Oral susceptibility of aedine and culicine mosquitoes (Diptera: Culicidae) to Batai orthobunyavirus

Luis Miguel Hernandez-Triana (Luis.Hernandez-Triana@apha.gov.uk)
Animal and Plant Health Agency https://orcid.org/0000-0001-7058-8848

Arran Folly
Animal and Plant Health Agency

Elsa Barrero
Animal and Plant Health Agency

Sarah Lumley
Public Health England Porton

Maria del Mar Fernández de Marco
Animal and Plant Health Agency

Sanam Sewgobind
Animal and Plant Health Agency

Lorraine M. McElhinney
Animal and Plant Health Agency

Anthony R. Fooks
Animal and Plant Health Agency

Nicholas Johnson
Animal and Plant Health Agency

Short report

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Abstract

Background

A number of zoonotic mosquito-borne viruses have emerged in Europe in recent decades. Batai virus (BATV), orthobunyavirus, is one example having been detected in mosquitoes and livestock. We conducted vector competency studies on three mosquito species at a low temperature to assess whether *Aedes* and *Culex* mosquito species are susceptible to infection with BATV.

Methods

Colonised lines of *Aedes aegypti* and *Culex pipiens*, and a wild-caught species, *Aedes detritus*, were orally inoculated with BATV strain 53.2, originally isolated from mosquitoes trapped in Germany in 2009. Groups of blood-fed female mosquitoes were maintained at 20^{o}C for seven or fourteen days. Individual mosquitoes were screened for the presence of BATV in body, leg and saliva samples for evidence of infection, dissemination and transmission, respectively. Batai virus RNA was detected by RT-PCR and positive results confirmed by virus isolation in Vero cells.

Results

Aedes detritus was highly susceptible to BATV with infection prevalence at or above 80% at both time points. Disseminated infections were recorded in 30.7–41.6% of *Ae. detritus* and evidence for virus transmission with BATV detected in saliva samples (n = 1, dpi = 14) was observed. Lower rates of infection for *Ae. aegypti* and *Cx. pipiens* with no evidence for virus dissemination or transmission at either time point.

Conclusions

This study shows *Ae. detritus* may be a competent vector for BATV at 20^{o}C, whereas *Ae. aegypti* and *Cx. pipiens* were not competent. Critically, the extrinsic incubation period appears to be ≤ 7 days for *Ae. detritus*, which may increase the onward transmissibility potential of BATV in these populations.

Background

Batai virus (BATV) was originally isolated from *Culex gelidus* mosquitoes from the Batai area of Kuala Lumpa in Malaysia in 1955 [1]. Antigenic studies subsequently showed it to be identical to Čalovo virus, previously isolated from *Anopheles maculipennis s.l.* mosquitoes trapped in Southern Slovakia in 1960 [1]. Both of these isolation are now formally recognized as *Batai orthobunyavirus* and classified within the genus *Orthobunyavirus* of the family Peribunyaviridae [2]. The BATV genome consists of three

negative-sense single-stranded RNA segments, the 948 base pair (bp) small (S) segment, the 4448 bp medium (M) segment and the 6,874 bp large (L) segment [3] that all code for structural and non-structural proteins of the virus. Batai virus is transmitted by mosquitoes during feeding and is widely distributed throughout Africa, Asia and Europe [4]. Strains of BATV in India have been isolated from *Anopheline* and *Culex* mosquito species, and pigs (*Sus scrofa*) [4]. Although the zoonotic potential of BATV in Europe is unclear [5], in Africa, BATV has been isolated from humans with symptoms of a febrile illness [6] and Ngari virus, which has been isolated from patients in Africa with haemorrhagic fever, is considered to be natural a reassortant virus containing the M segment of BATV and the S and L segments from Bunyamwera virus [7].

Active surveys have detected evidence of BATV infection in mosquitoes across Central Europe, most recently in regions of Germany [8, 9] and Italy [10]. Furthermore, surveillance for anti-Batai virus neutralising antibodies in cattle sampled between 2011 and 2012 in Germany indicated a seroprevalence level of 0.55% [11]. However, more recent studies from Germany have identified seroprevalence levels of 36.4% [12] and 41.4% [13], suggesting either an underestimation of seroprevalence in 2011–2012 or that BATV has recently emerged in these areas and can be considered an epizootic in northern Europe. The identification of cattle as a key reservoir species is further corroborated by the isolation of BATV from cattle sera sampled from Inner Mongolia, China [14]. Initial isolations of BATV from mosquitoes strongly favoured transmission by *An. maculipennis s.l.* [8, 15], but studies in Europe have detected the virus in a range of species including *Cx. pipiens* and *Ae. vexans* [9]. This indicates that more than one genus of mosquito is susceptible to infection with BATV and might be capable of transmitting the virus to vertebrate hosts. Given that different mosquito species have different feeding preferences, multiple competent vectors may increase the likelihood of pathogen transmission, spillover and disease spread, all of which can impact emergent and endemic disease.

To investigate the vector competence of different mosquito genera, we have assessed the infection, dissemination and transmission rates of BATV in three mosquito species, two *Aedes* and one *Culex* species. Given that all three species are known vectors of arthropod-borne viruses we predict that all three species will be susceptible to BATV infection under our experimental conditions. Furthermore, as *Ae. detritus*, is a competent vector for a range of arthropod-borne viruses that infect domestic animals such as Japanese encephalitis virus [16], West Nile virus [17] and Rift Valley fever virus [18] and that *Ae. detritus* feeds on cattle in the UK, we predict that *Ae. detritus* will be a competent vector for BATV. Previously work has shown that temperatures above 25^{o}C can lead to increased mortality of virus infected mosquitoes indigenous to the United Kingdom [20]. In order to reflect a typical summer temperature in the United Kingdom (www.metoffice.gov.uk) [21], when mosquito activity is at its peak, all experiments were conducted at 20^{o}C.

Methods

Virus provenance and propagation

BATV (strain 5.3) was isolated in Germany from *An. maculipennis* s.l. mosquitoes [8]. All following procedures were carried out in a dedicated biosafety level 3 laboratory. BATV was propagated and titrated in Vero cells using a previously described protocol [22]. This resulted in virus stocks maintained in Eagles minimum essential media of suitable concentrations which were kept in a -80^{o}C freezer until required.

Mosquitoes and virus inoculation

Laboratory colonies of *Ae. aegypti* strain AEAE, West Africa, donated by the London School of Hygiene and Tropical Medicine, and *Cx. pipiens* strain Brookwood, UK (hybrid of forms *pipiens* and *molestus*), supplied by The Pirbright Institute, were maintained at 25^{o}C on sucrose solution. Pupae *Ae. detritus* were caught from Dee Marsh, Cheshire (53^{o} 16'39.48"N, 3^{o} 4'5.286"W) and reared to adult stage similar to protocols described in [22, 23].

Three to five day old, unfed, adult females of each mosquito species were tested for the susceptibility to infection by oral challenge and the competency to vector BATV at 20^{o}C. Prior to feeding, mosquitoes were transferred to an insect cage (22 x 22 x 22 cm, bugzaare.co.uk, Suffolk, UK) and starved of sucrose for 5 hours to stimulate feeding. Groups of mosquitoes were offered a blood meal containing defibrinated horse blood, adenosine 5'-triphosphate (final concentration 0.02 mM) and virus at a final concentration between 1.4 x 10^4 plaque-forming units (PFU)/mL and 5.5 x 10^6 PFU/mL (Table 1) through a membrane feeding system (Hemotek Ltd, Accrington, Lancashire, UK) and allowed to feed overnight. Following this, cages of mosquitoes were anaesthetized with trimethylamine (FlyNap®, Blades Biological Limited, Edenbridge, UK) and engorged mosquitoes separated from unfed individuals. Blood-fed mosquitoes were held in cages within an incubator set at 20^{o}C for seven or fourteen days. At the designated time point, mosquitoes were caught using a battery-powered, hand-held aspirator and placed for two minutes, whilst in the aspirator, at -80^{o}C to immobilise the specimens. They were then place on a surface chilled by ice to ensure they remained immobile during removal of legs/wings and saliva collection, bodies were retained, then RNA extracted as previously described [22]. A control group of *Ae. detritus* was provided with a blood-meal without a virus.

Table 1
Infection, dissemination and transmission rates of *Ae. aegypti*, *Ae. detritus* and *Cx. pipiens* following consumption of a blood meal containing BATV. Groups of mosquitoes were maintained at 20°C for the indicated time periods. Infection rate: number of positive mosquitoes/number of blood fed; Dissemination rate: number of mosquitoes with virus detected in legs/total number infected; Transmission rate: number of mosquitoes with virus detected in saliva/total number with disseminated infection.

Mosquito species	Blood-meal titre (ln PFU)	Blood feeding rate (%)	Rate	DPI 7 (%)	DPI 14 (%)
Aedes aegypti	5.5×10^6	145/320 (45)	Infection	4/16 (25)	3/44 (7)
			Dissemination	0	0
			Transmission	0	0
Aedes detritus	1.4×10^4	80/112 (74)	Infection	12/15 (80)	13/16 (81.2)
			Dissemination	5/12 (41.6)	4/13 (30.7)
			Transmission	5/5 (100)	1/4 (24)
Culex pipiens	5.5×10^6	60/188 (32)	Infection	1/15 (7)	1/28 (4)
			Dissemination	0/1 (0)	0/1 (0)
			Transmission	0	0

Molecular detection of Batai virus in bloodfed female mosquitoes

Batai virus RNA was detected using a semi-quantitative RT-PCR that targets a 99 bp sequence of the S segment using the primers BATV-Forward: 5'-GCTGGAA GGTTACT GTA TTTAAT AC-3'; BATV-Reverse: 5'-CAAGGAATCCACTGAGTCTGTG-3'; and BATV-Probe: 5'-FAM-AACAGTCCAGTTCCAGACG ATGGTC-BHQ-1-3' [8]. Reactions were performed with iTaqTM Universal Probes One-Step Kit (Bio-Rad, UK) using the following reaction mix per microtube: RNase-free water (7 µl); 2x iTaq universal probes reaction mix (12 µl); 1 µl of each primer and probe at 10 pmol/µl; and 1 µl of iScript reverse transcriptase, and 2 µl of extracted RNA. Amplification was conducted using a MxPro 3005 thermal cycler (Agilent Technologies, US) using the following reaction conditions: reverse transcription 50°C for 10 min; reverse transcriptase inactivation 95°C for 5 min; and PCR amplification and detection 40 cycles consisting of 95°C for 10 sec, 55°C for 30 sec. Amplification files were visualised and analysed in MX3000p v4. Software (Agilent Technologies, US).

Results

To determine the susceptibility of particular mosquito species to BATV infection, females of two *Aedes* species and one *Culex* species were each provided a bloodmeal containing a BATV strain recently isolated in Germany. Blood fed individuals from each species were divided into two groups and maintained at 20^{o}C for either 7 or 14 days. Individual mosquitoes were then tested for infection (virus detected in body), dissemination (virus detected in leg/wings) and transmission (virus detected in expectorated saliva). At 20^{o}C, 25% of *Aedes aegypti* mosquitoes (n = 16) were infected with BATV at day 7 (Table 1). This dropped to 7% at day 14 (n = 44). No evidence for virus dissemination or transmission was detected in this species at either time point.

For *Ae. detritus*, infection rates of 80% and 81.2% were detected at days 7 (n = 80) and 14 (n = 16), respectively. Dissemination occurred at both time points with 100% of mosquitoes in which dissemination had occurred expectorating BATV in saliva at day 7 (n = 5), although this dropped to 25% of disseminated infection at day 14 (n = 13). The presence of virus in *Ae. detritus* bodies, legs and saliva at dpi 14 was confirmed by isolation of virus in Vero cells and corroborated by RT-PCR from RNA extracted from the isolation culture. In *Ae. detritus*, comparison to a control group provided with a blood-meal with no virus, the BATV-infected group showed increased mortality from day 5 onwards with 40% surviving to day 14 (n = 112) compared to over 80% (n = 32) in the control group (Fig. 1). *Culex pipiens* showed low levels of infection at day 7 (7%, n = 15) and day 14 (4%, n = 28). However, no evidence for dissemination or transmission was shown in this species.

Discussion

The risk of mosquito-borne virus transmission in Europe has increased in recent years due to the spread of invasive mosquito species [24] and the introduction of pathogens through human travel, for example outbreaks of chikungunya and dengue fever [25], and bird migration [26]. Whilst benefitting from a cooler maritime climate and geographical separation from the European mainland that has limited the emergence of such viruses, increased summer temperatures have made the United Kingdom susceptible to the emergence of mosquito-borne viruses that are present in countries of north-west Europe [27]. Continued vigilance and the assessment of potential risk are needed to fully understand the likelihood of such virus emergence and their ability to spread [28]. In this study, we have shown that at low temperature (20°C), indigenous *Cx. pipiens* mosquitoes and the exotic species *Ae. aegypti* are not vector competent to transmit BATV. This may be due to the limited ability of BATV to replicate in these species, although evidence for infection was found in mosquito body samples. Alternatively, this could reflect that lower temperature (20^{o}C) at which the mosquitoes were maintained is limiting virus replication [20], although other factors such as variation in humidity and daily temperature are also important. Two different virus concentration were used in the experiments as they were undertaken with newly produced stocks at different time frames, 10^4 and 10^6 PFU. No difference in infection rates was recorded at higher titres (10^6 PFU) between *Ae. aegypti* and *Cx. pipiens* in comparison to lower titres in *Ae. detritus* (10^4 PFU) (Table 1).

A recent investigation of vector competence for Chittoor virus, an Asian variant of BATV, in *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus* and *Ae. aegypti* showed that the *Culex* species were vector competent but *Ae. aegypti* was not, although again infection was also observed in that species [29]. By contrast, we have shown that *Ae. detritus* was highly susceptible to infection with BATV, resulting in dissemination and potential transmission at both 7 and 14 days following ingestion of a bloodmeal. However, this was also associated with increased mortality compared to a non-infected control group. This suggests that virus replication, sufficient to enable dissemination, may be detrimental to mosquito survival.

Aedes detritus populations are found in many coastal regions of the UK. It also appears to be competent to transmit a growing list of exotic mosquito-borne viruses [16–18, 30] at temperatures between 20^{o}C and 25^{o}C, now including BATV. The mosquito is mammalophilic, aggressively biting a range of species including humans and ruminant livestock. As a result, it could play a critical role in maintaining and transmitting exotic mosquito-borne viruses to susceptible species including humans. The widespread distribution of BATV in mainland Europe [31], and its wide vertebrate host range, including its recent detection in harbour seals in northern Germany [32], suggests that the virus has the potential to emerge in the UK in the near future.

Conclusions

Of the three species studied, all species could be experimentally infected with BATV at 20^{o}C. However, there was no evidence that the virus could disseminate in *Ae. aegypti* or *Cx. pipiens* at this temperature at either 7 or 14 days post-infection. By contrast, *Ae. detritus* proved to be highly susceptible to infection as early as 7 days post-infection. Dissemination occurred in a proportion of those infected and BATV was detected in the saliva of these mosquitoes by RT-PCR and plaque assay (tested at dpi 14), suggesting the potential to transmit this virus. Considering the widespread presence of BATV across Europe and the host-feeding preference of *Ae. detritus* for livestock, these results highlight a potential epizootic risk should this virus be introduced into the United Kingdom.

Declarations

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Availability of data

All data generated by this study and used is presented within this published article.

Author contributions

ARF, LMM obtained funding for the study. LMHT, SL, AF conceived and designed experiments. LMHT, AF, EB, SL, MF performed the experiments. LMHT, AF, EB, SL, MF, SS, LMM, ARF and NJ analysed the data. LMHT wrote the first draft. NJ revised the draft and all authors contributed to and approved the final draft.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author Detail

[1]Vector-Borne Diseases Research Team, Virology Department, Animal and Plant Health Agency, Woodham Lane, Addlestone, Surrey, KT15 3NB, United Kingdom

[2]Microbiology Services Division, Public Health England, Porton Down, Wiltshire, United Kingdom

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Figures

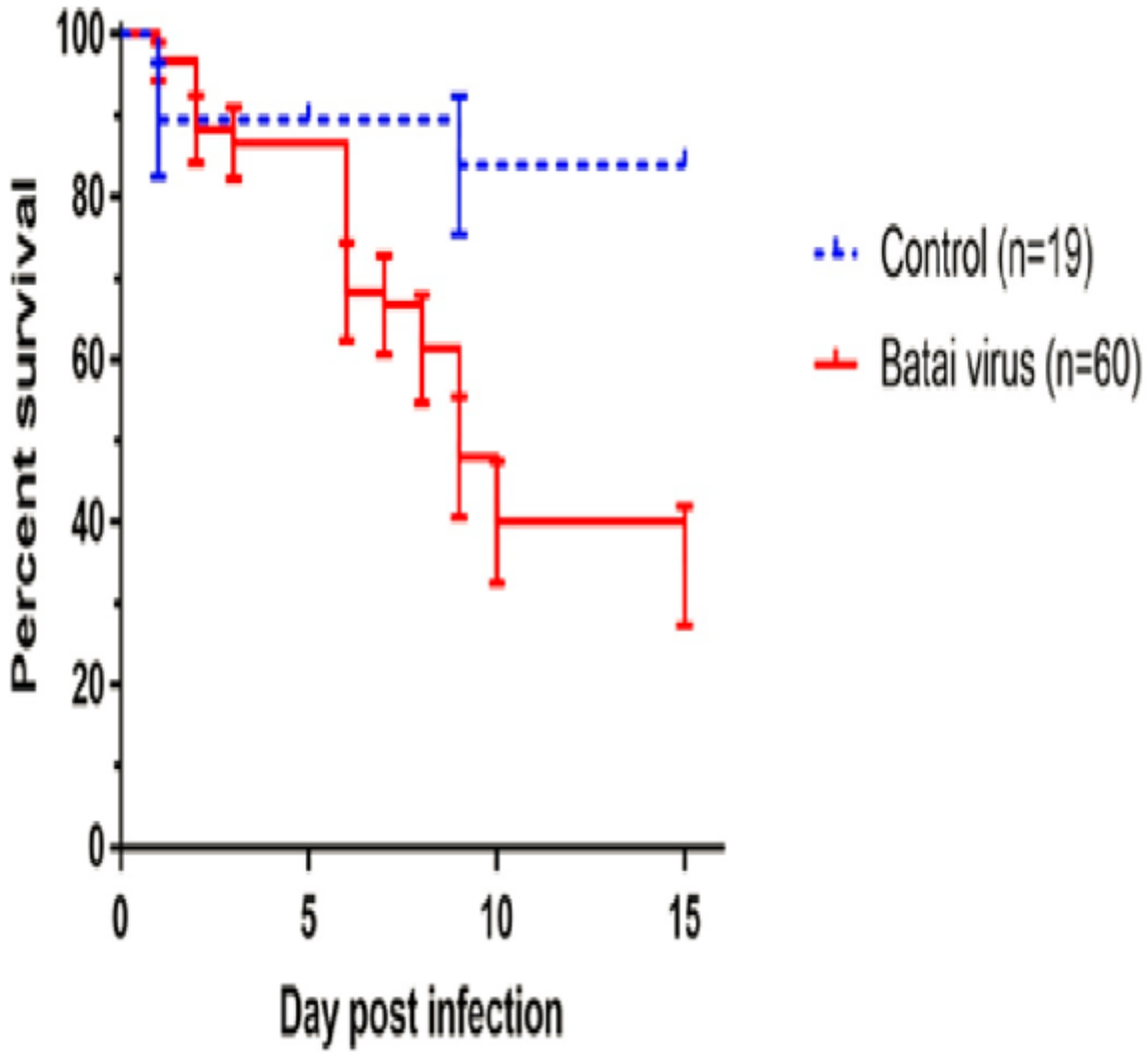

Figure 1

Survival curves for Ae. detritus at 20oC following ingestion of a blood-meal containing BATV (red) or without (blue) over a 14 day period.