One Health 18 (2024) 100670

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Arbovirus screening of mosquitoes collected in 2022 in Emilia-Romagna, Italy, with the implementation of a real-time PCR for the detection of Tahyna virus

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ARTICLE INFO

Keywords:
Entomological surveillance
Tahyna virus
West Nile virus
Usutu virus
Culex pipiens
Aedes caspius

ABSTRACT

Several Arboviruses (Arthropod-borne virus) are a concrete health risk. While some arboviruses, such as the West Nile virus (WNV) and the Usutu virus (USUV) are actively surveyed, others are neglected, including the Tahyna virus (TAHV). In this work, we tested – searching for all the three viruses – 37,995 mosquitoes collected in 95 attractive traps, baited by carbon dioxide, distributed in the lowlands of Emilia-Romagna, northern Italy, between 19 July and 12 August 2022.

Among the 668 pools obtained, WNV was detected in 45 pools of *Culex* (*Cx.*) *pipiens* and USUV was recorded in 24 pools of the same mosquito; ten of these *Cx. pipiens* pools tested positive for both WNV and USUV. Interestingly, we recorded a significant circulation of both WNV lineage 1 (WNV-L1) and lineage 2 (WNV-L2): WNV-L1 strains were detected in 40 pools, WNV-L2 strains in three pools and both lineages were detected in two pools.

TAHV was detected in 8 different species of mosquitoes in a total of 37 pools: *Aedes* (*Ae.*) *caspius* (25), *Ae. albopictus* (5), *Ae. vexans* (3), *Cx. pipiens* (2), *Ae. cinereus* (1) and *Anopheles maculipennis sl* (1). The significant number of *Ae. caspius*-pools tested positive and the estimated viral load suggest that this mosquito is the principal vector in the surveyed area. The potential involvement of other mosquito species in the TAHV cycle could usefully be the subject of further experimental investigation.

The results obtained demonstrate that, with adequate sampling effort, entomological surveillance is able to detect arboviruses circulating in a given area. Further efforts must be made to better characterise the TAHV cycle in the surveyed area and to define health risk linked to this virus.

1. Introduction

Arboviruses (arthropod-borne viruses) are viruses transmitted by arthropods, they do not represent a taxonomic category and include viruses of different orders, several of which are transmitted by mosquitoes [1].

Some mosquito-transmitted arboviruses are already monitored for their relevance in public health, such as West Nile virus (WNV) and Usutu virus (USUV) [2], both belonging to the *Flaviviridae* family and *Flavivirus* genus. These two flaviviruses circulate between wild birds (reservoirs) and mosquitoes – mainly of the *Culex* (*Cx.*) genus (vectors) – and can infect other vertebrates as dead-end hosts, for example humans and horses [3]. WNV can be divided phylogenetically into several lineages, of which the most widespread are lineage 1 (WNV-L1) and lineage 2 (WNV-L2), both pathogenic for humans [3]. In humans, WNV infections are usually asymptomatic or cause flu-like symptoms, but in a minority of cases (<1% of infected persons) they can cause a potentially fatal neuroinvasive disease. The clinical manifestations of USUV infection are similar but less common that those of WNV [3]. In addition to this direct risk, these viruses are a threat to the health of blood donations, due to the risk of infected but asymptomatic blood donors [4]. For this reason, these two viruses are the target of surveillance in Emilia-Romagna, Italy, with the principal aim of evaluating seasonal and spatial differences in the intensity of viral circulation to guarantee the safety of blood donations [5].

Other arboviruses pathogenic to humans may circulate in the environment but they are often neglected. One typical example is the Tahyna virus (TAHV), belonging to the *Peribunyaviridae* family and

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https://doi.org/10.1016/j.onehlt.2023.100670
Received 2 November 2023; Accepted 22 December 2023
Available online 26 December 2023

Orthobunyavirus genus. In humans, most TAHV infections are unapparent, while, when symptomatic, the infection produces an acute influenza-like disease, mainly in children. Meningitis or problems to the central nervous system have also been observed, but no TAHV attributed fatalities have been reported yet [6,7].

TAHV was the first ever arbovirus isolated in Europe from *Aedes* (*Ae*.) *vexans* and *Ae*. *caspius* collected in the villages of Tahyna and Krizany in East Slovakia in 1958 [8]. Then, the virus was isolated from several mosquito species and widely reported in Europe [9,10].

Lagomorphs are recognized as the main host of the virus [11] and *Ae*. *vexans* as the principal vector in Central Europe. However, the World Health Organization considers TAHV an important arbovirus agent with respect to public health in Europe [12].

In this study, we used an in-house developed real-time PCR to test mosquitoes collected for WNV and USUV surveillance in Emilia-Romagna also for the presence of TAHV, with the principal aim of characterising the presence and diffusion of the three arboviruses in the surveyed area.

2. Material and methods

2.1. Mosquito sampling and testing

The Arbovirus surveillance plan of Emilia-Romagna included 95 traps in 2022 sited in the lowland part of the region (Fig. 1). We retrieved mosquitoes sampled from 19 July to 12 August, which means that each site was sampled two times. Mosquitoes were collected overnight by modified CDC-light traps baited with carbon dioxide activated from roughly 5 pm to 9 am of the following day. The mosquitoes collected were refrigerated for transport to the lab and killed by freezing at −20 °C for 20 min. We then sorted mosquito by sex, identified them by using morphological keys [13,14] and made monospecific pools, with

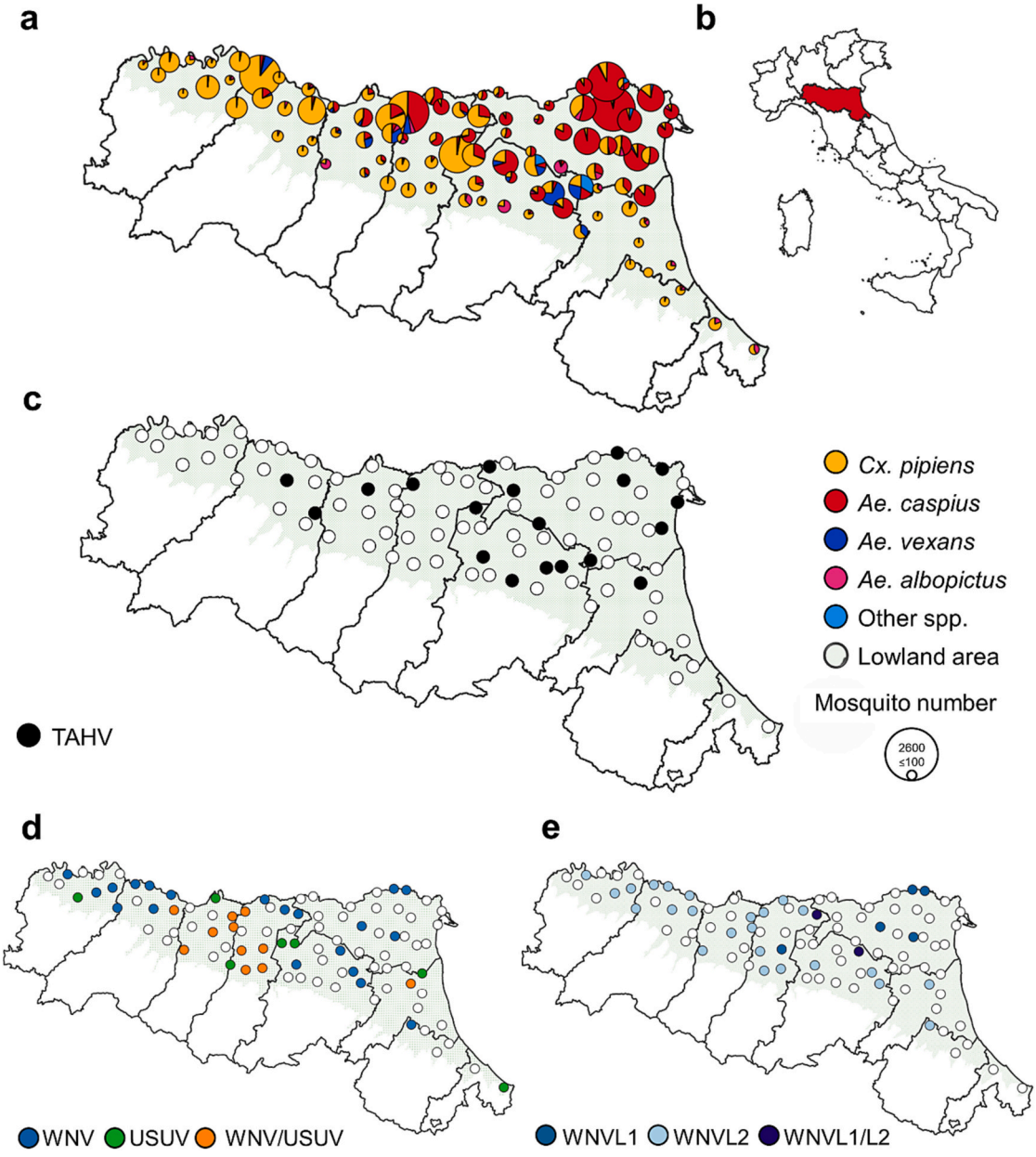

Fig. 1. Localization of sampling sites on the Emilia-Romagna map showing the sampled species (a), Tahyna virus positive sites (c), West Nile virus and Usutu virus positive sites (d), West Nile lineage 1 and lineage 2 positive sites. In (b) the location of Emilia-Romagna on a map Italy in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a maximum size of 200 females per pool.

The pooled mosquitoes were stored in 2 mL polypropylene cryotubes or, if >50, in 15 mL vials (Falcon ™). Two to four 4.5 mm diameter copper-plated round balls (Plinking, China) were added to the vials with variable amounts of phosphate buffered saline (PBS) (0.5 mL in pools <50 mosquitoes, 1 mL in pools from 51 to 100 mosquitoes, 1.5 mL in pools from 101 to 150 mosquitoes, 2 mL in pools from 150 to 200 mosquitoes). Then we ground the mosquito pools in a vortex mixer for 1 min and centrifuged at 4000 ×*g* for 7 min. Finally, aliquots were collected from the ground samples and subjected to biomolecular analysis.

Viral RNA was extracted from the mosquito homogenate in 96 well-plates using the BioSprint 96 One-For-All Vet kit (Qiagen, Milan, Italy) and the BioSprint 96 workstation (Qiagen), according to the manufacturer's instructions. The RNA was retro-transcribed using a 200 U/μL Super Script II™ Reverse Transcriptase (Invitrogen by Thermo Fisher Scientific, Milan, Italy) at 42 °C for 50 min.

The reverse-transcribed samples were examined for WNV [15] and USUV [16] and the WNV-positive pools were subjected to a protocol to discriminate WNV-L1 and WNV-L2 [17]. We also applied the in-house protocol described below for TAHV detection.

2.2. Real-time PCR for detecting Tahyna virus

To design the primers and probe of the real-time PCR for TAHV detection, we referred to the tract of viral genome amplified by a pan-orthobunyavirus PCR [18], to exploit the relevant number of sequences of this specific tract deposited in public databases. The sequences of TAHV (40) and affine orthobunyaviruses – Lumbo virus (2), California encephalitis virus (2), La Crosse virus (3), Snowshoe hare virus (3), Keystone virus (2) – were aligned to highlight the conserved nucleotides in TAHV. The primers and probes were designed as follows: forward primer F-TAHV 5′ AGG GTA TGT GGA CTT CTG T 3′, reverse primer R-TAHV 5′ TTT AGG ACT AGC CTT CCT CT 3′, probe P TAHV 5'FAM- TCC TTA ATG CCG CAA AAG CCA A 3'-BHQ1 (Fig. 2), the target sequence was 130 base long. The 20 μL reaction mix for every reaction was obtained using 4 μL of 5× QuantiFast® Pathogen Master Mix (Qiagen), 2 μM of each primer, 0.7 μM of probe, 3 μL of cDNA and distilled water. After a first step of denaturation at 95 °C for 8 min, 50 cycles were performed with the thermal profile of 94 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s. The real-time PCR was performed on the CFX96 real-time System (Bio-Rad, Milan, Italy) and the results were analysed by the CFX Manager™ Industrial Diagnostic Editor (Bio-Rad). The protocol was firstly applied to 10 pools of mosquitoes collected in 2021, in four of these pools TAHV was detected by the pan-orthobunyavirus PCR [19] followed by sequencing.

The positive control was obtained from the strain of TAHV 404118/8 isolated by IZSLER in 2021 and grown on VERO cells (4th passage), with the titre of $10^{6.2}$ TCID50/50 μL.

The obtained extract was ten-fold diluted to 10^{-5} and quantified by a digital PCR (QIAcuity ONE Digital PCR System, Qiagen). For this quantification, we used a 24-well plate containing 8500 partitions. The reaction mix was composed of 3 μL of a 4× QuantiTECT® Virus + ROX mix (Qiagen), 1.55 μL of primer and probe mix (50 μL of 100 μM primers, 35 μL of 100 μM probe and 365 μL of sterile water), 0.12 μL of a 100× reverse transcriptase and 1.33 μL of sterile water, per reaction. For each sample, the final volume was 12 μL: 6 μL of master mix and 6 μL of RNA. The thermal profile used was that reported for the real-time PCR.

The results were provided by the QIAcuity Software Suite 2.7.7.182, which returned the average value of the sample considering the generated partitions. The quantification results were used to build the calibration curve by plotting the concentration of the positive viral samples expressed in logarithmic scale and the Cq obtained from the analysis of the dilutions using real-time PCR. The obtained standard curve was used to estimate the viral load of TAHV per mosquito pool (Table S1).

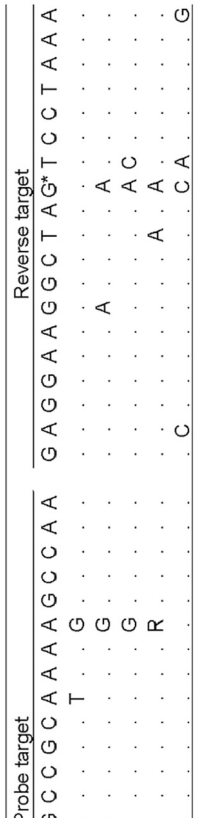

Fig. 2. Primers and probe target sequences recorded for Tahyna virus and in homologous sequences of other related orthobunyaviruses deposited in GenBank.
GenBank accession numbers: **Tahyna virus**: GQ480358, GU390669, GU390670, GU390671, GU390672, GU390673, GU390677, GU390678, GU390680, GU390682, GU390683, GU390685, GU390686, GU390687, GU390688, HM036208, HM036214, HM036217, HM068013, HM068014, HM068015, HM243139, HM243142, HQ541823, JN051146, JN051148, JN051150, KJ575081, KJ575082, MZ245724, MZ245725, NC055206, ON124935, ON124936, ON124937, ON124938, ON124939, ON124940, U47142, Z68497; **Lumbo virus**: NC043631, X73468; **California encephalitis virus**: MH830340, U12797; **La Crosse virus**: GU206111, GU206135, KP271104; Snowshoe hare virus: KM215562, KM215563, MK352486; **Keystone virus**: MG821231, MZ156793. * in one sequence (ON124939) A instead of G.

3. Results

3.1. Application of the Tahyna virus real-time PCR

The quantification by dPCR using four dilutions of the positive control showed a copy count of 21,322 copies/µL for 10^{-1}, 2059 copies/µL for 10^{-2}, 199 copies/µL for 10^{-3}, 16 copies/µL for 10^{-4}. The calibration curve has a slope of −3.726, with an R^2 of 0.993 and a reaction efficiency of 85.5% (Figs. S1, S2). The protocol was first applied to mosquitoes collected in 2021 and detected TAHV in 4 mosquito pools in which the virus had already been detected using the pan-orthobunyavirus PCR plus sequencing, while giving negative results in another 6 pools selected at random (Table S2).

3.2. Mosquito results

We analysed 37,995 mosquitoes, divided into 668 pools (Table 1). WNV was detected in 45 pools of *Cx. pipiens*, WNV-L2 viruses were detected in 40 pools and WNV-L1 in three pools, with both lineages detected in two pools. USUV was recorded in 24 pools of *Cx. pipiens*; ten of these pools tested positive to WNV-L2 and USUV. TAHV was detected in 6 different species of mosquitoes in a total of 37 pools: *Ae. caspius* (25), *Ae. albopictus* (5), *Ae. vexans* (3), *Cx. pipiens* (2), *Ae. cinereus* (1) an *Anopheles* (*An.*) *maculipennis sl* (1). TAHV and WNV-L2 were detected in two pools of *Cx. pipiens* (sampled in the same site on two different days of sampling).

WNV was detected at 31 sites (maximum four positive pools per site), USUV was detected at 18 sites (maximum five positive pools per site), TAHV was detected at 19 sites (maximum 9 positive pools per site) (Fig. 1). All three viruses were detected at two sites, WNV and USUV were detected at nine sites, TAHV and WNV were detected in six sites, TAHV and USUV were detected in two sites. Detection of WNV and USUV in the same site is significatively associated (of the 95 sites 19 were WNV+, 8 were USUV+, 10 WNV/USUV+; $\chi^2 = 6.56$, $p = 0.01$, d.f. = 1), while there is no meaningful correlation between TAHV and other detected viruses (data not shown).

Using the standard curve of our TAHV real-time PCR, we estimated the number of viral copies in the tested pools. Among the tested samples, four were from a single mosquito and showed the viral load of 1.4×10^6 for *Ae. caspius*, 7.2×10^5 and 7.5×10^5 for two specimens of *Ae. albopictus*, and 5.7×10^5 for one specimen of *Ae. vexans*.

Among species with more than one positive pool, *Ae. caspius* showed the highest viral load with a median value of 6.0×10^7 viral copies per pool, followed by *Ae. vexans* with a median value of 2.1×10^6, *Cx. pipiens* with 1.5×10^6 copies per pool and *Ae. albopictus* with 7.5×10^5 copies per pool (Fig. 3). The largest number of viruses were detected in a pool of 106 *Ae. albopictus* sampled in Bologna on 26 July, with an estimated number of 6.8×10^{10} viral copies. Other positive species, with only one TAHV-positive pool, included *Ae. cinereus* with a pool of four specimens with an estimated 2.1×10^7 viral copies and *An. maculipennis* sl, with a pool of 14 specimens with an estimated 2.9×10^5 viral copies. In 14 sites, TAHV was detected in one species of mosquito, in eight sites in *Ae. caspius* only, in two sites in *Ae. albopictus*, in two sites in *Ae. vexans*, in one site in *Ae. cinereus* and in one site in *Cx. pipiens*. In another five sites, TAHV was detected in *Ae. caspius* pools in association with another species: in three sites with *Ae. albopictus*, in one site with *Ae. vexans* and in one site with *Ae. maculipennis* sl.

Table 1
Mosquitoes tested by species and detected viruses.

	N	Pools	WNV	USUV	TAHV
Aedes albopictus	1456	120			5
Aedes caspius	16,746	191			25
Aedes cinereus	4	1			1
Aedes vexans	1924	73			3
Anopheles maculipennis sl	261	42			1
Anopheles plumbeus	4	2			
Coquillettidia richiardii	365	6			
Culex pipiens	17,235	233	45*	24	2
	37,995	668	45	24	37

* 40 L2 3 L1 2 L1/L2.

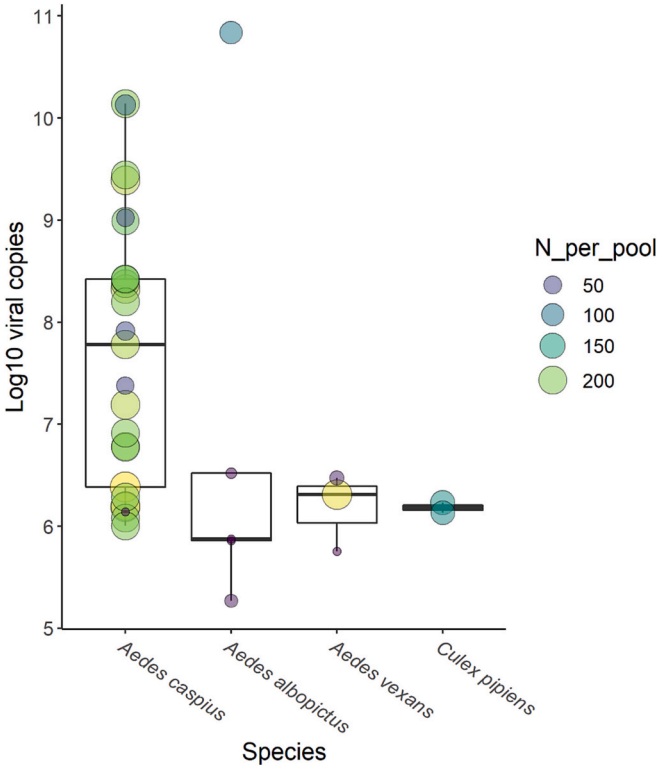

Fig. 3. Box plot of estimated number of viral copies of Tahyna virus per pool for the different species of mosquitoes for which more than one pool tested positive. The estimate for each single pool was also reported with reference to the number of mosquitoes for each pool (the size of the circle is proportional to the number of mosquitoes in the pool).

4. Discussion

The data collected showed an intense circulation of WNV in 2022 in Emilia-Romagna, which caused 69 human cases of neuroinvasive disease in the region. The screening launched after detection of the virus identified 17 asymptomatic blood donors [19], preventing the risk that infected blood donations represent.

Interestingly the co-circulation of WNV-L1 and WNV-L2 strains was recorded in the eastern part of the study area. Circulation of WNV-L1 was recorded in Emilia-Romagna from 2008 to 2010 [20,21]. From 2013, after two years in which the virus was undetected [5], the strains recorded during surveillance were almost exclusively WNV-L2, with just a single pool of mosquitoes testing positive for WNV-L1 in 2017. The co-circulation of both lineages, recorded also in the neighbouring region of Veneto in 2022, is an unusual finding and deserves to be explored further, since WNV-L1 strains seem more pathogenic for humans than WNV-L2 strains [22].

A significant circulation of USUV was also recorded. The detection of this flavivirus is relevant in the differential diagnosis, since it cross reacts with WNV, not only serologically but also in biomolecular tests [23]. Furthermore, the two viruses share many features, such as the vector, and show cycle affinity, as demonstrated by the association evidenced in this study. Moreover, this virus is pathogenic for humans, albeit to a lesser extent than WNV [24], and defining the viral circulation is relevant for characterising the health risk associated with this

virus. In fact, only one human case of USUV was reported in 2022 in Emilia-Romagna [19], though the circulation of the virus was significant, albeit less intense than for WNV, as demonstrated by the lower number of USUV-positive pools recorded in this work.

In this work, we tested only mosquitoes collected in a limited period of the 2022 surveillance, but the monitoring of TAHV was introduced and all mosquito species were tested for WNV and USUV. Despite this, WNV and USUV were detected only in pools composed of *Cx. pipiens*; this result further confirms this ornithophilic mosquito as the main vector of WNV and USUV in the surveyed area [2,21,25]. On the other hand, TAHV was detected in several mosquito species. The intrinsic biological ability of a vector to transmit a pathogen after exposure is known as vector competence and represents the main trait of a biological vector. The PCR-detection of the virus in a mosquito pool is a clue of vector competence of this mosquito, even if it cannot be considered a certain proof, since it does not provide evidence of mosquito infectivity. The described RT-PCR showed good performance in terms of sensitivity and specificity and its application allowed us to estimate the number of viral copies for the TAHV-positive pools. The variability of the amount of virus in a mosquito – which can vary in 3–4 logarithms in an experimental setting for TAHV [26] – does not allow us to calculate a precise number of infected mosquitoes per pool. However, a higher amount of virus recorded in the mosquito pool is strong evidence in favour of the ability of that mosquito to transmit the virus, i.e. to be a competent vector.

Interestingly, when two mosquito species tested positive for TAHV in one site, one was *Ae. caspius*. Moreover, this mosquito showed the highest number of TAHV-positive pools with the largest median viral load per pool. These findings strongly point towards *Ae. caspius* as the main vector of TAHV in the study area, as already suspected [21]. The low amount of virus and small number of TAHV-positive pools for *An. maculipennis* sl and *Cx. pipiens*, is probably due to their lower vectorial capacity. This seems particularly probable for *Cx. pipiens*, which was the most-sampled mosquito in this study, is ornithophilic and seem to have poor vector competence, as shown experimentally [26].

Despite the low number of specimens sampled, the role of *Ae. cinereus*, with a high viral load estimated for the only positive pool, likely indicates good vector competence and deserves further investigation. TAHV was already isolated from this mosquito in Moravia [27,28]. Our data also seem to indicate a less significant role as vector in the study area for *Ae. vexans*, the main vector in Central Europe [7]. While tiger mosquito showed the lowest media value for viral copies per pool, the highest amount of TAHV was found in a pool of this mosquito species, which are the second species in terms of number of positive pools. These findings suggest that *Aedes albopictus* is a competent vector of TAHV and is potentially able to transmit virus in an urban environment, where this mosquito is more widespread, if also competent hosts would be present. Experimental infections have been performed, with contrasting results [26,29]; similar experiments must be repeated using the virus isolated in the study area and in mosquito populations from the same area, in order to characterise the potential vector role of this mosquito.

Beyond primary vectors, *Ae. vexans* and *Ae. caspius* [30,31], the virus has been found in several other mosquito species in different areas [9,11] as recorded also in this study. The detection of TAHV in different mosquitoes implicates the local involvement of different mosquito species, mainly of the *Aedes* genus, as vector in different ecological settings. Different species seem involved in TAHV cycle at different latitudes, according to their relative abundance and host preferences; for instance, the virus was isolated from *Ae. communis* complex mosquitoes on the edge of the taiga on the Kolsky peninsula (Murmansk region) [32].

The main TAHV vectors are mammophilic mosquitoes, and this is in agreement with the observation that mammals are the main reservoirs; brown hares and rabbits are identified as the principal amplifying hosts in Central Europe [7,33]. High seroprevalence has been found in different mammals, such as deer and wild boar [34–36], sheep [37], bears [38], horses and domestic animals [39]. The possible involvement of different species in maintaining the virus in natural foci cannot be excluded a priori. While several wild birds tested positive for TAHV by serological methods [40,41], they appear not to be involved in the circulation and the ecology cycle of the virus [42]. In any case, these data seem to indicate a certain degree of plasticity in the cycle of the TAHV: for example, past serological studies hypothesized the role of rodents and insectivores as reservoirs in northern Italy [43]. The possible involvement of these animals and different species of domestic animals as occasional and local hosts has already been suggested [7], and the relative importance of different species in different areas requires more thorough investigation. While TAHV belongs to the California antigenic group, which includes viruses pathogenic for animals as Snowshoe hare virus and La Crosse virus [44], no diseases linked to this virus have been reported in animals. Experimental work is needed to obtain clearer information on the cycle of the virus in the study area, in particular on the characterization of competent hosts. The setup of the described TAHV real-time PCR for vertebrate samples would be useful to achieve this purpose.

5. Conclusions

Entomological surveillance, if applied with adequate sampling effort, may provide a reliable picture of the circulation intensity of the different flavivirus studied. This data is useful for assessing health risk and for adopting effective measures to limit the circulation of viruses. The detection of TAHV in the study area confirms the virus presence, as already reported [21,45]. Indeed, old serology studies have reported the likely human circulation of the virus in Italy, particularly in wetland areas [46]. The possible increase of TAHV after flooding events [11,28] makes this virus potentially more dangerous in climate change scenarios, which will cause a probable increase in extreme events, such as exceptionally heavy rainfall. The detection of TAHV at an important level testify a relevant circulation of the virus and implies the potential human infection in the surveyed area, this aspect needs to be investigated, to determine the potential impact of this virus on human health.

Funding

This research was supported by EU funding within the NextGenerationEU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases [Project no PE00000007, INF-ACT] and by the Arboviral Surveillance and Control Plan of Emilia-Romagna [DGR N. 531, 11/04/2022].

CRediT authorship contribution statement

Mattia Calzolari: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft. **Emanuele Callegari:** Investigation, Writing – review & editing. **Annalisa Grisendi:** Investigation, Data curation. **Martina Munari:** Investigation, Writing – review & editing. **Simone Russo:** Investigation, Validation. **Danilo Sgura:** Investigation. **Antonio Giannini:** Investigation. **Gastone Dalmonte:** Investigation, Writing – review & editing. **Mara Scremin:** Investigation, Data curation. **Michele Dottori:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

Author would like to acknowledge for the assistance in experimental

work Paolo Bonilauri, Matteo Ricchi and Katia Marzani.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2023.100670.

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