the microbiota (Ilmberger et al. 2014) and diet source (Pompanon et al. 2012, Jedlicka et al. 2016), and further to assign vertebrate species (Galan et al. 2012). Recently, arboviruses

such as the dengue viruses (DENVs), Ross River virus (RRV) and West Nile virus (WNV) have been detected in mosquito excreta by reverse-transcription real-time polymerase chain reaction (RT-rtPCR) by us and others (Fontaine et al. 2016, Ramírez et al. 2018b). Hepatitis B virus, which does not replicate in the mosquito vector, has been detected in mosquito excreta by RT-PCR and Southern blot up to 72 hours after the ingestion of an infectious blood meal (Blow et al. 2002).

In this study, we evaluated the application of NGS for unbiased detection of RNA viruses firstly in excreta harvested from experimentally infected mosquitoes and later from the excreta of field-collected mosquitoes from different locations across Queensland, Australia. This is the first reported study to investigate mosquito excreta as samples for NGS-based metagenomics.

6.4 MATERIALS AND METHODS

6.4.1 *Excreta samples*

6.4.1.1 *Laboratory studies*

Field-collected *Culex annulirostris* and laboratory-reared *Aedes vigilax* mosquitoes were exposed to defibrinated sheep blood (Institute of Medical and Veterinary Science, Adelaide, Australia) containing either WNV (Kunjin subtype; WNV_{KUN}) or RRV via the hanging drop method (Goddard et al. 2002) or using a Hemotek membrane feeder (Discovery Workshops, Accrington, Lancashire, UK), respectively. For each virus, three groups of five fully engorged females were placed in modified containers for excreta collection (Ramírez et al. 2018b). From day 4 to 7 post-exposure, a single excreta sample was collected from each container using a cotton swab moistened with growth media (GM; Opti-MEM, [Gibco, Invitrogen Corporation, Grand Island, NY] supplemented with 3% foetal bovine serum [In Vitro Technologies, Nobel Park North, VIC, Australia], antibiotics and antimycotics). The swab was then placed in a 2 mL free-standing tube containing 1 mL GM and stored at -80°C.

6.4.1.2 *Field studies*

Mosquito excreta samples were collected in March and April 2018 in South East Queensland (SEQ) and north Queensland (NQ), Australia, from different sites that encompassed a variety of vertebrate host species including flying foxes, wading birds, livestock and macropods (Fig. 6.1). Adult mosquitoes were collected using Centers for Disease Control and Prevention (CDC)- model 512 light traps (John

W. Hock Company, Gainesville, FL) fitted with collection containers modified from the design described in (Meyer et al. 2019). Collection containers housed two filter paper cards soaked with blue-dyed honey, and a removable polycarbonate substrate for excreta collection. All traps were baited with 1 kg of dry ice as a source of CO_2; traps deployed in NQ were also supplemented with 1-octen-3-ol to increase capture of mosquitoes (Ritchie and Kline 1995). Traps were operated for 14 h overnight, before being transported to the laboratory and placed in a humidified box where mosquitoes were allowed to feed on the honey-soaked filter paper cards for an additional 24 hours to increase the amount of excreta produced. Excreta were collected from the polycarbonate insert using a moistened swab as described above and stored at -80°C until processing. To avoid cross-contamination, the traps and polycarbonate inserts were handled with gloves during trap assembly and retrieval, and gloves were changed after each sample was collected. After collection, the polycarbonate inserts were soaked in 1% bleach, rinsed and wiped with 70% ethanol, while the pots and mesh inserts were wiped with 1% bleach followed by 70% ethanol.

6.4.2 *Virus assays*

Thawed excreta samples were mixed using a QIAGEN Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at 26 Hz and centrifuged at 14,000 g briefly (Ramírez et al. 2018b). RNA was extracted from the supernatant using the QIAamp One-For-All nucleic acid kit (Qiagen, Hilden, Germany) or the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) in duplicate: with and without carrier (to avoid interference in downstream NGS) according to the manufacturer's instructions. RNA extracted with carrier was immediately tested by RT-rtPCR and RNA extracted without carrier was stored at -80°C for sequencing.

Samples from experimentally infected mosquitoes were screened for either WNV_{KUN} or RRV by RT-rtPCR. Field-collected samples were tested for RRV and Barmah Forest virus (BFV), as these two alphaviruses are endemic in the sampling locations. Viral RNA was detected using RT-rtPCR assays specific for RRV (Hall et al. 2010), WNV_{KUN} and BFV (van den Hurk et al. 2014) on a Rotor-Gene 600 real-time thermocycler (Qiagen, Australia). Every run included synthetic primer and probe controls, a positive extraction control (bovine viral diarrhoeal virus, BVDV), a negative extraction control, and a no-template control (molecular grade water). The results were evaluated qualitatively: any sample with a threshold cycle number (C_t) > 40 indicated no RNA was detected (Pyke et al. 2004).

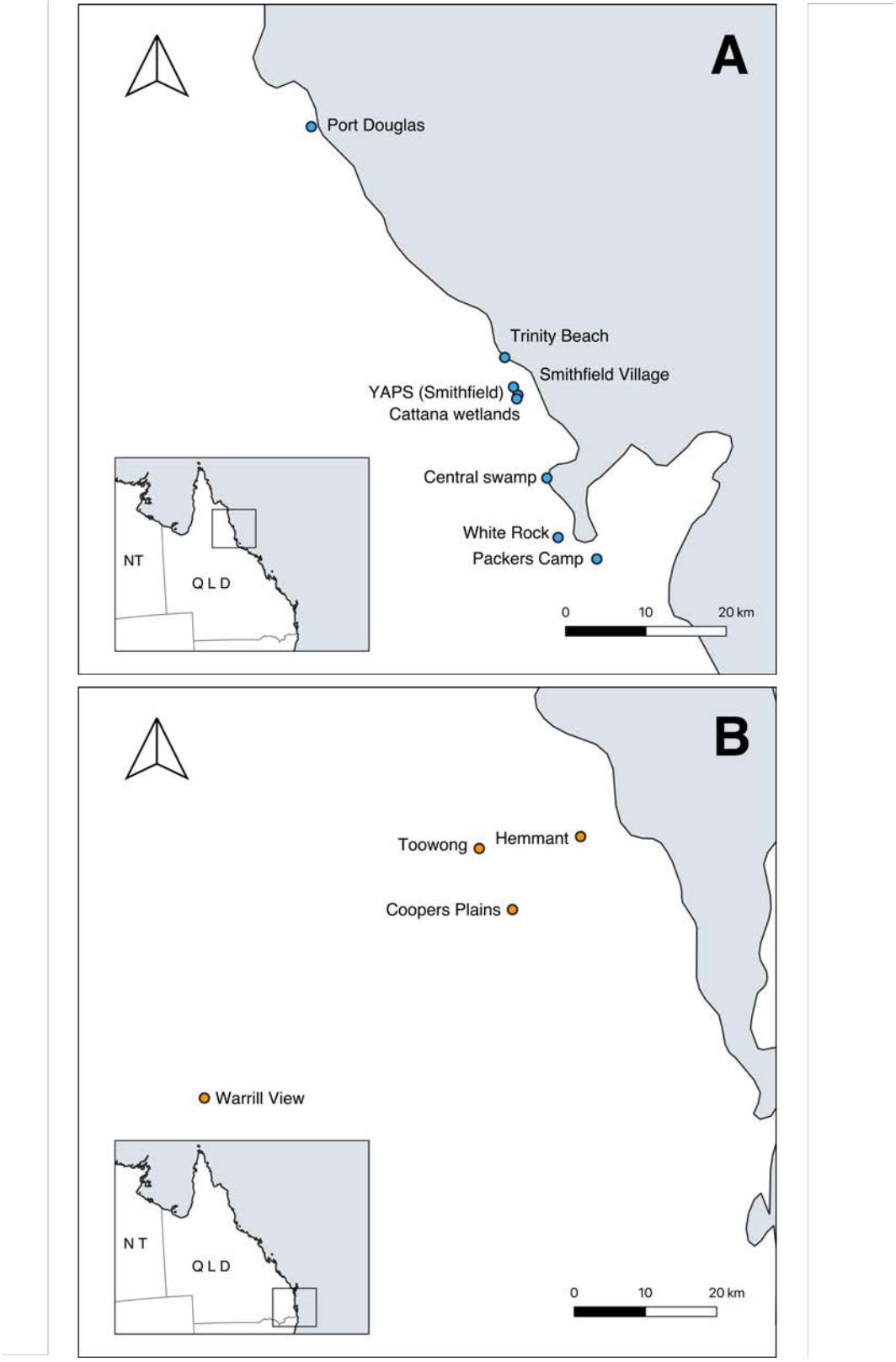

Figure 6.1: Study sites where mosquito excreta was collected from Queensland, Australia. A) north Queensland; B) south east Queensland.

6.4.3 *Sequencing*

Genomic DNA was removed from total RNA aliquots extracted without carrier RNA using the Heat & Run gDNA removal kit (ArcticZymes, Tromso, Norway). RNA was reverse transcribed and first strand cDNA was synthesized using the NEB Protoscript II® first strand cDNA synthesis kit (New England BioLabs, Ipswich MA) according to manufacturer's instructions, followed by second strand cDNA synthesis using NEB second strand synthesis enzyme buffer (New England BioLabs, Ipswich MA) and second strand DNA enzymes (DNA polymerase I (*E. coli*): 10 units, RNase H: 0.35 units and *E. coli* DNA ligase: 1.25 units; New England BioLabs, Ipswich MA). The newly synthesised DNA was purified by ethanol precipitation. DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina) and Illumina Nextera XT index kit. The resulting libraries were analysed and DNA sizing and quantification was performed using a 2200 TapeStation (Agilent Technologies). A foetal calf serum (FCS) library was prepared as described above from FCS RNA as a negative control. Libraries were diluted to 1 nM, pooled, denatured and diluted to a final concentration of 1.2 pM. Paired-end sequencing was performed using the Nextseq platform (Illumina) using a NextSeq 500 Mid Output V2 Kit (Illumina) (Huang et al. 2019).

6.4.4 *Sequence analysis and phylogenetics*

Sequence reads were demultiplexed and adapters were removed using bcl2fastq version 2.20 (http://sapac.support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html). An initial search of the raw sequences was conducted using Diamond (BLASTx) (Buchfink et al. 2015) against the NCBI-nr viral protein reference sequence database. Taxonomic binning of the reads was performed using MEGAN CE version 6.15.2 (Huson et al. 2016) using the naive LCA algorithm (Min Score=75.0, Max Expected=0.1, Top Percent=10.0, Min Support=10). Based on these results, RNA viruses sequences with at least 1,000 reads assigned as close relatives were selected as references for assembly. Viral sequences were assembled using Geneious Prime version 2019.0.4 either by (1) de novo assembly or (2) manually mapping the reads to a reference sequence obtained from GenBank. For de novo assembly, low quality (Q$<$ 30) and short reads ($<$ 100 nt) were trimmed using BBDuk before being assembled using SPAdes (Bankevich et al. 2012) with default parameters. Assembled sets of overlapping DNA sequences (contigs) were then mapped to a reference sequence or the longest contigs containing an open reading frame were compared against the NCBI-nr database using BLAST (Johnson et al. 2008) and used as a reference sequence for further assembly. Alternatively, raw

reads were mapped against a reference sequence using default settings, and the consensus sequence of this assembly was used for subsequent assembly (Colmant et al. 2017). This process was repeated until the length of the consensus sequence did not increase anymore.

Assembled sequences with $>$ 90% amino acid identity with existing viruses over the RNA-dependant RNA polymerase (RdRp) were considered strains of these viruses and assumed to phylogenetically group with them. Because of this, only complete sequences with less than 90% amino acid identity to the reference sequence were included in phylogenetic analyses. The translated contigs were aligned with protein sequences obtained from GenBank using the results from BLAST and previously published phylogenetic trees of the related viruses (Shi et al. 2016, Kobayashi et al. 2017). Multiple protein alignments were done using MAFFT v7.388 (Katoh and Standley 2013) and trimmed using TrimAl (Capella-Gutierrez et al. 2009). The optimal evolutionary model was selected using the Akaike information criterion in SMS (Lefort et al. 2017). Maximum likelihood phylogenies were generated using the Le Gascuel (LG) amino acid substitution model with 100 bootstrap replicates using PhyML v 3.3 (Guindon et al. 2010).

6.5 RESULTS

6.5.1 *Laboratory studies*

All six excreta samples collected from groups of experimentally infected mosquitoes were positive for either WNV_{KUN} or RRV by RT-rtPCR with C_t values ranging from 24.9 to 28.5. Based on these results, six libraries were subsequently prepared and sequenced. The mean ($\pm$ SEM) number of raw reads obtained from the libraries from mosquitoes exposed to WNV_{KUN} or RRV were $5,877,227 \pm 137,555$ and $9,020,173 \pm 515,578$ respectively. Preliminary Diamond/MEGAN analysis confirmed a correlation with WNV_{KUN} and RRV protein sequences in the respective WNV_{KUN} and RRV mosquito excreta samples. Results from subsequent assembly demonstrated that excreta collected from small numbers of mosquitoes infected with arboviruses provide sufficient template for NGS, allowing the assembly of near full-length genomes (Fig. 6.2).

6.5.2 *Field studies*

RRV RNA was detected by RT-rtPCR in 2 out of 46 (4%) excreta samples, which had been collected from White Rock and Cattana wetlands (NQ) with C_t values of

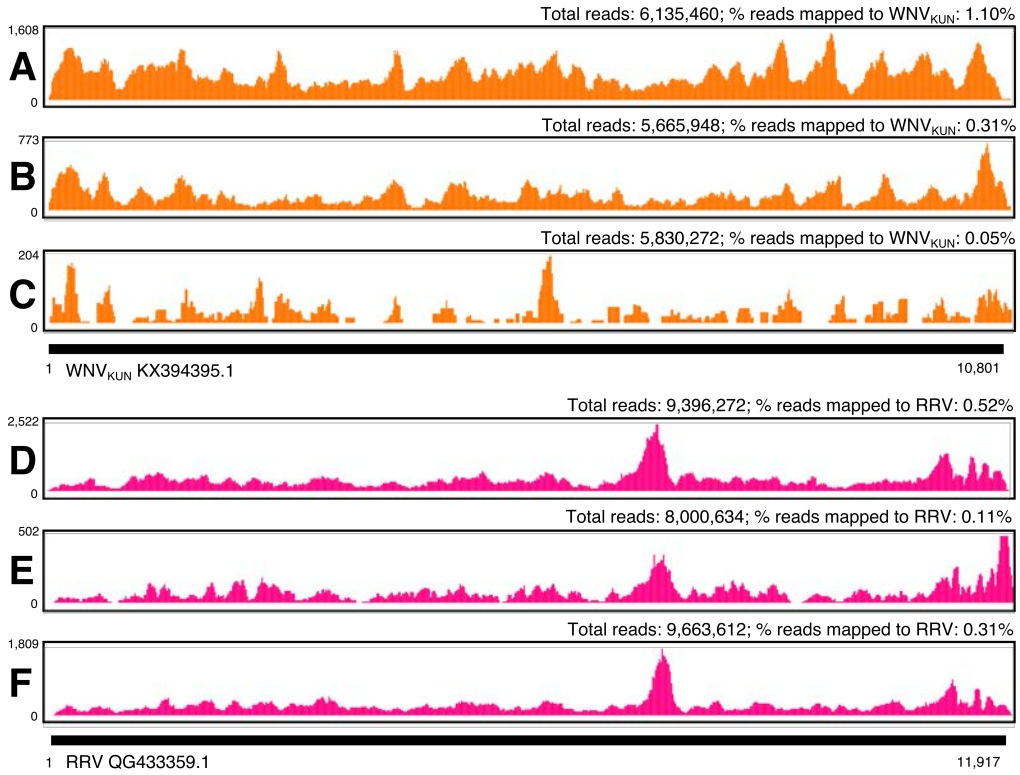

Figure 6.2: Sequence depth and coverage of virus genomes. Each sequence was obtained from excreta from groups of five experimentally infected mosquitoes exposed to either WNV_{KUN} (A-C) or RRV (D-F). Sequences were assembled to a reference sequence (GenBank accession numbers KX394395.1 and GQ433359.1 for WNV_{KUN} and RRV respectively). Sequence depth (y-axis) and coverage (x-axis) are shown.

36.9 and 36.0 respectively. BFV RNA was detected in a single sample from Cattana wetlands (2%, C_t=36.3), which coincidentally was also positive for RRV.

A total of 46 libraries, corresponding to 12 locations were sequenced, with a mean ($\pm$ SEM) of $12,750,316 \pm 583,655$ raw reads per sample. By performing preliminary Diamond/MEGAN analyses using a threshold of $1,000$ assigned reads, no sequences from known pathogenic arboviruses were obtained from field-collected samples, including those that were positive by RT-rtPCR for RRV and BFV. However, other RNA virus genomes were detected by NGS in 22 of the 46 excreta samples sequenced with some samples containing up to 3 different viruses (Table 6.1).

The thirteen viruses identified in this study were found to be related to members of the order *Picornavirales*, and previously described unclassified RNA viruses. For three of the viruses detected (Himetobi P virus (HiPV), Hubei tetragnatha maxillosa virus 2 (HBTMV2) and Hubei picorna-like virus 61(HBPLV61)) only partial sequences were obtained; for the rest of the viruses we were able to assemble near full-length genomes. Of these, Australian Anopheles totivirus (AATV), Wuhan insect virus 33 (WIV33) and Hubei odonate virus 5 (HBOV5) showed $>$ 90% amino acid (aa) identity over the RdRp to published sequences in GenBank, indicating that they correspond to strains of these viruses. The four HBOV5 sequences were $>$ 98% identical, indicating they were very closely related to strains of this virus obtained in China.

By constructing phylogenetic trees based on the RdRp sequences, we identified seven potentially novel virus species. A sample from SEQ contained sequences related to Drosophila C virus (DCV), a cripavirus belonging to the *Dicistroviridae* family (Figure 3). The sequence shared $\sim$ 84% aa identity with DCV. Sequences from three samples collected in SEQ were phylogenetically similar to Armigeres iflavirus (ArIFV, 71% aa identity), an iflavirus from the *Iflaviridae* family, which was first isolated from *Armigeres* spp. mosquitoes in the Philippines (Kobayashi et al. 2017). Between them, the three ArIFV sequences were $>$ 99 % identical.

We also obtained sequences closely related to unclassified RNA viruses identified as a part of a large-scale invertebrate virosphere survey conducted on samples from China by Shi and colleagues (Shi et al. 2016) (Fig. 6.3). A potentially novel virus from a sample from SEQ was related to Hubei odonate virus 7 (HBOV7), sharing 36% aa identity in the RdRp. Five samples from NQ contained sequences related to Hubei arthropod virus 1 (HBAV1; 59% aa identity), with $>$ 99% similarity between them. Four samples from NQ and a single sample from SEQ contained

Table 6.1: Virus genomes detected by NGS of field-collected mosquito excreta

Sample	Total reads	% Reads mapped to virus / % Nucleotide identity[a]												
		ArIFV	AATV	DV2	DCV	HiPV	HBAV1	HBOV5	HBOV7	HBPLV41	HBPLV61	HBTMV2	WIV33	ZJMV1
Smithfield 1	12,849,836	-	-	0.15/75	-	-	-	-	-	-	-	-	-	-
Smithfield 2	16,323,204	-	0.01/98	-	-	-	-	-	-	-	-	-	-	-
Smithfield 4	13,616,396	-	0.02/98	-	-	0.04/81	0.05/55	-	-	-	-	-	-	-
Packers Camp 1	15,409,794	-	-	-	-	-	1.24/56	-	-	< 0.01/NA	-	-	0.19/89	-
Packers Camp 2	8,743,892	-	-	0.15/76	-	-	0.07/57	-	-	-	-	-	-	-
White Rock 1	11,742,104	-	-	-	-	-	0.22/57	-	-	-	-	0.01/NA	-	-
White Rock 2	15,236,836	-	-	-	-	-	0.24/57	-	-	-	-	< 0.01/NA	-	-
Port Douglas 1	14,794,748	-	-	-	-	-	-	0.08/87	-	-	-	-	-	0.13/80
Port Douglas 2	13,773,766	-	-	0.02/80	-	-	-	0.29/87	-	-	-	-	-	0.05/80
Port Douglas 3	13,650,320	-	-	-	-	-	< 0.01/NA	0.04/87	-	-	-	-	-	1.86/80
Port Douglas 4	12,570,618	-	-	-	-	-	-	-	-	-	-	-	-	5.53/80
Port Douglas 5	12,121,104	-	-	-	-	-	-	-	-	-	-	-	-	2.68/80
Port Douglas 6	15,378,002	-	-	-	-	-	-	0.03/87	-	-	-	-	-	-
Warrill View 4	13,828,138	-	-	-	-	-	-	-	-	-	0.02/NA	-	-	-
Warrill View 6	11,277,978	3.91/66	-	-	-	-	-	-	-	-	-	-	-	-
Warrill View 7	7,115,728	-	-	-	-	-	-	-	-	-	0.09/NA	-	-	-
Warrill View 10	19,523,870	-	-	-	-	-	-	-	-	-	0.45/74	-	-	-
Warrill View 12	11,727,168	-	-	-	-	-	-	-	21.98/47	-	-	-	-	-
Warrill View 13	12,517,160	-	-	-	-	-	-	-	-	-	0.02/NA	-	-	-
Warrill View 14	9,031,916	-	-	-	3.68/78	-	-	-	-	-	-	-	-	-
Toowong 1	11,303,708	0.37/66	-	-	-	-	-	-	-	-	-	-	-	0.384/79
Toowong 2	8,748,950	9.87/66	-	-	-	-	-	-	-	-	-	-	-	-

[a]Viruses identified by DIAMOND/MEGAN and used for initial assembly. ArIFV: Armigeres iflavirus; AATV: Australian Anopheles totivirus; DV2: Daeseongdong virus 2; DCV: Drosophila C virus; HiPV: Himetobi P virus; HBAV1: Hubei arthropod virus 1; HBOV5: Hubei odonate virus 5; HBOV7: Hubei odonate virus 7; HBPLV41: Hubei picorna-like virus 41; HBPLV61: Hubei picorna-like virus 61; HBTMV2: Hubei tetragnatha maxillosa virus 2; WIV33: Wuhan insect virus 33; ZJMV1: Zhejiang mosquito virus 1. Only libraries with identified virus genomes are listed.
NA: not applicable, only partial sequences obtained.

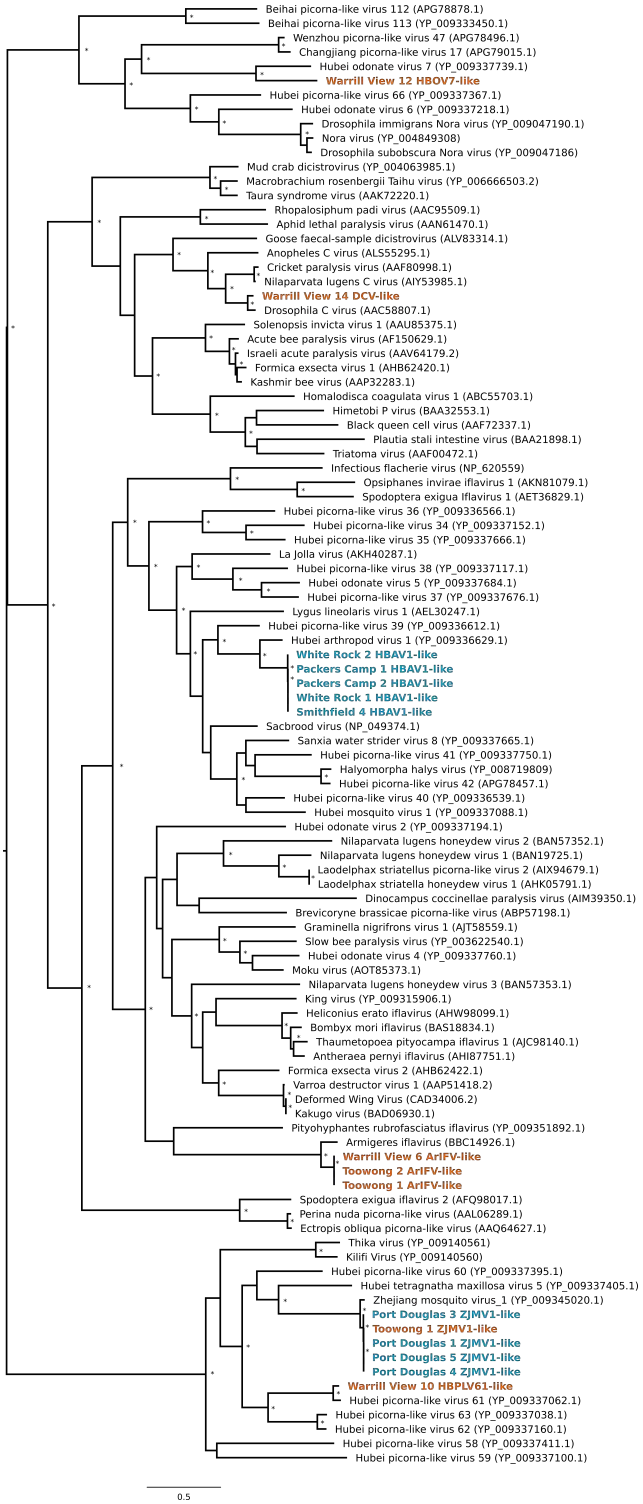

Figure 6.3: Phylogenetic relationships of viruses related to the order *Picornavirales* and other unclassified RNA viruses discovered in field mosquito excreta. A multiple-sequence alignment of the RNA-dependent RNA polymerase amino acid sequences was used to create a maximum likelihood phylogeny using 100 bootstrap replicates; an asterisk indicates node support of $> 70\%$ bootstrap support. The tree was mid-point rooted. The potential novel viruses discovered in this study are colour-coded: orange for samples collected in south east Queensland and blue for samples collected in north Queensland. Corresponding GenBank accession numbers for compared virus sequences are provided in parentheses.

sequences related to Zhejiang mosquito virus 1 (ZJMV1; 85% aa identity) with $>$ 97% similarity with each other. Sequences from one sample collected in SEQ were phylogenetically similar to Hubei picorna-like virus 61 (HBPLV61; 83% aa similarity), which had been previously identified in mosquitoes.

Finally, three samples from NQ contained sequences related to both Culex Daeseongdong-like virus and Daeseongodong virus 2 (DV2) (Figure 4). The sequences shared $\sim$ 84% aa identity with these unclassified RNA viruses, which are themselves highly similar ($>$ 99%) and have been identified in *Culex* mosquitoes from Korea and California respectively (Hang et al. 2016, Sadeghi et al. 2018). Between them, the sequences from these three samples were $>$ 99% identical.

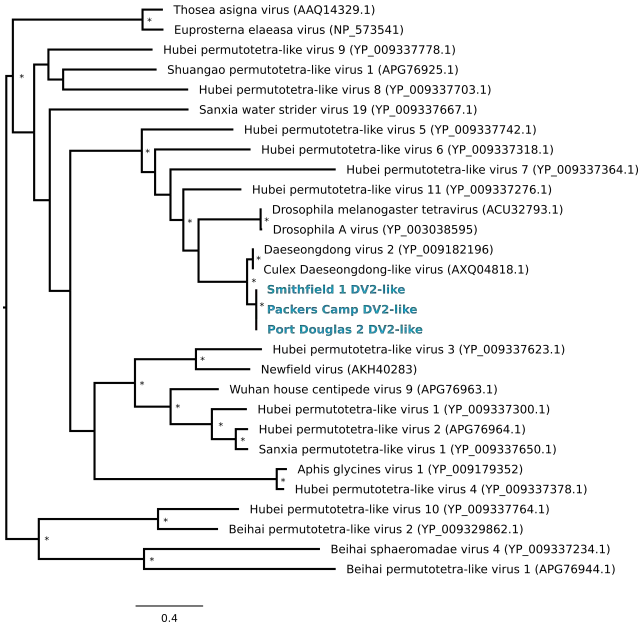

Figure 6.4: Phylogenetic relationships of unclassified RNA viruses discovered in field mosquito excreta. A multiple-sequence alignment of the RNA-dependent RNA polymerase amino acid sequences was used to create a maximum likelihood phylogeny using 100 bootstrap replicates; an asterisk indicates node support of $>$ 70% bootstrap support. The tree was mid-point rooted. The potential novel viruses discovered in this study are colour-coded blue for samples collected in north Queensland. Corresponding GenBank accession numbers for compared virus sequences are provided in parentheses.

6.6 DISCUSSION

Over the past decade, unbiased metagenomic analysis using NGS has become a valuable tool for virus discovery and surveillance (Forbes et al. 2017, Zhang et al. 2019). Our laboratory results demonstrate that excreta from experimentally infected

mosquitoes provides sufficient template for sequencing and assembly of near full-length arbovirus genomes. However, we were unsuccessful at sequencing RRV or BFV from the two field-collected samples that were positive by RT-rtPCR. A possible explanation for this is the likelihood that NGS is not as sensitive as RT-rtPCR for detection of viruses with low titer (Wylie et al. 2012). Additionally, in our study, samples from experimentally infected mosquitoes contained a higher amount of starting template (as evidenced by lower C_t values) and had less chance of sample degradation compared with field samples. With increased application of NGS, it is likely that new, improved protocols will be devised, increasing the efficiency and sensitivity of this sequencing platform. Coinciding with NGS technological advancements, mosquito excreta could be utililzed as a valuable sample alternative for routine arbovirus surveillance enabling the unbiased detection of arboviruses of public health importance. Further, this approach could potentially provide a wider and more comprehensive overview of pathogenic and non-pathogenic microbiota circulating in given locales.

By performing sequencing of field-collected mosquito excreta samples, we were able to show evidence of the circulation of 13 insect-borne viruses, of which five (AATV, ArIFV, DV2, HBPLV61 and ZJMV1) had been previously identified in mosquitoes (Hang et al. 2016, Shi et al. 2016, Colmant et al. 2017, Kobayashi et al. 2017). With the exception of AATV, these viruses had not been previously reported in Queensland. Although these insect-borne viruses are unlikely to be associated with disease in vertebrates, they can potentially affect the vector competence of mosquitoes for pathogenic viruses as it has been shown for a number of insect-specific viruses (Vasilakis and Tesh 2015). Because of this, elucidating the mosquito virome is critical for understanding the role a mosquito species plays in arbovirus transmission cycles and potential control strategies. In the future, novel insect-specific viruses could potentially be used as biological control agents or as platforms for vaccine and diagnostic development (Bolling et al. 2015).

At this stage, the costs associated with NGS and the time and bioinformatics skills required to analyse the results from 1,000s to 10,000s of mosquitoes can be prohibitive, especially in low resource settings (Souf 2016). We have shown that mosquito excreta can be used as a preliminary sample for virus discovery in field populations of mosquitoes. Using mosquito excreta has the advantage of reducing costs by sequencing only one sample from a trap, instead of multiple pools of mosquitoes. Based on the results obtained from excreta, the mosquitoes could be used for subsequent sequencing or to attempt virus isolation. It has been demonstrated that the low-cost hand-held portable sequencer (MinION, Oxford

Nanopore Technologies) can be used for metagenomic detection of arboviruses from experimentally infected mosquitoes, with results comparable to those obtained by commonly used sequencers (Batovska et al. 2017). Thus, mosquito excreta could be coupled with portable sequencers like the MinION to further reduce costs; its application for this purpose still needs to be evaluated.

Due to resource limitations, we focused on identifying RNA viruses only. However, the sequences generated could be used to identify DNA viruses, bacteria, fungi and protists from mosquito excreta. For example, recently a bioinformatic approach has been used to assemble *Wolbachia* genomes from publicly available data sets generated from arthropods (Pascar and Chandler 2018). Other information that could be obtained from sequencing mosquito excreta is the identification of mosquito species. Bioinformatic analysis of field-collected mosquito excreta could be used to obtain evidence of the circulation of biosecurity important mosquito species such as *Aedes aegypti* or *Aedes albopictus* without time-consuming speciation efforts.

Sequencing of mosquito excreta could also be used for xenosurveillance, that is identifying pathogens that are not necessarily transmitted by the mosquito but that could be present in the blood meal. Interestingly, a recent study of the mosquito virome in China and Kenya identified vertebrate and even plant viruses, some of which are not vectored by mosquitoes, from *Culex* mosquitoes suggesting that the mosquitoes might have ingested the viruses during blood- or sugar-feeding (Atoni et al. 2018). Furthermore, it has been demonstrated that viruses present in the host blood can be detected by RT-PCR for up to 24 hours post feeding in engorged mosquitoes (Grubaugh et al. 2015). Methods used to enhance the capture of blood-fed mosquitoes, such as backpack aspirators and resting boxes (Ramírez et al. 2018a), could be combined with collection and sequencing of mosquito excreta to detect pathogens circulating in vertebrate hosts or even the source of the blood meal.

A limitation of our field study is that it would be impossible to attribute the excreta deposited on the polycarbonate substrate to a particular insect. Although mosquitoes comprise the majority of the collections, traps used to capture mosquitoes also attract non-target insects (Li et al. 2015), which could feed on the honey and excrete on the substrate. This can be reflected by the detection of HBOV5, which is associated with dragonflies and damselflies and HBTMV2 which is associated with spiders (Shi et al. 2016). To overcome this technical limitation, the mosquitoes could first be sorted and transferred to clean containers in the laboratory from which excreta would be obtained and sequenced to confirm the

origin of each virus.

It is evident that NGS technologies have many applications for the study of both vectors and the pathogens they transmit (Rinker et al. 2016). Here we have demonstrated that metagenomic analysis of mosquito excreta can be used in the near future for virus discovery and, as costs decrease and technologies become more accessible, for unbiased unbiased environmental virome sampling with applications to arbovirus surveillance.

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7

GENERAL DISCUSSION AND FURTHER DIRECTIONS

The overarching focus of my research was to evaluate the use of mosquito excreta for the detection of mosquito-borne pathogens, such as flaviviruses, alphaviruses and *Plasmodium* in laboratory and field studies, with the ultimate goal of enhancing mosquito-borne disease surveillance. As each data chapter already contains a comprehensive discussion, this chapter aims to synthesise the main findings of the experiments described in this thesis and to provide possible directions for future research.

It appears that excretion of pathogens by infected mosquitoes is a general phenomenon that can be exploited for research and surveillance applications. My research supports previous findings that indicate that mosquitoes excrete DENV RNA after sugar feeding which can be detected by molecular methods (Fontaine et al. 2016). Through my experiments, I was able to expand on this knowledge and demonstrate that this is not an exclusive occurrence for DENVs. I designed and performed experiments that demonstrated that mosquitoes also excrete nucleic acids from other flaviviruses, as well as alphaviruses. Similarly, I conducted experiments with *Plasmodium falciparum*, to evaluate whether *Anopheles* excrete parasite RNA over time, expanding on previous research which suggested that parasites could be detected in mosquito excreta after blood feeding (Pilotte et al. 2016).

The results presented in my thesis indicate that analysing mosquito excreta provides a simple and efficient method for assessing virus dissemination or parasite development in vector competence experiments. I was able to detect viral RNA in mosquito excreta continually from day 2 until day 15 post-exposure (PE). Supporting the results of Fontaine et al. 2017, I observed a significant correlation between the detection of arboviruses in excreta and virus dissemination in the mosquito (Chapter 3). In fact, only 6% of mosquitoes without a disseminated infection showed evidence of arbovirus RNA in their excreta, which could be explained by the lower sensitivity of cell culture used to assess the infection status of the mosquito. In the case of *P. falciparum* (Chapter 5), detection of the parasite in mosquito excreta occurred as early as 4 days after ingesting an infectious bloodmeal. Although the mechanism by which this occurs is unclear, it appears to be related to the parasite establishing a midgut infection. As with the arboviruses, the nucleic

acid continued to be detectable over the course of infection until at least day 19 PE. Traditional methods for assessing viral dissemination or parasite midgut infection usually require sacrificing the mosquitoes. In the case of arboviruses, mosquito's heads, legs and wings are removed and tested by either cell culture or molecular methods. Similarly, dissection of mosquito midguts and microscopic observation of *Plasmodium* oocysts is used to estimate mosquito infection in malaria experiments. My results suggest that mosquito excreta could be analysed throughout the extrinsic incubation period, providing a measure of time to arbovirus dissemination or estimation of the potential of a parasite to establish a midgut infection in a non-destructive manner without need to sacrifice the mosquito. As any person who has conducted experiments with infected mosquitoes can attest, the outcome of the infection (and the success of the experiment) cannot be known until even weeks after the experiment has finalised. In this context, mosquito excreta could be monitored early in the extrinsic incubation period to determine if the infection was successful or to select susceptible mosquitoes for subsequent experiments.

With the goal of enhancing mosquito-borne disease surveillance, I compared the detection of arboviruses and *P. falciparum* in excreta and expectorate. As described in Chapter 2, current sugar-based surveillance methods rely on the detection of viral RNA expectorated by mosquitoes into filter paper cards or wicks during sugar feeding (Hall-Mendelin et al. 2010, Lothrop et al. 2012). Similarly, *Plasmodium* sporozoites can be detected using this method (Brugman et al. 2018). Although this approach provides a better estimate of transmission risk, it requires that the mosquitoes transmit the pathogen, which can take up to two weeks. In the case of the arboviruses, when used as a proxy for viral transmission, there was a 4-fold increase in sensitivity of detection of viral RNA for excreta compared with saliva. As discussed in Chapter 3, this would be expected given that detection of viral RNA in excreta and saliva result from dissemination and transmission respectively, and not all mosquitoes with a disseminated infection succeed at transmitting the virus (Forrester et al. 2014, Franz et al. 2015). Not surprisingly, given that mosquitoes excrete 300 times more fluid than what they expectorate ($\sim$ 1.5μl vs 4.7 nl), the median C_t values from positive excreta samples were significantly lower than those from positive saliva samples. These findings suggest that detection of arboviruses in excreta can be used to enhance the sensitivity of currently used honey-based surveillance methods (van den Hurk et al. 2014b, Johnson et al. 2015). Interestingly, for *P. falciparum*, once sporogony (the development of sporozoites) had occurred, no difference was observed between the detection of the parasite in excreta or saliva or the amount of RNA detected, suggesting that once mosquitoes are transmitting

the parasite, both approaches are equally sensitive at detecting it.

Since dissemination for many arboviruses can take as little as 2 days, an important advantage of analysing mosquito excreta over saliva for mosquito-borne disease surveillance in the field is that it enables earlier detection, increasing the window of opportunity to detect the pathogen. For example, in the case of West Nile virus (WNV), the virus is detectable in saliva using sugar-based methods or sentinel animals from days 7 to 10 for the rest of the insect's life (van den Hurk et al. 2014a), whereas with excreta, detection would be possible since day 2 PE. This period would be even longer for *Plasmodium* since it takes at least 10 days for sporozoites to develop and reach the salivary glands (Beier 1998), while detection of the parasite could be possible since day 4 PE by analysing excreta. I have demonstrated that testing mosquito excreta allows for detection of mosquitoes with the potential to transmit. Likewise, as described above, *Plasmodium* detection in excreta allows for an estimation of the potential of a parasite to establish a midgut infection. It is important to note that the detection of pathogens in excreta does not facilitate the incrimination of vectors or the calculation of entomological metrics, such as minimum infection rates or sporozoite rates. However, it provides an alternative to the use of animals as part of a sentinel system to obtain evidence of circulation of viruses or parasites in a given area. Integration of excreta into trapping systems provides an attractive approach for malaria surveillance, as there are no suitable animals that can be used as sentinels. A positive result in these sentinel systems can be used to trigger intensive trapping to collect mosquitoes for analysis of key entomological measures, such as infection rates, population dynamics, host feeding patterns and genotypic characterisation. An exciting novel application is xenosurveillance, where mosquitoes are used as "flying syringes" and their blood meals are tested for the presence of vertebrate pathogens ingested during feeding (Grubaugh et al. 2015). This approach can be used to detect pathogens that do not necessarily replicate in the mosquito but might be present in the ingested blood meals. In my experiments, I did not test excreta collected < 48 hours after the mosquitoes acquired an infectious blood meal to allow for digestion and thus avoid false positives resulting from detecting the pathogen in the blood meal. Regardless of this, traps used for excreta collection in the field capture mosquitoes from different physiological stages, allowing the collection of excreta from mosquitoes who are fully or partially blood fed. Therefore, mosquito excreta could potentially be used for xenosurveillance applications, since a positive resulting from blood meal digestion indicates pathogen circulation in the population.

From an operational perspective, the use of mosquito excreta for mosquito-borne disease surveillance has several advantages when it comes to the deployment and handling in the field. First, only low amounts of viable virus are present in the sample, suggesting a relatively low risk to personnel handling the samples. Second, mosquito excreta is easy to collect and, like the collection of expectorate, does not require a cold chain to preserve virus infectivity. As I demonstrated in Chapter 4, viral RNA in mosquito excreta deposited on FTA® cards and polycarbonate plastic is stable under tropical conditions for at least 24 hours. After that, viral RNA is stable in excreta deposited on FTA® cards for up 14 days whilst RNA stability decreases in excreta deposited on polycarbonate after 7 days. Based on these results, I propose the use of polycarbonate substrates which are then wiped with an FTA® card for overnight collection, and the use of FTA® cards as a direct substrate for long term deployment in passive box traps (Meyer et al. 2019). Additionally, since the excretion of arboviruses is associated with viral dissemination, it circumvents the need of using water reservoirs that provide the humidity necessary to keep the mosquitoes alive to increase the chances of detecting transmitting mosquitoes, as would be required from saliva-based systems (Johnson et al. 2015). In parallel with working on the experiments described in my thesis, I participated in the development of field methodology for collecting mosquito excreta and testing field-collected samples from the Northern Territory and Queensland (Meyer et al. 2019). One of the main findings of that study was the detection of Murray Valley encephalitis (MVEV), WNV and Ross River viruses (RRV) in excreta harvested from field populations of mosquitoes. This is the first report of detection of arboviruses in field-collected mosquito excreta, supporting the laboratory-derived results I presented in Chapter 3 and Chapter 4. Importantly, the methods I have developed for harvesting and analysing excreta can be easily integrated into laboratories that already conduct sugar-based surveillance, since the samples are processed using the same protocols, molecular assays and equipment.

As described in Chapter 2, over the last decade there have been advances in technologies used to detect and characterise mosquito-borne pathogens, with an expansion of instruments, chemistries and techniques used to obtain genomic information (Levy and Myers 2016). Frequently used assays used for surveillance, such as RT-rtPCR, require a priori knowledge and the use of specific primers and probes that target characterised pathogens. Because of this, other viruses and microorganisms (pathogenic or not) go undetected. Next-generation sequencing (NGS)-based metagenomics overcomes this limitation, by allowing the unbiased detection of all the pathogens, endosymbionts and even mosquito species from a single reaction without prior sequence knowledge. In the last chapter of my thesis, I investigated if

mosquito excreta could be used as a sample for NGS-based metagenomics. Results from my laboratory study with experimentally infected mosquitoes demonstrated that mosquito excreta provided sufficient template for NGS and facilitated the assembly of near full-length genomes from RNA viruses. Based on these promising results, I examined whether the excreta from field-collected mosquitoes could be used as a sample type for metagenomic analysis for environmental virome sampling. At this stage, NGS has less sensitivity than RT-rtPCR for detecting viruses with low titer (Wylie et al. 2012) and I was not able to detect RRV or Barmah Forest virus (BFV) from two field-collected mosquito excreta samples that were positive by RT-rtPCR. Excitingly, I was able to identify several insect-borne viruses from these samples, including seven potentially novel viruses, suggesting that mosquito excreta can be used for virus discovery. Using mosquito excreta as a preliminary sample for virus discovery in field populations of mosquitoes has the advantage of reducing the number of samples that need to be sequenced from a single trap (one excreta sample vs multiple pools of mosquitoes), thus reducing costs. As mentioned above, this approach could be used to identify areas where more intense trapping and investigation can be conducted. Although the resources and bioinformatic skills associated with this technology can be prohibitive especially in low resource settings, as cost decreases and technologies become more accessible, NGS of mosquito excreta could be integrated for mosquito-borne disease surveillance in the future. Indeed, it is not hyperbole to imagine a future where detailed information about all circulating viruses and microorganisms could be routinely obtained from mosquito excreta in a single reaction. Currently, there is a low-cost hand-held portable sequencer available in the market (MinION, Oxford Nanopore Technologies) which has been already used for metagenomic detection of arboviruses in experimentally infected mosquitoes (Batovska et al. 2017) and could potentially be used to analyse mosquito excreta. Ultimately, findings from this chapter demonstrate that mosquito excreta is a versatile sample type, which depending on the application, can be analysed by molecular methods and NGS.

While my research answers several questions, it also highlights avenues for further research. Although I hypothesised that the excretion of pathogen material results as a consequence of viral dissemination or degradation of parasite life-stages, little is known about the physiological process that leads to the pathogens (or their nucleic acid) being deposited in the mosquito excreta. At least for the malaria study, I was unable to observe any distinguishable life-stages from excreta samples from *P. falciparum* life stages that were positive by RT-rtPCR. Clearly, additional analyses are required to elucidate what is happening in the digestive tract of the mosquito. This could be done by using immunohistochemistry (IHC) or immunofluorescence

assays from mosquito tissues associated with excretion such as malpighian tubules and hindguts collected at frequent intervals (Clements 2000, Girard et al. 2004).

As with any new technology, the use of mosquito excreta to enhance mosquito-based disease surveillance in routine applications needs to be thoroughly assessed in the field. For this, the use of excreta needs to be compared with current sugar-based surveillance protocols (detection in saliva) in the same trap, as well as traditional modes of surveillance, namely processing pools of mosquitoes and sentinel animals. Based on the field results described in Chapter 6 and in Meyer et al. 2019, it seems unlikely that the excreta from numerous uninfected mosquitoes and other contaminants (such as non-target insects or spider webs) would interfere with the detection of viruses or parasites from a single infected mosquito. However, this effect needs to be further evaluated with both laboratory and field studies. Finally, with the goal of using mosquito excreta for mosquito-borne disease surveillance in low resources settings or remote locations, the application of portable automated rapid diagnostic tests or microfluidic devices (Ryan et al. 2001, Vontas et al. 2016, Wasik et al. 2017) for detection of pathogens in excreta needs to be assessed.

Concerning the use of mosquito excreta for NGS-based metagenomics, some points still need to be evaluated. To increase the likelihood of detection by NGS, in Chapter 6 I collected excreta samples from field-collected mosquitoes using the protocols developed for laboratory applications, which included wiping the excreta with a cotton swab and placing it in growth media before storage at -80°C. However, this method requires a cold chain, so the use of excreta collected with FTA® cards or other protocols that preserve RNA, such as RNAlater®, needs to be assessed. The amount of sequence data produced in my study is vast and would take time to analyse entirely. Because of this, I decided to focus on looking for RNA viruses. However, NGS-based metagenomic analysis of mosquito excreta could also be used to identify DNA viruses, bacteria, mosquito species and even blood meal analysis. As bioinformatic pipelines become more efficient, the future will allow for faster analysis of sequence data.

Finally, the analysis of excreta for the study of pathogens could also be expanded to other hematophagous arthropods such as ticks, *Culicoides* biting midges and phlebotomine sand flies that transmit vector-borne diseases of public health and veterinary importance such as Lyme disease, bluetongue virus and leishmaniasis among many others (Mellor et al. 2000, Mead 2015, Akhoundi et al. 2016).

CONCLUSIONS

In conclusion, the results presented in my thesis from researching the applications of mosquito excreta advance our understanding of mosquito-borne diseases and their surveillance in a number of ways. Firstly, I have expanded the number of viruses and microorganisms that can be found in mosquito excreta. Combining my results with previous findings, we now know that mosquitoes excrete RNA from DENV (Fontaine et al. 2016), RRV, WNV (Chapter 3 and Chapter 4), MVEV (Meyer et al. 2019), BFV (Chapter 6), *P. falciparum* (Pilotte et al. 2016, Cook et al. 2017, Chapter 5), filarial parasites (Pilotte et al. 2016) and insect-borne viruses (Chapter 6) at levels which can be detected by RT-rtPCR or by next-generation sequencing. The experiments in Chapter 5 were the first to provide a comprehensive assessment of excretion and expectoration of *Plasmodium*, in conjunction with observation of sporozoites in salivary glands from the same mosquitoes. In direct reference to handling excreta samples in the field, I present the first study to analyse the viability of arboviruses in mosquito excreta demonstrating that only low levels of infectious virus are present. Although the virus in excreta has low levels of infectiousness, I revealed that viral RNA in mosquito excreta is stable in tropical conditions and provided recommendations to optimise the detection of arboviruses from excreta collected using traps for overnight and long-term deployment. Finally, and particularly with a view to future modes of surveillance, this is the first study to investigate mosquito excreta as a sample type for next-generation sequencing-based metagenomics, showing that this technology can be used for the detection of arboviruses and virus discovery. It can be said with confidence that mosquito excreta is the latest addition to the array of sample types available to study and ultimately prevent the spread of vector-borne diseases.

A

APPENDIX

A.1 DATA AVAILABILITY

Chapter 3: Ramirez Lopez, A. (2019): Dataset describing detection or RRV and WNV in mosquito excreta. James Cook University. (dataset). http://dx.doi.org/10.25903/5d5f31376caa4
Digital Object Identifier (DOI):10.25903/5d5f31376caa4

Chapter 4: Ramirez Lopez, A. (2019): Dataset describing stability of WNV RNA in mosquito excreta. James Cook University. (dataset). http://dx.doi.org/10.25903/5d5f2a38e823f
Digital Object Identifier (DOI):10.25903/5d5f2a38e823f

Chapter 5: Ramirez Lopez, A. (2019): Dataset describing the detection of *Plasmodium falciparum* in mosquito excreta and saliva. James Cook University. (dataset). http://dx.doi.org/10.25903/5d5de1e49aae9
Digital Object Identifier (DOI):10.25903/5d5de1e49aae9

A.2 SIGNED STATEMENT OF CONTRIBUTION BY OTHERS

Chapter	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis
2	**Ramírez AL**, van den Hurk AF, Meyer DB, Ritchie SA. 2018. Searching for the proverbial needle in a haystack: advances in mosquito-borne arbovirus surveillance. Parasit Vectors 11(1):320	Ana Ramírez reviewed the literature and drafted the first version of the manuscript. Andrew van den Hurk, Dagmar Meyer and Scott Ritchie edited the manuscript	Andrew van den Hurk: Dagmar Meyer: Scott Ritchie
3	**Ramírez AL**, Hall-Mendelin S, Doggett SL, Hewitson GR, McMahon JL, Ritchie SA, van den Hurk AF. 2018. Mosquito excreta: A sample type with many potential applications for the investigation of Ross River virus and West Nile virus ecology. PLoS Negl Trop Dis 12(8):e0006771	The study was conceptualized by Ana Ramírez, Scott Ritchie and Andrew van den Hurk. Stephen Doggett provided *Aedes vigilax* eggs and Scott Ritchie provided field collected mosquitoes used in this experiment. Ana Ramírez completed the laboratory infections, sample collection, vector competence experiments and cell culture assays with advice provided by Andrew van den Hurk and Sonja Hall-Mendelin. Glen Hewitson and Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Data curation, formal analysis, visualization and writing of the original manuscript was done by Ana Ramírez. Sonja Hall-Mendelin, Stephen Doggett, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Scott Ritchie and Andrew van den Hurk acquired funding for this experiment.	Sonja Hall-Mendelin: Stephen Doggett: Glen Hewitson: Jamie McMahon: Scott Ritchie: Andrew van den Hurk:

4	**Ramírez AL**, Hall-Mendelin S, Hewitosn GR, McMahon JL, Staunton KM, Ritchie SA, van den Hurk AF. 2019. Stability of West Nile virus RNA in mosquito excreta. J Med Entomol 56(4): 1135-1138	The study was conceptualised by Ana Ramírez, Scott Ritchie and Andrew van den Hurk. Ana Ramírez and Andrew van den Hurk collected the mosquitoes used in this experiment. Ana Ramírez completed the laboratory infections and sample collection with advice provided by Andrew van den Hurk and Sonja Hall-Mendelin. Glen Hewitson and Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Ana Ramírez analysed the samples using RT-rtPCR and cell culture assays. Kyran Staunton assisted Ana Ramírez with data analysis. Data curation, formal analysis, visualisation and writing of the original manuscript was done by Ana Ramírez. Sonja Hall-Mendelin, Kyran Staunton, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Scott Ritchie and Andrew van den Hurk acquired funding for this study.	Sonja Hall-Mendelin: Glen Hewitson: Jamie McMahon: Kyran Staunton: Scott Ritchie: Andrew van den Hurk:

5	**Ramírez AL**, van den Hurk AF, Mackay IM, Yang ASP, Hewitson GR, McMahon JL, Boddey JA, Ritchie SA, Erickson SM. 2019. Malaria surveillance from both ends: concurrent detection of *Plasmodium falciparum* in saliva and excreta harvested from *Anopheles* mosquitoes. Parasit Vectors 12(8):e0006771	The study was conceptualized by Ana Ramirez, Andrew van den Hurk, Scott Ritchie and Sara Erickson. Sara Erickson exposed the mosquitoes to gametocyte cultures. Ana Ramirez and Sara Erickson performed the experiments and collected the samples. Ian Mackay developed the molecular assay used in this study. Glen Hewitson and Jamie McMahon assisted Ana Ramirez in the analysis of samples using RT-rtPCR. Data curation, formal analysis, visualization and writing of the original manuscript was done by Ana Ramirez. All authors read and approved the final manuscript. Andrew van den Hurk, Justin Boddey, Annie Yang and Sara Erickson provided the study materials. Scott Ritchie, Justin Boddey and Andrew van den Hurk acquired funding for the research.	Andrew van den Hurk: Ian Mackay: Annie Yang: Glen Hewitson: Jamie McMahon: Justin Boddey: Scott Ritchie: Sara Erickson:

6	**Ramírez AL**, Colmant AMG, Warrilow D, Huang B, Pyke AT, McMahon JL, Meyer DB, Graham RMA, Jennison AV, Ritchie SA, van den Hurk AF. Metagenomic analysis of mosquito excreta for environmental virome sampling. *In preparation*	The study was conceptualized by Ana Ramírez, Andrw van den Hurk and Scott Ritchie. Ana Ramírez and Andrew van den Hurk completed the laboratory mosquito exposures, conducted the field work and collected the samples. Dagmar Meyer assisted Ana Ramírez in different aspects of sample collection in north Queensland. Ben Huang and Alyssa Pyke assisted Ana Ramírez with library preparation and sequencing. Rikki Graham and Amy Jennison provided advice and equipment used for sequencing. Jamie McMahon assisted Ana Ramírez in the analysis of samples using RT-rtPCR. Ana Ramírez completed the bioinformatic analyses with advice provided by Agathe Colmant, David Warrilow and Alyssa Pyke. Data curation, formal analysis, visualization and writing of the original manuscript was done by Ana Ramírez. Agathe Colmant, David Warrillow, Ben Huang, Alyssa Pyke, Amy Jennison, Scott Ritchie and Andrew van den Hurk reviewed and edited the final manuscript. Andrew van den Hurk and Scott Ritchie acquired funding for this study.	Agathe Colmant: David Warrilow: Ben Huang: Alyssa Pyke: Jamie McMahon: Dagmar Meyer: Rikki Graham: Amy Jennison: Scott Ritchie: Andrew van den Hurk:

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