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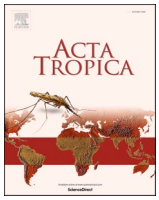

West Nile virus in adults and larvae of *Culiseta longiareolata* and *Culex hortensis* (Diptera: Culicidae) captured in Hamedan, western Iran

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ABSTRACT

West Nile virus (WNV) is an emerging arbovirus transmitted by mosquitoes. Although it is considered the most widespread mosquito-borne arbovirus in Iran, vectors of this zoonotic pathogen remain unknown in many regions. This study aimed to assess the presence of WNV in mosquitoes collected in the western city of Hamedan in 2022. Adult mosquitoes were captured using light traps, and mosquito larvae were collected by dipping technique from 45 diverse habitats, including urban, suburban, and rural sites. Specimens were identified and pooled into 69 batches based on their species for viral RNA extraction and Real-Time PCR. In total, 3243 mosquitoes (2209 larvae and 1034 adults) were captured and identified as *Culiseta longiareolata, Culex hortensis, Anopheles maculipennis* s.l., *Culex theileri, Culex pipiens, Anopheles claviger*, and *Anopheles superpictus* s.l. in decreasing order. Molecular screening revealed seven WNV-positive pools of *Culiseta longiareolata* and *Culex hortensis* in rural (n = 5) and urban areas (n = 2). Detection of WNV RNA indicates active circulation in mosquitoes and risk of transmission to humans and animals in Hamadan. These findings identify putative vectors in Hamadan, though vectors likely vary regionally in Iran. Further surveillance is needed to elucidate local WNV epidemiology and transmission dynamics fully. Nonetheless, this study provides important baseline evidence of WNV activity to guide prevention strategies in this area.

1. Introduction

West Nile virus (WNV) is the most medically important zoonotic arbovirus of the family Flaviviridae. This mosquito-borne neuropathogen for humans, horses, and birds is the most geographically widespread arbovirus in the world and the leading cause of arboviral encephalitis globally (Denman and Hart, 2015; Chancey et al., 2015). Most human infections with WNV are asymptomatic or patients may experience mild symptoms such as fever, headache, muscle aches, fatigue, and skin rash. In moderately affected patients, WNV can also cause neurological and psychological symptoms, such as dizziness, balance disorder, cognitive and memory disorders, anxiety and depression, seizures, and muscle paralysis. In severe cases, however, brain inflammation, meningitis, and encephalomyelitis may lead to death. Generally, about 20% of infections lead to an acute systemic febrile illness called West Nile fever, while less than 1% progress to severe to fatal neuroinvasive disease (D'Amore et al., 2022).

WNV is maintained in nature in a mosquito-bird-mosquito transmission cycle, with human infection and disease resulting from enzootic spillover (Ciota, 2017). Members of the crow family (Corvidae) are particularly susceptible, but the virus has been detected in dead and dying birds of more than 250 species as for instance in a previous study from Iran, molecular detection of WNV in samples from 519 birds representing 26 different species in six Iranian provinces showed that the common coot *Fulica atra* Linnaeus, 1758 was the main reservoir although the virus was detected in great cormorant *Phalacrocorax carbo* (Linnaeus, 1758), great white egret *Egretta alba* (Linnaeus, 1758), gray heron *Ardea cinerea* Linnaeus, 1758 and common teal *Anas crecca*

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Linnaeus, 1758 (Fereidouni et al., 2011). Given the serological and molecular evidences of WNV presence in humans, equines, birds, and mosquitoes the virus is circulating in 26 out of 31 Iranian provinces (Malkinson and Banet, 2002; Petersen and Marfin, 2002).

Despite the ubiquity of its competence, WNV is still predominantly vectored worldwide by *Culex pipiens* Linnaeus, 1758, *Culex quinquefasciatus* Say, 1823 and *Culex tarsalis* Coquillett, 1896 in Africa, Asia, and the Americas, and both *Cx. australicus* Dobrotworsky & Drummond, 1953 and *Cx. globcoxitus* Dobrotworsky, 1953 in Australia (Garrigós et al., 2023; Kilpatrick et al., 2006). It has been shown that WNV can be maintained in mosquito populations through vertical transmission (adults to eggs) (Chianese et al., 2019). Although mosquitoes within the genus *Culex* have been implicated as the principal vectors efficient transmission of the virus has been experimentally demonstrated in several mosquito species and the virus has been detected in over 150 mosquito species though mosquitoes with mammalian feeding behavior are responsible for most cases of human infection with transmitting WNV from the amplifying bird hosts through opportunistic feeding behavior so acting as "bridge vectors" (Fros et al., 2015; Jansen et al., 2008; Jiang et al., 2010; Turell et al., 2005).

The current number of mosquito species in Iran stands at 73, representing 8 genera (Paksa et al., 2024; Azari-Hamidian et al., 2019; Azari-Hamidian et al., 2024). To date, WNV RNA has been detected in three species of mosquitoes across 4 out of 31 provinces (Shahhosseini et al., 2017; Bagheri et al., 2015; Shahhosseini et al., 2020). Considering that understanding the interrelated ecology of vectors, suitable habitats, and preferential hosts is of prime importance to predict the emergence and amplification of WNV infection (Kramer and Styer, 2008) and the lack of information about WNV vectors in Hamedan, a western city in Iran with ca. 706,000 inhabitants, this study aimed to determine the infection of Culicidae mosquitoes with WNV in urban and peri-urban areas.

2. Materials and methods

2.1. Study area and sampling

This study was carried out in urban and rural areas in central parts and subordinate villages of Hamedan (34.7989°N, 48.5150°E) in the west of Iran from late May to November 2022. Hamedan has a cold semi-arid climate with an average annual precipitation of 384 mm, and an annual average temperature of 11.3 °C. To ensure a comprehensive and representative sample of the mosquito population in the region, all four geographical directions of Hamedan were selected for the collection of larvae and adult mosquitoes (Fig. 1).

Larval specimens were captured from each identified larval habitat by standard dipping method using a dipper to collect approximately 100 mL of water per dip (Hong et al., 2023). Larvae were transferred to the laboratory under a cold chain in plastic jars filled halfway with the habitat's water. Pertinent details such as the date and environmental characteristics of the habitat were recorded on the containers. Adult mosquitoes were mainly captured by CDC light traps installed in determined locations. Collected adults and larvae were immobilized by chilling and mounted on glass slides using Hoyer's medium, then identified to species level using taxonomic keys (Azari-Hamidian and Harbach, 2009). Following identification, specimens were transferred to the virology laboratory for RNA extraction and further analyses.

2.2. Genome extraction

Based on a pooling protocol described previously (Bagheri et al., 2015) mosquitoes were formed and stratified by date of collection, geographic area, and species. Mosquitoes of each pool were transferred into conical centrifuge tubes supplemented with 1 mL of complete Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS; Gibco, Grand Island, NY, USA), amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), 100 IU penicillin, and 100 μg streptomycin (Gibco, Grand Island, NY, USA). Mosquitoes were incubated in the medium for 30 min at room temperature, then smashed using glass rods and centrifuged at 2500 rpm for 10 min. The supernatant was collected into microcentrifuge tubes and used for viral genomic RNA extraction using LabPrep TM Viral DNA/RNA Mini Kit (General Biologicals Corporation, Hsinchu County, Taiwan). RNA samples were finally aliquoted in RNAse-free microtubes stored at −70 °C until further use.

2.3. Real-time PCR

Viral RNA samples were tested with a commercial qualitative real-time PCR kit (Kian Gene Azma, Tehran, Iran) which specifically

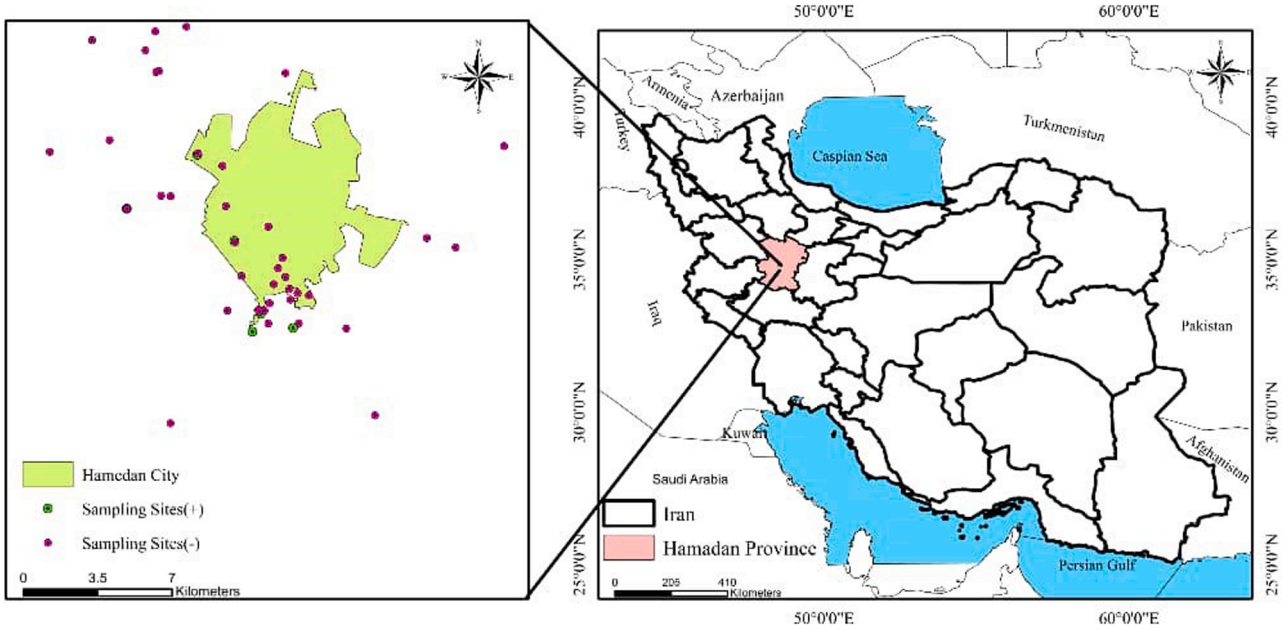

Fig. 1. Location of sampled sites showing localities with WNV positivity.

detects WNV. To confirm the results, all of the samples were tested by a multiplex TaqMan-based Real-Time PCR kit (GA Arbo Encephalitis One Step Real Time PCR Kit, Geneova, Tehran, Iran) which simultaneously detects WNV, tick-borne encephalitis (TBE) virus, Usutu virus, and Zika virus. Fluorescence signals were measured by QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).

3. Results

3.1. Mosquitoes

A total of 3243 mosquito specimens including 2209 larvae and 1034 adults were captured and identified as *Culiseta longiareolata* (Macquart, 1838), *Culex hortensis* Ficalbi, 1889, *Anopheles maculipennis* s.l. Meigen, 1818, *Culex theileri* Theobald, 1903, *Culex pipiens, Anopheles claviger* (Meigen, 1804), and *Anopheles superpictus* s.l. Grassi, 1899 in decreasing order. The composition of species varied between urban and rural areas for instance *Culiseta longiareolata* was the predominant species in rural areas (70% of rural specimens) while *Culex pipiens* was most abundant in urban areas (45% of urban specimens). *Anopheles* species were primarily captured in rural habitats. In total, 69 pools were generated each containing 47 female and male individuals from 45 representative locations across urban (22 pools) and rural areas (47 pools) (Table 1).

3.2. WNV positivity

WNV was detected in 7 out of 69 examined pools (10.1%); five *Culiseta longiareolata* (2 larvae and 3 adults) and 2 *Culex hortensis* pools (both larvae). Positive pools were from both rural ($n = 5$; 10.6% of rural pools) and urban areas ($n = 2$; 9.1% of urban pools) (Fig. 1).

4. Discussion

We report molecular detection of WNV in *Culiseta longiareolata* and *Culex hortensis* mosquitoes from rural and urban areas of a western region in Iran which adds both species to vectors of the virus in Iran along with *Culex pipiens, Culex theileri* and *Aedes caspius* (Pallas, 1771) (Shahhosseini et al., 2017; Shahhosseini et al., 2020; Bagheri et al., 2015). *Cs. longiareolata* has been shown to carry WNV RNA in different countries (Balasubramanian and Nikhil, 2015) but to the best of our knowledge herein we report WNV RNA in *Cx. hortensis* for the first time. Although it is generally speculated that because *Cx. hortensis* primarily feeds on reptiles, it cannot have a significant role in the epidemiology of WNV, several studies have shown avian and human bloodmeal in this species (Grisenti, 2016; Schönenberger, 2015) suggesting the potential bridge-vectorial role of *Cx. hortensis.* Although the primary vectors of WNV in urban areas of the United States, southeastern Canada, Europe, and Africa is *Culex pipiens* (Monath, 1988; Macan, 1950; Minar, 1981), in some studies including two previous studies from Iran (V V Moin-Vaziri et al., 2019; Moosa-Kazemi et al., 2022) similar to present research, *Cx. pipiens* pools scored negative for WNV possibly due to environmental conditions, mosquito genetics, or the presence of symbiotic bacteria like *Wolbachia* that can block virus transmission (Soto et al., 2023).

There is a scarcity of information about WNV vectors in western Asia but in neighboring Turkey, WNV RNA has been identified in *Cx. pipiens, Ae. caspius,* and *Aedes albopictus* (Skuse, 1894) (Akıner et al., 2019; Ergunay et al., 2013), and in Israel, *Culex perexiguus* Theobald, 1903, *Cx. pipiens*, and *Ae. caspius* species were shown to be the WNV vectors (Orshan et al., 2008). In Europe where many studies have been conducted, four mosquito species i.e. *Ae. albopictus, Aedes detritus* (Haliday, 1833), *Culex modestus* Ficalbi, 1947 and *Cx.pipiens* have been confirmed to possess the competence required for the transmission of WNV. In contrast, *Aedes caspius* and *Aedes japonicus* (Theobald, 1901) have been found to lack the competence necessary to serve as vectors for WNV. Among the competent species, *Cx. pipiens* exhibits the highest rates of transmission, coupled with elevated field infection rates and a high degree of abundance during the summer months. Consequently, *Cx. pipiens* is regarded as the most significant vector for WNV in Europe (Vogels et al., 2017). Considering that several mosquitoes distributed all over Iran and in particular Hamedan (Paksa et al., 2024; Azari-Hamidian et al., 2019) are known to be involved in the epidemiology of WNV e.g. *Ae. albopictus, Coquillettidia richiardii* (Ficalbi, 1889), *Culex modestus, Culex perexiguus, Culex pipiens, Culex quinquesfasciatus* Say, 1823, *Culex tritaeniorhynchus* Giles, 1901 and *Mansonia uniformis* (Theobald, 1901) (V. V Moin-Vaziri et al., 2019) continuous surveillance and research for effective control and prevention strategies are necessary.

Culiseta longiareolata was the most abundant mosquito species captured in this study and had the most WNV positivity rate. *Culiseta* mosquitoes are suspected as essential vectors in the transmission cycle of eastern equine encephalitis and potentially the WNV (Cupp et al., 2003; Huang et al., 2013; Lubelczyk et al., 2014) as well as western equine encephalomyelitis (Muul et al., 1975) and Sindbis Virus (Melaun et al., 2014). Members of the genus *Culiseta* have also been documented as vectors of *Dirofilairia immitis* (Leidy, 1856) Railliet & Henry, 1911, a parasite primarily infecting domestic and wild canines as definite hosts (Huang et al., 2013; Zittra et al., 2014). They are also considered as possible vectors of Malta fever (Maslov, 1989). *Culiseta longiareolata* in particular, is a vector of avian malaria (Schoener et al., 2017), tularemia (Maslov, 1989), and arboviruses such as West Nile fever (Medlock et al., 2007). It is a multivoltine and thermophilic species distributed in forest-free regions in the plains and hills of Asia, Europe, and Africa, as well as in the Mediterranean Sea. It mainly develops in small water bodies like artificial containers and fountains, rich in decaying organic materials, and is known to be an ornithophilic and batracophilic species but may enter houses and attack humans making it a potential bridge vector. Generally, an efficient WNV bridge vector should: 1) exhibit a propensity to blood feed on both avian and mammalian (including human) hosts, 2) be competent to transmit the virus, 3) survive long enough to acquire at least two bloodmeals, and 4) be relatively abundant (Rochlin et al., 2019). Studies have shown that as preferred avian hosts such as American robins leave in the late summer *Culex* mosquitoes are more likely to feed on mammals. These changes in feeding behavior generally coincide with peaks in mosquito populations and human outdoor activities and likely contribute to peaks in human infection in late summer and early fall which may also be the case for *Cs. longiareolata*. Another point about *Cs. longiareolata* is that it can share larval breeding sites with *Cx. pipiens* and could be possibly involved in the maintenance of the enzootic cycle (Fois et al., 2012). In addition, although typically, the development of eggs in blood-sucking insects is normally dependent on a blood meal (Service, 2012); nonetheless, some female mosquitoes can develop their eggs without a blood meal (Laurence, 1964), a trait called autogeny. Autogeny has been shown in *Cs. longiareolata* in laboratory conditions in Iran (Khaligh et al., 2020). Hence, *Cs. longiareolata* is an important player in the epidemiology of WNV. Nonetheless, since *Cs. longiareolata* is capable of consuming 2nd and 4th instar larvae of the "usual suspect" *Cx. pipiens* as well as *Ae. caspius* and *Anopheles multicolor* Cambouliu, 1902 (Shaalan, 2012) detection of viral RNA in wild-captured *Cs. longiareolata* can be due to predation on competent vectors and not necessarily vertical transmission of the virus although it is known that WNV is preserved in

Table 1
Mosquito samples collected in this study.

Species	*n* Mosquito (Larvae / Adults)	Frequency	*n* Pools
Cs. longiareolata	2200 (1253 / 947)	67.8 %	46
Cx. hortensis	396 (396 / 0)	12.2 %	7
An. maculipennis s.l.	180 (180 / 0)	5.5 %	5
Cx. pipiens	188 (128 / 60)	5.8 %	4
Cx. theileri	243 (243 / 0)	7.5 %	4
An. claviger	27 (0 / 27)	0.8 %	2
An. superpictus s.l.	9 (9 / 0)	0.3 %	1
Total	3243		69

nature by vertical transmission within several mosquito species including *Cx. pipiens* (Saiyasombat et al., 2011), *Ae. vexans (*Meigen, 1830) (Anderson et al., 2020), *Ae. albopictus, Ae. aegypti* (Linnaeus, 1762) and *Culex tritaeniorhynchus* (Baqar et al., 1993), *Culex tarsalis* (Nelms et al., 2013), *Culex univittatus* Theobald, 1901 (Miller et al., 2000), *Culex salinarius* Coquillett, 1904 (Anderson et al., 2012), *Culex tarsalis* (Anderson et al., 2012), *Culex erythrothorax* Dyar, 1907 (Phillips and Christensen, 2006), and *Aedes triseriatus* (Say, 1823) (Unlu et al., 2010).

Culex hortensis was the second most prevalent species collected in Hamedan which was also positive for WNV although in previous studies from Iran and Italy *Cx. hortensis* scored negative (Shahhosseini et al., 2017). According to older literature, as an adult, *Cx. hortensis* gorges itself in nature on *Rana dalmatina* Fitzinger, 1838 then called *Rana agilis* Thomas, 1855, and the common toad *Bufo bufo* (Linnaeus, 1758) then called *Bufo vulgaris* Laurenti, 1768 (Purpari et al., 2018) so undoubtedly other amphibians. In addition, molecular analysis of the blood meal of different mosquito species showed that *Cx. hortensis* feeds on lizard reptiles (Roiz et al., 2012) such as the common wall lizard *Podarcis muralis* (Laurenti, 1768) (Martínez-de la Puente et al., 2015). As a stenogamous species, the complete evolutionary cycle of *Cx. hortensis* could be obtained in restricted space, and a single blood meal is enough to achieve egg-laying albeit it gorges itself extremely slowly and a complete meal takes 25 to 40 min. In regards to WNV epidemiology, it is widely accepted that *Culex* species with a predominantly ornitophilic diet serve as the main amplifiers and/or bridge vectors of WNV to humans (Giesen et al., 2023), and prevalent and widespread species *Cx. hortensis* does not bite humans so is not known to vector the agents of any human disease (Gutsevich et al., 1974) although they can be found in the house (Gutsevich et al., 1974). But it should be noted that experimental infection of the lake frog *Rana ridibunda* Pallas, 1771 (Kostiukov et al., 1985) with a Russian strain of WN virus resulted in viremia sufficient to infect *Cx. pipiens* and that reptiles have been observed to sustain long-term viremias for mosquito-borne viruses (Bowen, 1977). Hence, the role of *Cx. hortensis* in WNV circulation may not be negligible. However, *Cx. hortensis* level of competence for WNV needs further investigation.

While this investigation provides important initial data, there were some limitations. The first was that because of financial and logistic reasons samplings were performed only during spring and summer which limited our understanding of year-round or interannual variations in WNV activity and mosquito populations. Two others were limitations in the number of real-time PCR examined samples and that sequencing was not performed to find out WNV lineage(s) present in the study area. Since avian hosts are key to the WNV cycle because of their ability to rapidly amplify the virus and usually survive the infection even in high levels of viremia, monitoring resident and migrating birds for the virus is strongly recommended.

5. Conclusion

Detection of WNV RNA herein reported in *Culiseta longiareolata* and *Culex hortensis* in both urban and rural areas indicates active circulation of the virus and risk of transmission to humans and animals in this western region of the country. Continued surveillance of the virus in mosquitoes, birds, and breeding sites in the area is strongly recommended. Since there are no specific treatments or vaccines for human WNV infection and prevention of this mosquito-borne disease relies on controlling the mosquito population and personal protective measures to avoid mosquito bites community efforts should focus on increasing public awareness to decrease mosquito breeding sites and implement effective control strategies.

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Ethical approval

The study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Ref. No: IR.UMSHA.REC.1401.614).

CRediT authorship contribution statement

Mehran Khaledian: Writing – original draft, Resources, Methodology, Investigation. **Iman Owliaee:** Writing – original draft, Resources, Investigation. **Alireza Sazmand:** Writing – review & editing, Writing – original draft, Investigation. **Behroz Davari:** Writing – original draft, Investigation. **Amir Hossein Zahirnia:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Farid Azizi Jalilian:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Alireza Sazmand is editorial board member of Acta Tropica. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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