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Project title: **Mosquito-borne viruses in Canton Ticino: Evaluation of the public health risk for autochthonous transmissions and surveillance using sugar-baited nucleic acid preservation cards**

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1 General considerations

Over the past decade, epidemics of mosquito-borne diseases such as chikungunya and dengue fever have been occurring several times in Europe highlighting the real threat that these diseases constitute for public health (Rezza 2016; Schaffner *et al*. 2013). Obviously, the risk of autochthonous transmission of mosquito-borne diseases depends on the presence of competent vectors on the territory. *Aedes albopictus* (*Ae. albopictus*) (Skuse), also known as Asian tiger mosquito, is able to transmit a multitude of viruses, including chikungunya, dengue and Zika viruses (Schaffner *et al*. 2013). In the last 30-40 years, this highly invasive mosquito has been spreading from Southern Asia to almost all continents (Bonizzoni *et al.* 2013). Currently, populations of *Ae. albopictus* are established in several European Countries, including Southern Switzerland, where it was firstly reported in 2003 (Flacio *et al*. 2004). The establishment and spread of these mosquitoes and other invasive species in Switzerland are constantly monitored by the Working Group for Mosquitoes (Gruppo Lavoro Zanzare, LMA-SUPSI), and due to the importance of this issue the Federal Office for the Environment started a Swiss Monitoring Program and created a Swiss Mosquito Network with regional stations on the territory. The goal is to provide help to the Cantons and information on invasive mosquitoes in Switzerland and suggestions to prevent spread. The LMA is the coordination centre of this network.

The first relevant autochthonous transmission of mosquitoes-borne viruses associated to *Ae. albopictus* in Europe occurred in Italy in 2007, when hundreds of chikungunya cases were reported (ECDC 2007). After this epidemic, France experienced an analogous situation in 2010 (Grandadam *et al*. 2011). In 2017, a second outbreak of chikungunya occurred in Italy (Lazio and Calabria regions) and in France (Var department) (ECDC 2017). In 2010 also the first autochthonous transmission of dengue virus by *Ae. albopictus* occurred in Europe (in France and Croatia) (La Ruche *et. al* 2010; Schaffner *et al*. 2013) and in both 2018 and 2019 cases of dengue were reported again in France and in Spain (ECDC 2018a, ECDC 2019).

Arboviruses are not only transmitted by invasive mosquitoes but also by autochthonous mosquitoes, especially *Culex* spp. West Nile virus, the most widespread flavivirus (Tran *et al.* 2014) mainly transmitted by *Culex* mosquitoes, is spreading in continental Europe and autochthonous human cases are regularly reported in Italy since 2011 (Bellini *et al.* 2014; Rizzo *et al.* 2016). In 2018, a high increase in the number of human and equine infections has been reported. (ECDC 2018b).

In Switzerland, autochthonous cases of mosquito-borne viruses transmission have not been reported so far (Federal Office for Public Health 2019). However, the consolidated presence of the vector *Ae. albopictus* in densely populated urban areas of Canton Ticino increases the risk of indigenous transmissions following imported cases. An efficient surveillance of vectors and emerging viruses, as well as the investigation of the risk of autochthonous transmission would allow preventing the spread of arboviruses across Switzerland.

2 Aim

The project consisted in two main parts.

In the ***first part of study*** we aimed to assess the potential risk of an outbreak of arboviral diseases in Canton Ticino where the vector *Ae. albopictus* is established. To estimate the risk of a local transmission it is essential to know the mean number of female mosquito bites per human per day. Therefore, specific objectives of this part of study were:

- to evaluate the *Ae. albopictus* population density in six urban areas of Canton Ticino during the seasonal peak period;
- to calculate the level of correlation between ovitrap data and human landing collection data in Canton Ticino;
- to estimate the risk of outbreak in case of introduction of chikungunya, dengue, or Zika viruses throughout viremic travellers.

This risk assessment was carried out during summer 2017.

The circulation of arboviruses in the local mosquito population increases the risk for outbreaks. Therefore, in the ***second part of the study*** we aimed to survey for the presence of arboviruses known to be pathogenic for humans and animals and potentially transmitted by mosquitoes. Specific objectives were:

- the use of honey-soaked, nucleic acid preserving Flinders Technology Associates filter paper cards (FTA cards) for arboviruses surveillance in the Cantons of Ticino and Graubünden;
- the use of FTA cards in combination with mosquito traps.

Arboviruses surveillance was carried out during years 2018 and 2019.

The Federal Office of Public Health (FOPH) and the Cantonal Health Authority (Canton TI) provided financial support for the study. A further financial help from the Federal Office for Civil Protection (FOCP) allowed in 2019 to expand the surveillance to Canton Graubünden.

3 Methods

3.1 Part I: Risk assessment

The estimation of the potential risk of an outbreak of arboviral diseases was carried out in 2017. Detailed methods are described in the first intermediate report (Guidi 2017a). Briefly, in order to estimate the risk of a local arbovirus transmission, the population densities of the Asian tiger mosquito were assessed in six different urban areas in Canton Ticino by two different indices:

- number of bites/human/day estimated by the number of host-seeking females captured in green areas in 15 minutes in the peak activity period of mosquitoes (*Human Landing Collection, HLC*);

- number of eggs laid by female mosquitoes during a 14 days period (*ovitraps*).

Human landing collections were performed from 29th of August to 8th of September 2017, whereas ovitrap data were collected from week 19 until week 37, 2017. The relationship between the mean numbers of bites/human/day estimated by HLC and the mean numbers of eggs collected in each location in the same period was assessed.

The basic reproduction number R_0 (defined as the number of secondary cases that originate from a primary case) was calculated for chikungunya (non-mutant and A226V mutant strains), dengue and Zika (Asian and African lineages) viruses for the six different localities.

3.2 Part II: Arbovirus surveillance

A surveillance for arboviruses was carried out during two consecutive years, 2018 and 2019. The arbovirus surveillance in 2018 was performed within the Mendrisiotto, Bellinzonese and Locarnese districts, using FTA cards associated with ovitraps, BG-Gravid Aedes traps (GAT) (Biogents AG, Germany) and Box-Gravid Mosquito trap (BOX) (BioQuip Products, USA) placed over the period from end of July to end of September. In 2019 the surveillance was enlarged to Canton Graubünden and only the BOX-Gravid traps coupled with FTA-honey baited cards were used. A detailed description of the methodology used in 2018 is reported in the second intermediate report (Ruinelli 2018), whereas the study design and methods used in the 2019 surveillance are depicted hereafter.

3.2.1 Field sampling 2019

Samplings were performed in 11 municipalities of Canton Ticino and Canton Graubünden. For Canton Ticino, 10 sampling locations within six municipalities were selected based on the abundance of *Ae. albopictus* registered in 2018 (Table 1). For Canton Graubünden, additional 10 sampling sites within five municipalities were selected in the Mesolcina Valley, where people complained for mosquitoes, as well as in Bregaglia and Poschiavo Valleys, near the Italian border (Table 1).

In order to survey for the presence of West Nile, dengue, chikungunya and Zika viruses, Box-Gravid Mosquito traps (BOX) (BioQuip Products, USA) were selected for the sampling of *Ae. albopictus* (main potential vector for dengue, chikungunya and Zika in our country) and *Culex pipiens* (main potential vector for West Nile). Previous works showed a good catching ability of this trap for both species (Guidi 2017b; Wipf & Guidi *et al.* 2019). Each BOX trap was supplied with a honey-soaked FTA card (prepared as described in section 3.2.2) (Figure 1) and run three times for two consecutive weeks using lead acid 6V, 60Ah batteries. The sampling started on the 9th August 2019 and finished on the 27th September 2019 following the scheme presented in Table 1. During the last sampling round, the BOX located at the border post Castasegna, Bregaglia, was found tampered with. Hence, for the two locations in Bregaglia a GAT coupled with a FTA card (Figure 1) was also placed in parallel at a distance of about 2m during the last

sampling round. Throughout the sampling period, a total of 62 traps (60 BOX and 2 GAT) coupled with FTA cards were used.

Table 1. Sampling scheme of the FTA cards based arbovirus surveillance in 2019. For each location and sampling round, the trap and the FTA card numbers are reported.

SAMPLING LOCATION	ROUND 1	ROUND 2	ROUND 3
LUGANESE	**13.08 - 27.08.2019**	**27.08 - 10.09.2019**	**10.09 - 24.09.2019**
Via Norello 1, 6928 Manno	*MAN1-1, FTA1*	*MAN1-2, FTA21*	*MAN1-3, FTA41*
Via ai Boschetti 9, 6928 Manno	*MAN2-1, FTA2*	*MAN2-2, FTA22*	*MAN2-3, FTA42*
Via Belvedere 2, 6942Savosa	*SAV1-1, FTA3*	*SAV1-2, FTA23*	*SAV1-3, FTA43*
Via Bruglino (cemetery), 6942 Savosa	*SAV2-1, FTA4*	*SAV2-2, FTA24*	*SAV2-3, FTA44*
MENDRISIOTTO	**13.08 - 27.08.2019**	**27.08 - 10.09.2019**	**10.09 - 24.09.2019**
via Poma 45, 6832 Seseglio (Chiasso)	*CHI1-1, FTA5*	*CHI1-2, FTA25*	*CHI1-3, FTA45*
Via Como 7, 6832 Chiasso	*CHI2-1, FTA6*	*CHI2-2, FTA26*	*CHI2-3, FTA46*
Via Marcetto 33, 6883 Novazzano	*NOVA1-1, FTA7*	*NOVA1-2, FTA27*	*NOVA1-3, FTA47*
Via Mulini 31, 6883 Novazzano	*NOVA2-1, FTA8*	*NOVA2-2, FTA28*	*NOVA2-3, FTA48*
LOCARNESE	**14.08 - 28.08.2019**	**28.08 - 11.09.2019**	**11.09 - 25.09.2019**
Via Pentima 22a, 6596 Gordola	*GOR1-1, FTA9*	*GOR1-2, FTA29*	*GOR1-3, FTA49*
BELLINZONESE	**13.08 - 27.08.2019**	**27.08 - 10.09.2019**	**10.09 - 24.09.2019**
Via Portaccia 7b, 6500 Bellinzona	*BEL1-1, FTA10*	*BEL1-2, FTA30*	*BEL1-3, FTA50*
MESOLCINA	**13.08 - 27.08.2019**	**27.08 - 10.09.2019**	**11.09 - 25.09.2019**
In Rugn 9, 6535 Roveredo	*ROV1-1, FTA11*	*ROV1-2, FTA31*	*ROV1-3, FTA51*
Strada de Carasoo 189, 6535 Roveredo	*ROV2-1, FTA12*	*ROV2-2, FTA32*	*ROV2-3, FTA52*
Strada Cantonale 182, 6534 San Vittore	*SANV1-1, FTA13*	*SANV1-2, FTA33*	*SANV1-3, FTA53*
Via Monticello 14, 6533 Lumino	*SANV2-1, FTA14*	*SANV2-2, FTA34*	*SANV2-3, FTA54*
Piazza Stazione 7, 6537 Grono	*GRO1-1, FTA15*	*GRO1-2, FTA35*	*GRO1-3, FTA55*
Via al Mot 7, 6537 Grono	*GRO2-1, FTA16*	*GRO2-2, FTA36*	*GRO2-3, FTA56*
BREGAGLIA	**09.08 - 23.08.2019**	**23.08 - 06.09.2019**	**6.09 - 20.09.2019**
Strada Cantonale 4, 7608 Bregaglia	*CAS1-1, FTA17*	*CAS1-2, FTA37*	*CAS1-3, FTA57* *CAS3-3, FTA61**
Via Principale 327, 7608 Bregaglia	*CAS2-1, FTA18*	*CAS2-2, FTA38*	*CAS2-3, FTA58* *CAS4-3, FTA62**
POSCHIAVO	**19.08 - 30.08.19**	**30.08 - 13.09.2019**	**13.09 - 27.09.2019**
Via Li Canvi, 7743 Brusio	*BRU1-1, FTA19*	*BRU1-2, FTA39*	*BRU1-3, FTA59*
Via Ca Zur 93, 7744 Brusio	*BRU2-1, FTA20*	*BRU2-2, FTA40*	*BRU2-3, FTA60*

*GAT (BG-Gravid *Aedes* traps).

At time of collection, BOX and GAT traps were transported to the laboratory and the collection chamber was placed into dry ice for 2 minutes prior to removal and counting of adult mosquitoes. Each FTA card was carefully removed from the plastic sleeve using forceps and placed into a plastic bag at -20°C until further analysis. The identification of mosquito species and the evaluation of their feeding on the FTA card (based on the blue color) (Figure 2) were not a main goal. Since the traps remained for two consecutive weeks in the field, some dead mosquitoes were difficult to identify and the observation of the blue color was not reliable (color digested, some specimens in a bad state). However, as far as possible, we identified captured individuals to species level and we checked whether at least some mosquitoes in the trap had a blue coloration.

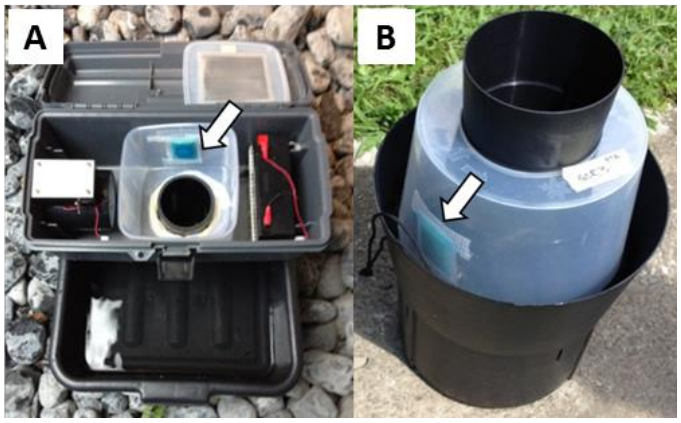

Figure 1. Types of traps used in the present study in association with FTA cards. A) Box-Gravid Mosquito trap (BOX) (BioQuip Products, USA) B) BG-Gravid *Aedes* trap (GAT) (Biogents AG, Germany). The position of the FTA card in each trap type is shown (arrow). (Pictures: Nadja Wipf, 2016)

Figure 2. Blue coloration of *Aedes albopicuts* (left) and *Culex* spp. (right) observed under the binocular. Figures are not in scale. (Pictures: Michela Ruinelli, 2018).

3.2.2 FTA cards preparation, elution and extraction of RNA

Preparation of FTA cards was performed one day prior to placement of the traps in the field as described elsewhere (Wipf 2016, Wipf & Guidi 2019). Few drops of food blue dye were added into a plastic box filled with honey (possibly bio) and mixed thoroughly with a spoon. Each FTA™ classic card (GE Healthcare Life Sciences, Cat. No. WB120305) was cut into four pieces and placed on blue colored honey overnight, with the matrix side facing down. The following day residual blue colored honey was removed from the FTA matrix side using a spatula. Each FTA card quarter was placed on a sponge of the same size and inserted into dedicated plastic sleeves with the matrix side exposed to the opened side. Afterwards, few milliliters of blue colored water were added to the sponge in order to keep the humidity on the FTA card over the sampling period. The so prepared FTA cards were attached in the inner side of the collecting chamber of the trap as shown in Figure 1. For elution, FTA cards were placed into a RNase-free 5 ml microcentrifuge tube and 10 µl of Mengo Virus (1:10 dilution of stock solution of inactivated Mengo virus vMC0 in HeLa cells) were added onto the matrix side of the FTA card as internal extraction control. After addition of 1 ml of FTA purification reagent (Sigma, WHAWB120204), the tube was vortexed for 15 seconds, placed on ice horizontally and shaken at 250 rpm for 20 minutes. Subsequently, the liquid was transferred from the 5 ml tube to a 1.5 ml Eppendorf. The eluate was stored at -80°C until RNA extraction, which was performed on 560 µl of eluate using the QIAamp Viral RNA Mini Kit (QIAGEN, Cat. No. 52906) following the instruction protocol. After extraction, RNA was stored at -80°C.

3.2.3 Extraction of RNA from mosquitoes

Viral RNA was extracted from mosquitoes retrieved from traps gaveling a positive results for FTA cards. Mosquitoes, spiked with 10 µl of a Mengovirus solution as extraction and inhibition control, were homogenized in QIAzol lysis solution (Qiagen, Switzerland) using a TissueLyser system (Qiagen,

Switzerland) and the upper aqueous phase used for RNA extraction with the commercial kit RNeasy Plus Universal kit (Qiagen, Switzerland) with elution in 2 x 30 µl RNase-free water. After extraction, RNA was stored at -80°C until real-time PCR analysis.

3.2.4 Molecular Analyses

RNA templates FTA cards were analysed by a real-time PCR system targeting the Mengovirus (extraction and inhibition control) (see 3.2.2), as well as with a nested PCR for the detection of alphaviruses (PanAlpha PCR) and a semi-nested endpoint PCR for the detection of flaviviruses (PanFlavi PCR) with subsequent sequencing of the amplified product for identification. Positive samples were also confirmed by specific real-time PCR on RNA extracts from the FTA card as well as from the mosquitoes retrieved from the corresponding trap.

Mengovirus Real-time PCR. Each reaction consisted of a 20 µl-solution containing 5 µl TaqMan® Fast Virus 1-Step Mastermix (Life Technologies), 0.4 µM of the Mengo F2 and Mengo R2 forward and reverse primers, 0.25 µM of Mengo P1 probe (Table 2), and 4 µl of template RNA. The real-time PCR reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems), with the following thermal cycling conditions: 5 min at 50°C, 20 sec at 95°C followed by 45 cycles of 3 sec at 95°C and 30 sec at 60°C.

Nested endpoint PCR for alphaviruses. The PanAlpha RT-PCR was performed in a Veriti AB Prism instrument (Applied Biosystems) in a total volume of 25 µl containing 5 µl 5x OneStep RT-PCR Buffer, 1 µl OneStep RT-PCR Enzyme Mix (Qiagen), 0.6 µM of the Alpha 1+ and Alpha 1- forward and reverse primers (Table 2), 400 µM of dNTP, and 5 µl of template RNA. Thermal cycling conditions were 30 min at 50°C, 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1min at 50°C and 1min at 72°C, followed by a final extension step at 72°C for 10 min. The nested PCR amplifications were carried out in a 20 µl-reaction containing 10 µl Fast Cycling PCR Mastermix 2x (Qiagen), 0.5 µM of the Alpha 2+ and Alpha 2- forward and reverse primers (Table 2), and 1.5 µl of the Alpha 1+/ Alpha 1- PCR product. The thermal cycling conditions were 5 min at 95°C, followed by 45 cycles of 5 sec at 96°C, 5 sec at 49°C and 30 sec at 68°C, followed by a final extension step at 72°C for 1 min. Amplification products were analysed by gel electrophoresis using 1.2% agarose gel stained with GelRed (Biotium).

Semi-nested endpoint PCR for flaviviruses. All mosquito pools and FTA cards were also analysed by semi-nested endpoint PCRs for the detection of flaviviruses (PanFlavi PCR) with subsequent sequencing of the amplified product. Reactions consisted of a 25 µl-solution containing 10 µl 2.5x OneStep *Ahead* RT-PCR Master Mix, 1 µl 25x OneStep *Ahead* RT-Mix (Qiagen), 0.5 µM of the MAMD and cFD2 forward and reverse primers (Table 2), and 5 µl of template RNA. The PCR reactions were performed in a Veriti AB Prism instrument (Applied Biosystems), with the following thermal cycling conditions: 10 min at 50°C, 5 min at 95°C followed by 40 cycles of 10 sec at 95°C, 10 sec at 50°C and 10 sec at 72°C, followed by a final extension step at 72°C for 2 min. One and a half µl of the 100-fold diluted PCR product was subsequently used as template for the semi-nested PCR. The 20 µl-reaction contained 10 µl Fast Cycling

Mastermix (Qiagen), 0.5 µM of the FS778 and cFD2 forward and reverse primers (Table 2), and 1.5 µl of PCR product. The reactions were carried out in a Veriti AB Prism instrument (Applied Biosystems). The thermal cycling conditions were 5 min at 95°C, followed by 45 cycles of 5 sec at 96°C, 5 sec at 50°C and 30 sec at 68°C, followed by a final extension step at 72°C for 1 min. Amplification products were analysed by gel electrophoresis using 1.2% agarose gel stained with GelRed (Biotium).

Sequencing. PCR products were purified using Sephadex® G-100 (Sigma) columns by centrifugation at 770 *xg* for 3 min. The sequence reactions were performed in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The reactions consisted of a final volume of 10 µl containing 1 µl BigDye Terminator Ready Reaction Mix, 1.5 µl of 5x BigDye Buffer, 2 µl of either forward or reverse primer, and 3 to 10 ng DNA. The temperature profiles were as follow: 1 min at 96°C, followed by 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Sequencing products were subsequently purified using Sephadex® G-50 (Sigma) columns by centrifugation at 770 *xg* for 3 min. and mixed with 5 µl of HiDi Formamide (Applied Biosystems) and resolved on an Applied Biosystems 3500 Genetic Analyser (Applied Biosystems). Sequences were analysed using the Mega6 Software and compared to BLAST Nucleotide database.

Usutu virus Real-time PCR. Positive samples were analysed by a real-time PCR system targeting the Usutu virus RNA. Usutu virus PCR was performed with the same reagents volumes and conditions as for Mengovirus PCR, using the primers and probe described in Table 2.

Table 2. Overview of the PCR methods used for detection of arboviruses in FTA cards RNA extracts

		Oligonucleotides	Reference
PanAlpha target gene *nsP4*	RT-PCR	**Alpha1+**: 5' GAYGCITAYYTIGAYATGGTIGAIGG 3' **Alpha1-**: 5' KYTCYTCIGTRTGYTTIGTICCIGG T 3'	Sánchez-Seco *et al.* 2001
	Nested PCR	**Alpha2+**: 5' GIAAYTGYAAYGTIACICARATG 3' **Alpha2-**: 5' GCRAAIARIGCIGCIGCYTYIGGICC 3'	
PanFlavi target gene NS5	RT-PCR	**MAMD**: 5' AAC ATG ATG GGR AAR AGR GAR AA 3' **cFD2**: 5' GTG TCC CAG CCG GCG GTG TCA TCA GC 3'	Scaramozzino *et al*. 2001
	SemiNested PCR	**FS778**: 5' AAR GGH AGY MCD GCH ATH TGG T 3' **cFD2**: 5' GTG TCC CAG CCG GCG GTG TCA TCA GC 3'	
Mengovirus	Real time PCR	**Mengo F2:** 5'- TGCCAACCCAAAACCACAT -3' **Mengo R2:** 5'- ACGCACACCGCCTTATTC -3' **Mengo P1:** 5'- (FAM) CTCACATTACTGGCCGAAGCCGCT (BHQ1) -3'	Labor Spiez in-house PCR, C. Beuret.
Usutu	Real time PCR	**UsuE F2:** 5'- CAAGCTCACATCTGGTCATCTCA -3' **UsuE R2:** 5'- GTCAGCCGGATTTTTTGCAA -3' **UsuE P2:** 5'- (FAM)-AAGTTGACACTAAAAGGCACCACCTACGGC-(BHQ1) -3'	Labor Spiez in-house PCR, C. Beuret.

4 Results and discussion

4.1 Part I: Risk assessment

The estimation of the potential risk of an outbreak of arboviral diseases was carried out in 2017. *Aedes albopictus* females were sampled in six urban localities within the Locarnese and Luganese districts by 6 different trained biologists by HLC, allowing to calculate the mean number of bites per human per day. The number of host-seeking females derived from the HLC and the number of eggs retrieved from ovitraps could not be correlated (Spearmann correlation, rho = 0.54, p = 0.26), in contrast to what observed in northern Italy (Carreri *et al.* 2012). Therefore, the risk of outbreak in case of introduction of chikungunya, dengue, and Zika viruses throughout viremic travellers could not be estimated for the whole mosquito season using ovitrap data of the different round checks but only for the period of the study using HLC data. Table 3 shows the basic reproduction numbers R_0 calculated for the six localities included in the study. Only Agno and Magliaso presented a low to moderate risk for mutated chikungunya, dengue and the Uganda Zika lineage. In all the other localities, the risk for epidemics resulted to be absent, with possibly only isolated cases (Table 3). The tiger mosquito is a sedentary species, flying actively over short distances from its breeding site (about 50-200m) (Marini *et al.* 2010). Therefore, the high abundance of adults is relied to the proximity of important breeding hotspots. Indeed, during HLC evident *Ae. albopictus* breeding hotspots were observed near the sampling sites both in Agno and in Magliaso. Those hotspots were not removed for scientific correctness. Highly productive breeding sites are usually found on private lands and their presence is not homogenous within the territory, depending a lot on the effectiveness of interventions by citizens. Results obtained in the present study indicate that the risk for epidemic exist mainly in presence of important breeding hotspots.

Table 3. Reproduction numbers (R0) estimated for different viruses in the six localities included in the study.

		Manno	Agno	Magliaso	Cugnasco-Gerra	Tenero-Contra	Gordola
Chik	Lower IC95%	0.05	0.37	0.40	0.01	0.04	0.04
	Medium	0.08	0.76	0.61	0.07	0.09	0.07
	Upper IC95%	0.12	1.16	0.82	0.12	0.15	0.10
Chik A226V	Lower IC95%	0.16	1.22	1.32	0.05	0.13	0.15
	Medium	0.28	2.54	2.02	0.22	0.31	0.25
	Upper IC95%	0.41	3.87	2.72	0.39	0.48	0.35
Den2	Lower IC95%	0.12	0.95	1.03	0.04	0.10	0.11
	Medium	0.22	1.98	1.57	0.17	0.23	0.19
	Upper IC95%	0.32	3.01	2.12	0.29	0.36	0.26
Zika African	Lower IC95%	0.12	0.94	1.02	0.04	0.10	0.11
	Medium	0.22	1.97	1.57	0.17	0.23	0.19
	Upper IC95%	0.32	3.00	2.11	0.30	0.37	0.26
Zika Asian	Lower IC95%	0.05	0.38	0.41	0.01	0.04	0.04
	Medium	0.09	0.78	0.62	0.07	0.09	0.07
	Upper IC95%	0.13	1.19	0.84	0.12	0.15	0.10

$R_0<1$
$1\leq R_0<2$
$2\leq R_0<3$
$R_0\geq 3$

Results of this risk assessment are reported and discussed in detail in the first intermediate report (Guidi, 2017a).

4.2 Part II: Arbovirus surveillance

4.2.1 Surveillance 2018

The arbovirus surveillance in 2018 was performed in Canton Ticino in six municipalities within the districts of Mendrisiotto, Bellinzonese and Locarnese, using FTA cards associated with ovitraps, GAT and BOX traps. A total of 75 traps supplied with an honey-soaked FTA card were used over the surveillance period from end of July to end of September. No alphaviruses or flaviviruses were detectable in the selected locations during the surveillance period.

Results obtained in 2018 are reported and discussed in detail in the second intermediate report (Ruinelli, 2018).

4.2.2 Surveillance 2019

The circulation of arboviruses was checked for 2019 in 11 municipalities of Cantons Ticino and Graubünden using mainly BOX traps coupled with FTA honey-baited cards, for a total of 62 traps.

For arbovirus surveillance, mosquito traps placed in the field are usually ran for 24 hours and collected mosquitoes are successively screened for viruses. In our study each trap with an FTA card was left in the field for two consecutive weeks, increasing the number of mosquitoes caught per trap and thus the possibility to find viruses. The screening for viruses on FTA cards rather than on mosquito pools allow decreasing the number of samples to be analysed. In order to let BOX traps to run for two consecutive weeks, the usual battery supplied with the trap was substituted with a lead acid 6V, 60Ah battery. Despite the trap operation time with this battery should have been of 28 days (according to the manufacturer), during our surveillance some traps were unfortunately found with the fan off, with a consequent trapping period of less than two weeks.

A total of 2’480 mosquitoes were sampled using BOX traps and had possibly fed on FTA cards. No mosquitoes were found in three BOX (SANV1-3, GRO1-3, and GRO2-3) and in the two GAT placed in Bregaglia. Since GAT are passive traps, mosquitoes could have escaped from the traps. It is therefore not possible to know if some mosquitoes fed on FTA cards placed inside the GAT translucent chamber. Table 4 report the numbers and species of mosquitoes retrieved from BOX traps. The highest number of mosquitoes collected belonged to the tiger mosquito species (N=1622), followed by *Culex* species (N=617).

In order to know whether mosquitoes fed on the FTA cards and possibly released viruses, the blue coloration of specimens (Figure 2) was also checked, despite this was not evaluated for every single

specimen. Only in six traps (GRO1-1, CAS1-1, NOVA2-2, ROV2-2, SANV2-2, and CAS2-2) any specimens were not blue-colored or this was not visible.

Table 3. Number and species of mosquitoes collected with BOX traps during the arbovirus surveillance in 2019

	#Traps	*Ae. albopictus*	*Ae. japonicus*	*Ae. koreicus*	*Culex* sp.	*Other* sp.**	*Total # mosquitoes*
Luganese	*12*						
Females		243	1	2	129	0	375
Males		148	0	0	6	0	154
Unknown*		316	13	1	70	0	400
Total		*707*	*14*	*3*	*205*	*0*	*929*
Mendrisiotto	*12*						
Females		183	4	7	51	0	245
Males		49	0	0	16	0	65
Unknown*		145	24	3	153	1	326
Total		*377*	*28*	*10*	*220*	*1*	*636*
Locarnese	*3*						
Females		65	1	0	6	0	72
Males		19	0	0	2	0	21
Unknown*		9	0	0	10	0	19
Total		*93*	*1*	*0*	*18*	*0*	*112*
Bellinzonese	*3*						
Females		184	0	0	0	0	184
Males		14	0	0	0	0	14
Unknown*		0	9	0	0	11	20
Total		*198*	*9*	*0*	*0*	*11*	*218*
Mesolcina	*18*						
Females		127	29	1	46	15	218
Males		13	0	0	9	0	22
Unknown*		97	27	0	102	7	233
Total		*237*	*56*	*1*	*157*	*22*	*473*
Bregaglia	*6*						
Females		2	28	6	5	0	41
Males		0	0	0	1	0	1
Unknown*		3	30	3	1	3	40
Total		*5*	*58*	*9*	*7*	*3*	*82*
Poschiavo	*6*						
Females		2	0	5	4	0	11
Males		0	0	0	4	0	4
Unknown*		3	0	9	2	1	15
Total		*5*	*0*	*14*	*10*	*1*	*30*
TOTAL		***1622***	***166***	***37***	***617***	***38***	***2480***

* The sex identification was not possible since specimens were too damaged (missing antennas)
**Mosquito species that could not be morphologically identified.

Every FTA card was screened for flaviviruses and alphaviruses. Out of the 62 FTA cards analyzed, only one (FTA27) was positive for a flavivirus, that was further identified as Usutu virus by both sequencing and specific real-time PCR. Mosquitoes retrieved from the trap with the positive FTA card, which was located in Novazzano (NOVA1-2), were also analyzed and confirmed to be positive for the same arbovirus. It is not possible to know how many and which species of mosquitoes were infected with Usutu virus. In fact, despite mosquitoes were analyzed in pools sorted by species and sex, every pool gave a positive signal for Usutu. The viral concentration was however much higher in the pool consisting in *Culex* mosquitoes (39 specimens of unknown sex and one male). In past years, mosquitoes sampled in Novazzano and other

localities within the Mendrisiotto district, as well as in the Magadino plain (Locarnese) were already found positive for Usutu virus (Wipf & Guidi *et al.* 2019; Engler et al., 2013). This virus is closely related to the West Nile virus and the Japanese encephalitis virus, and spreads through mosquitoes and infected birds (Ashraf *et al.* 2015). At present, Usutu virus is considered endemic in several European countries (Gaibani and Rossini 2017). Usually pathogenic for birds (mainly blackbirds), it can cause neuroinvasive infections in immunocompromised patients (Vazquez et al., 2011). The first human cases in Europe have been reported in Italy in 2009 (Pecorari *et al.* 2009; Cavrini *et al.* 2009). Reports of human infections in several European countries are on the rise (e.g. Vilibic-Cavlek *et al.* 2014; Simonin *et al.* 2016; Grottola *et al.* 2017; Aberle *et al.* 2018; Nagy *et al.* 2019), while remaining relatively low and mostly asymptomatic (Roesch *et al.* 2019).

Results of the real-time PCR system on Mengovirus RNA was satisfactory for all samples, indicating successful RNA extractions.

5 Deviations from original research plan

The applicability of the FTA cards for arbovirus surveillance was assessed in previous studies (Wipf & Guidi *et al.* 2019; Hall-Mendelin *et al*. 2010). This project aimed at implementing the utilization of FTA cards for large scale monitoring in combination with ovitraps, which are routinely used for monitoring *Ae. albopictus* population in Ticino. During the 2018 surveillance, the choice of oviposition traps in combination with FTA cards was not considered as optimal. In fact, FTA cards placed in ovitraps are not protected from rain and other environmental factors, influencing a lot the choice of the trap positioning (sheltered locations). This could have reduced the number of females attracted by the trap. Moreover, ovitraps are optimised for the monitoring of *Aedes* mosquitoes in urban areas, which are not the unique potential vector in our Country. Considering the presence of West Nile virus transmitted by *Culex* spp. in Northern Italy and the increase of human and equine cases in 2018 (ECDC, https://www.ecdc.europa.eu), we decided to use for the 2019 surveillance a different trap type coupled with FTA cards. In previous work we found that the Box-Gravid trap collected the highest variety of species, with *Aedes albopictus* and *Culex pipiens* being the most abundant (Guidi 2017b).

To expand the surveilled area and increase the chance to find arboviruses, in particular West Nile virus, near the border with Italy, during the 2019 surveillance we also decided to include the Canton of Graubünden.

6 Conclusions

The research project carried out from 2017 to 2019 comprised a risk assessment analysis for arbovirus transmission in Canton Ticino and a two-year surveillance using mosquito traps coupled with FTA cards.

In 2017 we wanted to assess the potential risk of an outbreak of arboviral diseases in Canton Ticino where the vector *Ae. albopictus* is established. The *Ae. albopictus* population density in six urban areas of Canton Ticino between end of August-beginning of September was evaluated both by mean number of *Ae. albopictus* bites/human/day (estimated by human landing collection) and by the number of eggs laid by female mosquitoes during a 14 days period. Unfortunately, we could not find any correlation between these two indices and thus the risk of outbreak in case of introduction of chikungunya, dengue, or Zika viruses could be estimated only for the period of the study using HLC data. We could assess that in case of introduction of mutated chikungunya, dengue (serotype 2), or Zika (african lineage) during that period, an epidemic could have occurred in the municipalities of Agno and Magliaso. This study served as a pilot for a risk assessment carried out in 2018 over the whole mosquito season (mid-May to end of September) (Ravasi *et al.* 2019).

In 2018, the conducted surveillance did not reveal presence of arboviruses (flaviviruses or alphaviruses) in the selected municipalities over the surveillance period. In contrast, an Usutu virus positive trap was found in Novazzano during the second trapping round in 2019 (from 27th August to 10th September 2019). In the study areas, no other relevant arbovirus was found. The finding of Usutu confirmed again the applicability of the FTA card method for arbovirus surveillance.

Due to the presence of autochthonous transmission of arboviruses in neighboring countries, it would be of putative importance to continue the surveillance and, ideally, to expand it on a larger scale including also other cantons, especially those close to the borders with Italy and France, which were affected in the past by outbreaks of mosquitoes borne diseases.

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