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Mosquito-Borne Diseases and 'One Health': The Northwestern Italian Experience

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Abstract

In Italy, the surveillance of Mosquito-Borne Diseases (MBDs) is regulated by two national preparedness plans: (1) for West Nile and Usutu viruses, integrating human and veterinary surveillance in order to early detect viruses circulation and to quickly apply control measures aimed at reducing the risk of transmission through blood and blood components and (2) for Arbovirosis transmitted by *Aedes* mosquitoes, mainly Chikungunya, Dengue and Zika viruses, based on surveillance of both imported and autochthonous human cases. This chapter reports the results of the application of these National Plans in Northwestern Italy and their impact for human health. In detail, we present the coordinated activities enforced in Piemonte and Liguria Regions, as a good example of the 'One Health approach' to control MBDs and prevent human transmission.

Keywords: mosquito-borne diseases, Italy, one health

1. Introduction

Mosquito-borne diseases (MBDs) are dangerously increasing in prevalence, geographical distribution and severity, representing a worldwide emerging threat for both humans and animals. Of great concern are the infections caused by viruses belonging to the *Flavivirus*

genus. This genus includes viruses considered endemic in Italy as West Nile Virus (WNV) and Usutu virus (USUV) transmitted by *Culex* sp. and viruses as Dengue virus (DENV) and Zika virus (ZIKV), transmitted by *Aedes* sp., not endemic in Italy but with the potential to spread to new areas where the mosquito vector is present.

Furthermore, in the last 10 years (2007/2017), two autochthonous Chikungunya virus outbreaks (CHIKV_ *Alphavirus* genus, family *Togaviridae* transmitted by *Aedes* sp.) with hundreds of cases due to an imported case followed by local vectorial transmission occurred in Italy, showing the impact that this virus could have even in a non-endemic country [1, 2].

Given the complex epidemiology of MBDs, Public Health Authorities are encouraging the interaction between multiple disciplines to implement an effective early warning.

WNV and USUV are antigenically close flavivirus with similar enzootic birds-mosquitoes transmission cycle where co-circulation has been reported in several studies [3–6].

WNV can be occasionally transmitted, through mosquito bites, to vertebrates other than birds as humans and horses that are considered dead-end hosts. Human infection through blood transfusion and solid organ transplantation is also demonstrated [7].

WNV risk for human health is well recognized: the majority (*80%) of infections in humans are asymptomatic, and symptomatic infections are mostly characterized by a mild, self-limiting febrile illness. WNV neuroinvasive disease develops in <1% of WNV-infected persons [8].

USUV is generally correlated with high mortality rates in its bird reservoirs. Mosquitoes infected with USUV can incidentally transmit the virus to other vertebrates, including humans, which can result in neuroinvasive disease [9].

In Italy, an increasing number of outbreaks of West Nile disease, with occurrences of human cases, have been reported since 2008, mainly in the North East part of the Country.

WNV lineage 1 (Lin1) was the only strain detected until 2011 when, for the first time, the presence of both WNV Lin1 and WNV lineage 2 (Lin2) was demonstrated. Since 2013, WNV Lin2 was the main strain detected, and a west bound spread of the virus started [8].

USUV has been detected in Italy since 2007 in mosquitoes, birds and humans [10–13].

Although characterized by a lower pathogenicity than WNV, USUV pathogenic potential for human is not completely characterized, and knowledge about this crucial aspect is constantly evolving [14–16]. In 2009, two cases of encephalitis in immunocompromised patients were reported in Italy [10, 12, 17], and additional USUV neuroinvasive infections in humans were described in Croatia in 2013 even in one healthy patient [18].

Finally, retrospective analyses to monitor the spread and to evaluate the role for public health of USUV in an endemic area conducted in the city of Modena showed a high seroprevalence in humans with or without neurologic impairments, underlying that USUV infection in humans should not be considered a sporadic event [16].

DENV and ZIKV are characterized by a human-to-mosquito-to-human cycle of transmission.

DENV is the most prevalent arthropod-borne viral disease in tropical and subtropical countries. The disease manifestations range from an influenza-like disease known as dengue fever

to a severe, sometimes fatal disease characterized by haemorrhage and shock, known as dengue haemorrhagic fever/dengue shock syndrome [19].

The classic clinical picture of ZIKV infection resembles that of dengue fever and is manifested by fever, headache, arthralgia, myalgia, conjunctivitis and maculopapular rash. Recently, a possible association between ZIKV infection in pregnancy and fetal malformations has been hypothesized [20].

Up to date, all the DENV and ZIKV human cases reported in Italy have been related only with returning travellers from endemic countries and not associated with transmission through local potentially competent vectors [21].

However, if vectors are present, infected returning travellers could initiate a local virus transmission as in the CHIKV outbreak occurred in the Emilia-Romagna Region, Italy, in 2007 [22].

CHIKV is characterized by a human-to-mosquito-to-human cycle of transmission.

Clinical onset is abrupt, with high fever, headache, back pain, myalgia and arthralgia; the latter can be intense, affecting mainly the extremities (ankles, wrists, phalanges) but also the large joints. Skin involvement is present in about 40–50% of cases and consists of a pruriginous maculopapular rash predominating on the thorax. The symptoms generally resolve within 7–10 days, except for joint stiffness and pain: up to 12% of patients still have chronic arthralgia, 3 years after onset of the illness [23].

Italy experienced two CHIKV autochthonous outbreaks, in Emilia Romagna Region in 2007 and in Lazio Region in 2017, due to returning infected travellers during the vectors' season with hundreds of cases.

Given the complex epidemiology of MBDs, determined by the interaction between pathogens, hosts, vectors and ecosystem, the cooperation of multiple disciplines (veterinarians, epidemiologists, entomologists, biologists and doctors) is needed for an effective early warning, surveillance and control [24]. A deep knowledge is required to better perform the surveillance plans: viruses with different cycles need different surveillance approaches. The syndromic surveillance on human cases could be considered more adequate to detect the introduction of new viruses in which humans are the reservoir. However, the entomological surveillance can be a valuable additional tool, considering that the presence of risk factors as competent vectors and suitable climatic conditions significantly increases the potential risk for the possible autochthonous transmission of MBDs.

1.1. West Nile virus national surveillance programme: origin and evolution towards an integrated system

Among MBDs, since almost 20 years, surveillance activities and control measures against WNV represent a deal both in human and in veterinary medicine, due to its complicated transmission cycle, the zoonotic potential and the risk of human-to-human transmission mainly through blood transfusion and organ transplantation.

In Italy, the WNV circulation was firstly detected in the late summer of 1998, in horses that displayed neurological clinical symptoms in a wetland area located in Toscana Region (West-central Italy); no cases of human encephalitis were reported [25].

After this outbreak, a national veterinary surveillance plan was implemented in 2001 under the coordination of the Italian Ministry of Health (MoH) and of the National Reference Centre for Exotic Diseases of Animals (Centro Studi Malattie Esotiche; CESME), with the aim to early detect new incursions of WNV. Throughout the whole Italian territory, 15 risk wetland areas were identified, considering the presence of migrating birds as a possible risk of virus introduction. During the transmission season, active surveillance was conducted by the periodic serological screening of sentinel-chicken flocks and sentinel horses, in order to detect antibodies against WNV. Moreover, passive surveillance was conducted by virological screening of carcasses of dead birds and by clinical surveillance of neurological signs in equines all over the year [26].

In 2008, after 10 years of disappearance, a large WNV epidemic affected regions in the Northeast of Italy surrounding the Po river Delta. The virus was identified in mosquitoes, birds, horses and humans.

Following these events, the surveillance system was updated with the aim to detect as early as possible the WNV circulation. Three epidemiological areas were identified with different surveillance approach (**Figure 1**) [27]:

1. Area with Virus Circulation (AVC): areas of Northern Italy with WNV circulation during 2008. The aim of surveillance in this area was to evaluate WNV spread. Active surveillance was conducted by virological testing on synanthropic birds and/or by serological testing on sentinel-chicken flocks or free-range poultry flocks throughout the epidemiological season; entomological surveillance was enforced in 10 horse stables during the whole year with monthly mosquito trapping, in order to evaluate the role of mosquitoes as WNV vectors;
2. Surveillance Zone (SZ): areas surrounding the AVC for an extension of 20 km. The aim of surveillance in this area was to monitor WNV spread over the AVC. Active surveillance was conducted by virological testing on synanthropic and/or by serological testing on chickens or poultry flocks as enforced in AVC and by recruitment of sentinel horses for repeated serological tests in May, August and September in order to detect seroconversion against WNV. Entomological surveillance was enforced in three horse stables with monthly mosquito trapping throughout the year, in order to evaluate the role of mosquitoes as WNV vectors;
3. Areas at Risk (AR): 14 wetlands inherited from the previous surveillance plan. The aim of surveillance in this area was to detect WNV new introduction by migration routes. Active surveillance was conducted on synanthropic and chickens or poultry flocks as enforced as in AVC, and the same testing of sentinel horses as in SZ; entomological surveillance was enforced in one horse sentinel stable by monthly mosquito trapping during the epidemic season, in order to evaluate the composition of mosquito fauna.

Passive surveillance was carried throughout the year in the whole Italian area by the detection of neurological signs in equines and increased mortality among wild birds.

Since 2008, WNV has expanded to a wide geographical area in Europe and the Mediterranean basin, with an increasing number of outbreaks, including several human cases of West Nile Neuroinvasive Disease (WNND).

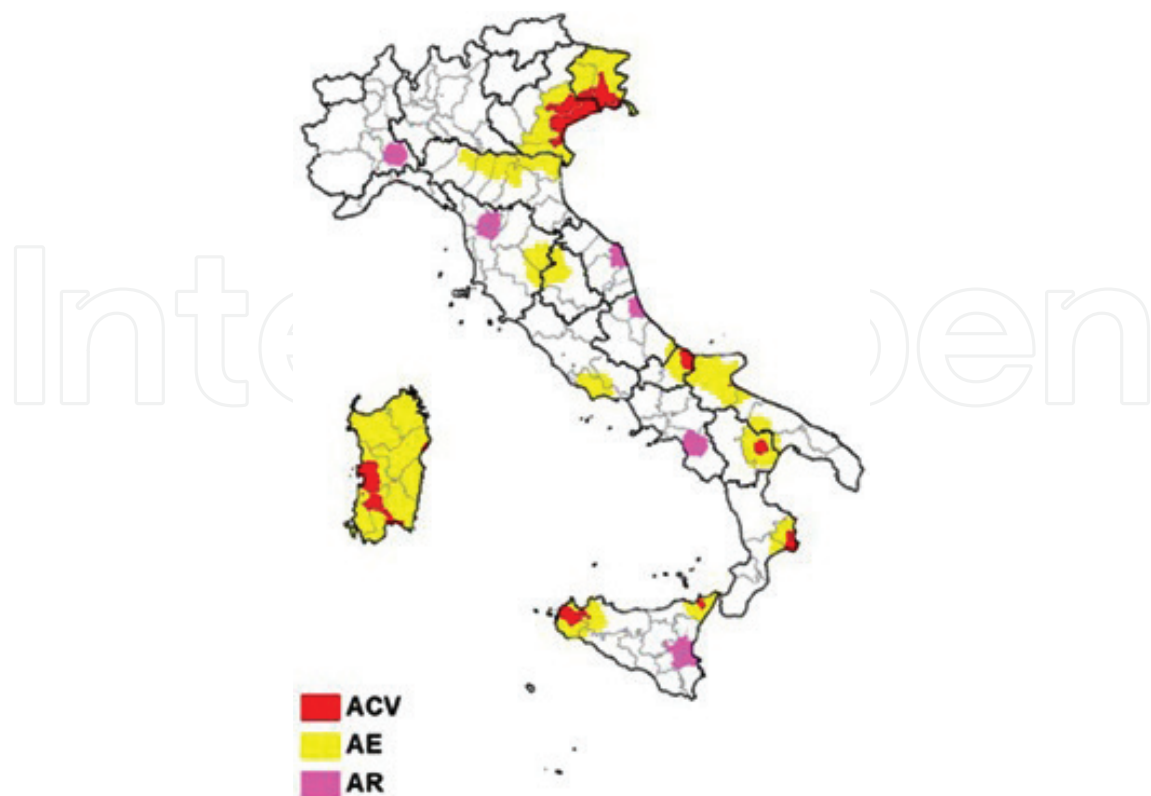


Figure 1. Map of Italy with WNV surveillance areas.

In Northern Italy, the virus has become endemic, progressively extending the range from the East to the West in the Po river valley area. The establishment of overwintering cycle was due to the presence of the main mosquito vector *Culex pipiens* at high density and the evidence of virus transmission in resident wild bird species susceptible to WNV infection [28].

In 2010, the MoH published a National Plan for WNND Human Surveillance in Italy, which refer to the WNV veterinary Surveillance Plan. This was the first step towards the adoption of a comprehensive human and veterinary (animals and vectors) surveillance system against WNV in Italy.

The national definition of WNND suspected human case included every patient presenting with fever $\geq 38.5^{\circ}\text{C}$ and neurological symptoms: encephalitis, meningitis or acute polyradiculoneuritis (Guillain-Barré syndrome) or acute flaccid paralysis. Every suspect case of WNV infection was promptly reported to the Public Health Department and laboratory investigated. Clinical suspected cases of WNND or West Nile Fever (WNF) were classified as confirmed cases if they fulfilled at least one of the following laboratory criteria: (1) isolation of WNV from blood or cerebrospinal fluid (CSF), (2) the presence of IgM antibodies in CSF by ELISA, (3) the detection of WNV RNA by RT-PCR in blood and/or CSF and (4) the detection of increased levels of IgM and IgG antibodies against WNV by neutralization testing [29].

Human or veterinary WNV confirmed cases have to be notified by the Regional authorities to the national level, both to the MoH and to the National Blood Center (NBC).

In line with the EU directive for blood safety (Directive 2004/33/EC, Annex III), from 2009 to 2014, the NBC implemented, as a preventive measure, a 28-day deferral period for blood

donor, leaving areas with ongoing transmission of WNV and WNV Nucleic Acid Test (NAT), testing of all donations (peripheral blood, bone marrow and cord blood stem cell donations) generally from July to November [30] and coming from Provinces in which human cases of WNND had been reported the previous year.

The NAT screening approach changed in 2015 for five Northern Italian Regions (Piemonte, Lombardy, Friuli Venezia Giulia, Veneto and Emilia-Romagna) concerned by WNV circulation in previous years. These regions guaranteed an active standardized integrated entomologic and veterinary WNV surveillance from June to October. In addition to the notification of human cases, the date detection of WNV in mosquitoes and wild birds or detection of WNV-IgM antibodies in horses was introduced as new trigger criteria for the implementation of WNV NAT testing for the screening of blood donors [31].

Since 2009, a total of 103 WNV positive samples in blood donors have been reported in Northern Italy (Veneto, Lombardia and Emilia-Romagna Regions).

Comprehensive epidemiological, virological and entomological surveillance system is crucial for the timely detection of the spread of WNV, implementation of control measures and prevention of virus transmission in humans. Both national WNV veterinary and human surveillance plan have undergone further revisions year by year, in order to adapt to the seasonal evolution of the epidemiological scenario.

In 2016, the MoH has released a National WNV veterinary-entomological-human integrated Surveillance Plan. According to 'One-Health' approach, real-time cross-sectorial collaboration by Public and Veterinary Health Institutions is crucial to timely achieve and share information in WNV surveillance as a key to improve the management of WNV outbreaks and mitigate the risk of human transmission.

The aim of the programme was to early detect WNV circulation, reducing the risk of infection in the human populations. The steps to achieve this goal are as follows:

1. Veterinary surveillance: the Italian territory was subdivided into two distinct epidemiological territories:
 - a. Endemic areas, where WNV was detected in the previous 2 years (mainly Regions of the Po river Valley, Sicilia e Sardegna islands): reinforcement of the virological screening in migratory and resident wild birds, mainly belonging to *Corvidae* genus. Alternatively, serological testing of rural poultry or sentinel chickens groups.
 - b. Non-endemic areas (the remaining Italian Regions): serological testing of horses sera, in order to detect WNV-specific IgM early antibodies (recent infection).
2. Entomological surveillance: active mosquitoes fortnightly trapping during the epidemic season. Sampling sites selection was made according to risk factors for WNV cycle establishment: proximity to wetland areas, the presence of hosts, previous outbreaks. Moreover, a standardized approach was suggested, mapping provinces into equal quadrants (20 x 20 km), with at least one mosquito trap per quadrant. The aim was to establish seasonal mosquito patterns and detect WNV in mosquito pools by virological screening.

3. Human surveillance: active identification of all possible, probable and confirmed WNV infection human cases, including WNND.

The integrated plan was updated in 2017, including activities to control USUV and assess the risk for public health (MoH) [32]. Indeed, in the last years in Europe and northern Italy, the co-circulation of WNV and USUV was largely demonstrated [4–5, 33]. The two viruses seem to show biological cycle similarities, but while the WNV risks for human health are well recognized, knowledge about the medical importance of USUV is not fully understood and the transmission risk throughout blood transfusion has been recently suggested [34, 35].

This underlined the need to put in place surveillance measures to detect USUV activity and to assess the risk for public health. As in northern Italy, USUV shows a substantial geographic overlap with WNV circulation, surveillance was enforced in WNV endemic areas, by routine differential diagnosis of all cases of WNV-positive test (serological/virological).

2. Human MBDs national surveillance plan

After the CHIKV outbreak in 2007 in Italy, a national plan on imported and autochthonous human vector-borne disease (including CHIKV, ZIKA, DENV and WNV) has been implemented and annually updated on the basis of the epidemiological changes-based evidences.

The plan has been annually revised to 2017 with minor changes except for the exclusion of WNV and USUV in 2016/2017 from the human plan, due to the birth of the WNV/USUV National integrated Plan.

Here, we summarize the main concepts.

Epidemiological surveillance of human cases.

The main objectives of human surveillance are the following:

- to monitor imported cases in Italy, in particular in areas where there are competent mosquitoes, for the assessment of the risk of possible autochthonous transmission of the virus;
- the early identification of outbreaks and the monitoring of local transmission in order to implement control measures (prevention and response activities);
- to prevent accidental transmission through blood or organs transfusion and to identify other potential transmission pathways (e.g. sexual).

The human surveillance is carried out throughout the year. However, during the period of a major vector activity (June–October), the surveillance system will have to be strengthened (in terms of timeliness and sensitivity) in mosquito-infested areas, to allow the identification of cases, for the immediate adoption of the necessary control measures (in relation to entomological surveillance) and to reduce the risk of transmission.

Therefore, from the beginning of June to the end of October, particular attention must be paid to:

- early identification of suspected cases (symptomatic cases returning from an endemic Country);
- identification of people with compatible clinical symptoms but who did not travel to endemic countries, to early detect autochthonous outbreaks (two or more cases occurred within 30 days in a restricted area).

The plan defines, in a given area, three situations with different risk levels depending on vectors presence and density and occurrence of human cases:

Area A: the vector is present/absence of human cases; Area B: the vector is present/one or more imported human cases; Area C: the vector is present/isolated autochthonous cases/outbreaks.

For each area, the actions to carry out during the vector season and the rest of the year are defined:

Area A

December–May and November

- no activities

June to October

- monitoring and treatment protocols, if already existing, following Regional or national legislation;
- prevention activities: health education, methods of vector control including the elimination or management of breeding sites, larviciding with insecticides, the use of biological agents avoiding the application of adulticides.

Area B

December–March

- no activities

April–May and November

In the presence of human cases (probable and confirmed) **depending on the seasonal climatic conditions**, the following activities must be activated:

- activation or enhancement of entomological surveillance around the house of the patient for at least 2 weeks from reporting;
- treatments on private and public land, in an area within a radius of 200 m around the positive house;
- elimination of breeding sites;

- adulticide treatment (1 cycle);
- treatment of non-removable breeding sites with larvicidal products;
- information to the inhabitants about preventive measures to apply to avoid contact with vectors;
- follow-up for the week following the alert.

Area C

December–March

- no activities

April–May and November

In the presence of human cases (probable and confirmed) **regardless of the seasonal climatic conditions**, the following activities must be activated:

- activation or enhancement of entomological surveillance around the house of the patient for all the vector season;
- treatments on private and public land, in an area within a radius of 200 m around the positive house;
- elimination of breeding sites;
- adulticide treatment (1 cycle);
- treatment of non-removable breeding sites with larvicidal products;
- information to the inhabitants about preventive measures to apply to avoid contact with vectors;
- replication of all interventions in case of rain or poor efficacy of the first treatment cycle;
- in case of outbreak, repeat the disinfestation protocol after the first week;
- follow-up for all the vector season.

Human cases are notified by Regional and local authorities to the MoH and to the Istituto Superiore di Sanità (ISS, national public health institute) using a specific password-protected web-based system, which permits to report probable and confirmed cases, adding available epidemiological (including the province of exposure), clinical and laboratory information. The web-based system is accessible also to the National Blood Center (NBC) and to the National Transplant Center (NTC), which in cases of WNV human cases will implement precautionary measures on blood donation and transplant activities.

Measures for human cases

In order to reduce the disease spread, the home isolation of the case is recommended up to the exclusion of the disease and, in any case, not beyond the time course of viremia. The adoption of protective measures against vector bites is essential to interrupt the transmission cycle.

Other family members and people should use general precautions for parenterally transmitted diseases, such as:

- wash hands with soap and water, before and after assisting the patient, and, in any case, after removing the gloves if used;
- use gloves, not sterile, if contact is made with the patient's blood;
- do not use sharp products used for patient care or assistance.

Risk communication

Risk communication, training, information and health education have a decisive role in obtaining people collaboration.

Since there are currently no vaccines and/or therapies for the prevention and treatment of diseases as Chikungunya and Dengue (authorized only in some endemic countries) and Zika, the most effective prevention is to reduce people exposure to mosquito bites.

Currently, therefore, the key message is: 'Protect yourself from mosquito bites' which includes both the active control of the vector (use of insecticides, reduction of breeding sites) and adoption of individual protection measures (clothes, mosquito nets, repellents and also preventive measures to avoid sexual transmission, e.g. for Zika virus).

Risk communication to travellers going to or returning from endemic areas is of primary importance:

1. People travelling to endemic areas or endemic countries should inform about the circulation of ongoing epidemics (consulting ECDC and WHO sites and the Safe Travel website of the Ministry of Foreign Affairs and International Cooperation) and protect themselves from mosquito bites.
2. People travelling from endemic areas or endemic countries who develop suspected symptoms within 2 weeks of returning home should seek medical attention.

3. Materials and methods

3.1. West Nile virus integrated surveillance system in Northwestern Italy

Up to 2011, according to the national WNV surveillance programme, just a small area in Piemonte, defined as a 'risk area', in the Provinces of Alessandria and Asti, had to be monitored by entomological surveillance and checking for WNV seroconversion in horses residing in the area. No active surveillance activities were planned in Liguria. Even if any animal nor human cases had not been reported before, geographical and environmental features were conducive to maintaining competent vectors for MBDs.

Since 2011, supplementary surveillance activities have been put in place to control MBDs, with particular attention to WNV.

3.1.1. Area under surveillance: Piemonte and Liguria regions

The study area includes Piemonte and Liguria regions, in Northwestern Italy (**Figure 2**).

Piemonte region is the second largest Italian Region by geographical area (25.402 km²) and comprises eight Provinces. It is surrounded on three sides by the Alps. About 41% of the Region is mountainous (prealpine and alpine) and 59% is hilly or flat. It is crossed from the West to the East by the Po river and bounded to the East by the Ticino river. Surrounding highly urbanized areas are intensive agriculture farmlands; they cover approximately 960,000 hectares where cereals and forage are predominantly cultivated; extensive rice fields dominate the landscape in the northeast part of the Region.

Liguria is one of Italian smallest Regions. It is divided into four Provinces. It is a narrow strip of land (5.416 km²), highly urbanized, overlooking the Mediterranean Sea: the ring of Maritime Alps and Ligurian Apennines beyond the narrow strip of coast descends almost immediately to a considerable marine depth. It represents an important touristic and commercial area.

Characteristic rocky coasts and seascape attract many travellers to spend the holidays in the most famous tourist resorts along the Italian Riviera. The Port of her capital City Genova, with a trade volume of 58.6 million tonnes, is the first port of Italy. The invasive alien tiger mosquito (*Aedes albopictus*), which is an important vector of viral MBDs, was firstly detected in Italy in the city of Genova in 1990.

Piemonte and Liguria Region neighbours on areas where WNV is historically endemic: Camargue and Var in Southeastern France [36] to the West, Lombardy and Emilia Romagna Regions in Italy to the East.



Figure 2. Map of Italy with regional boundaries. Piedmont and Liguria in the upper left, in white colour.

In both Regions, the climate is warm temperate, and socioeconomic and geographic features described earlier provide ideal habitats for potential mosquito vector species for MBDs.

3.1.2. Entomological surveillance on mosquitoes

Entomological surveillance was activated from July to the end of October through adult mosquito collection with fortnight samplings. After every daily catch, mosquitoes were collected from each trap, and then they were transported in refrigerated boxes to the laboratories. Adult females were counted and identified to the species level, using a stereomicroscope and morphological standard classification keys [37]. After identification, mosquitoes were pooled in groups of up to 100 specimens each, sorted by species, date and site of collection, frozen, and then stored at -80°C for virological investigation.

3.1.2.1. Piemonte region

Prior to 2011, Piemonte had no a widespread entomological surveillance system for public health purposes. A local mosquito-fighting programme was carried out since 1997 for nuisance issues, only in eastern areas where rice is extensively cultivated nearby human settlements [38].

Besides the active surveillance in the 'risk area' established by the WNV National Surveillance Plan, a Region-wide systematic mosquito collection system has been carried out during seasonal mosquito activity since 2011 to gather entomological and virological data to support the Regional MDBs monitoring programme.

The Region was mapped into 73 equal quadrants (20 km x 20 km) as a proxy for geographical area, where the quadrant was defined as the epidemiological unit.

Trapping sites were selected according to risk factors of mosquito spread and the epidemiological cycle for MBDs: proximity to wetland zones, the presence of hosts, commercial areas and touristic routes (ports, airports and freight terminals), different land use and habitat features. At least one trap was placed in each quadrant at an elevation below 600 m. a.s.l., because most anthropophilic mosquito species, potential vectors of MBD, generally inhabit areas below 600 m. a.s.l. in Italy [39].

All trapping sites were mapped by latitude and longitude on a geographical positioning system (**Figure 3**).

Two types of traps were employed to collect adult mosquitoes:

- CDC traps baited with dry ice as a source of carbon dioxide (CO_2) (approximately 0.5 kg/trap): they attract adult females searching for a blood meal and are routinely used as the most common sampling method in WNV surveillance programmes in many Regions in the world. Each trap worked for a minimum of 12-h periods from sunset to sunrise.
- BG sentinel traps baited with BG-Lure and CO_2 (approximately 0.5 kg/trap): the BG-Lure attractant contains a combination of substances found on human skin that strongly attract anthropophilic mosquitoes (mainly belonging to the *Aedes* genus). Nevertheless, with the addition of CO_2 , they are adapted to the collection of *Culex* mosquito species [40]. They were

located in sampling stations considered at a higher risk level for the introduction of exotic species and exotic pathogens. Each trap worked for a 24-h period.

3.1.2.2. Liguria region

Prior to 2011, no entomological surveillance activities were conducted in Liguria Region.

Since 2011, a Regional entomologic surveillance plan was carried out during seasonal mosquito activity with the goals to gather entomological and virological data and early detection of the introduction of invasive vectors and MDBs.

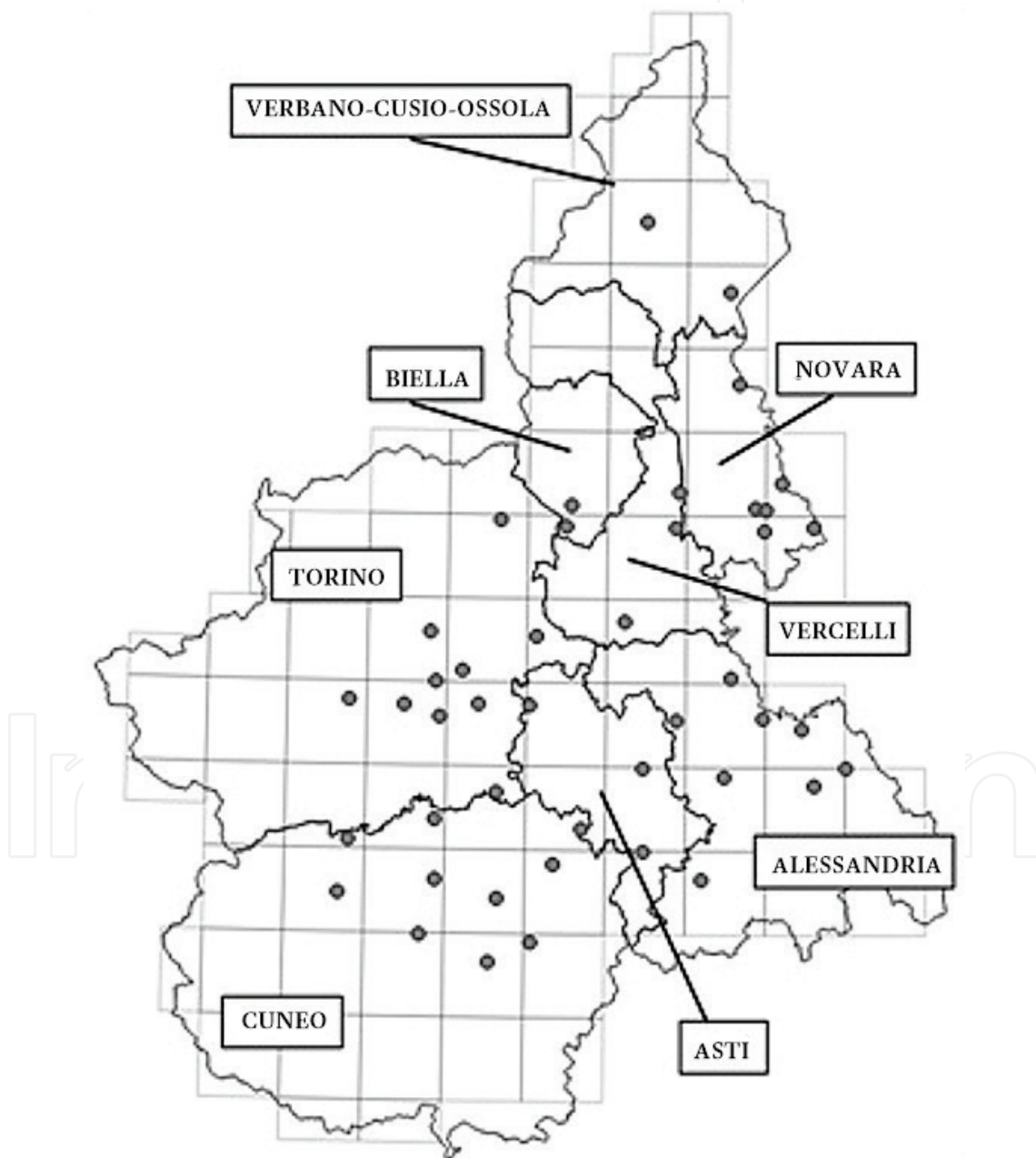


Figure 3. Map of Piemonte region with the grid of 20 x 20 km quadrants. Grey dots represent trapping sites. The eight provinces are named in uppercase letters in textboxes.

As the Regional territory displays characteristic feature of a narrow hilly coastal strip, trapping sites were located along the coastline. Locations were selected according to risk factors for the introduction and spread of exotic invasive mosquitoes and MBDs: habitat features, proximity to commercial areas and touristic routes (ports, airports and freight terminals) and presence of hosts.

The surveillance system has undergone further updates over time, increasing the number of trapping sites in order to best adapt to the evolving epidemiological scenario of the Region.

Particular attention was paid to the city of Genova, due to the presence of several risk factors of importing invasive mosquitoes and viral diseases: the port, the airport and the largest ethnic Latin America Ecuadorian community outside Ecuador itself. In the last decade, South America recorded changes in spatio-temporal distribution of infectious diseases, including MBDs due to globalization and climate changes [41, 42].

All trapping sites were mapped by latitude and longitude on a geographical positioning system (**Figure 4**).

Three types of traps were employed to collect adult mosquitoes:

- CDC traps baited with CO₂ and BG sentinel traps baited with BG-Lure and CO₂, with the same rules as in Piemonte Region;
- gravid traps baited with hay infusion added with yeast (almost 2.5 l): these traps attract *Culex* females that have blood fed and are ready to lay their eggs [43] in sites containing water high in organic matter, increasing together the likelihood of collecting infected mosquitoes. Each trap worked for a 24-h period.

3.1.3. Virological surveillance on mosquitoes in Piemonte and Liguria regions

Virological surveillance was performed since 2011 in Piemonte and 2013 in Liguria, focusing on mosquitoes collected from a selection of traps, updated every year, according to risk-based factors. In 2016 and 2017, mosquitoes collected at least from a trap in each quadrant (20 × 20) were analysed. Pools were homogenized in phosphate-buffered saline (600 µl if pool of <30 mosquitoes or 1200 µl if pool of >30 mosquitoes) in a 2-ml microtube with round copper beads.

Total RNA was extracted from mosquito samples according to the RNeasy Mini kit (Qiagen) manufacturer's instructions, with an *automated QIAcube protocol*.

All pool were analysed by a TaqMan® One-Step RT-PCR protocol distinctive for WNV Lineage 1 and Lineage 2 with WN-LCV-F1 and WN-LCV-R1 primers [44] and a TaqMan® One-Step RT-PCR protocol for USUV with USU F and USU R primers [45].

On positive pools, two traditional RT-PCRs for the amplification of WNV [46] and USUV [47] were carried out. Amplicons of the expected size (408 and 425 bp, respectively) were sequenced using the Big Dye Terminator kit v 3.1 (Lifetecnologies) and run on a ABI3130 Genetic Analyzer (Applied Biosystems). The related sequences were employed to perform a basic local alignment search tool (BLAST) in the GenBank library to confirm the specificity of positive reaction and to estimate the degree of identity of detected strains.

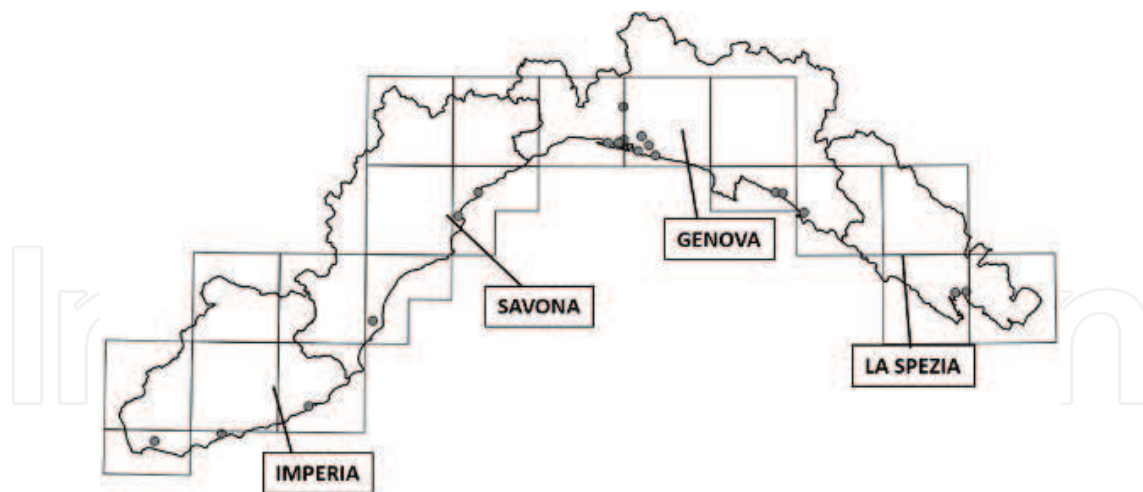


Figure 4. Map of Liguria region with the grid of 20 x 20 km quadrants. Grey dots represent trapping sites. The four provinces are named in uppercase letters in textboxes.

All WNV and USUV positive pools were sent to the National Reference Centre for Animal Exotic Diseases (CESME, Teramo) for confirmation, sequencing and determination of Lineage.

In addition, *A. albopictus* pools collected in sites considered at a higher risk level for the introduction of exotic invasive species and exotic pathogens (annually reevaluated) were tested using a panflavivirus end-point RT-PCR, targeting the conserved Region of the NS5 gene sequences.

The SuperScript_{III} Platinum_{One-Step} qRT-PCR Kit (Thermo Fisher Scientific) was used to detect other flaviviruses of extreme importance, such as Dengue or Zika as previously described by Scaramozzino et al. [48].

3.1.4. Veterinary surveillance

According to WNV National Surveillance system [32], a passive clinical surveillance of all neurological signs in equine, and monitoring of wild birds found dead have been carried out in the study area. Necropsy of dead animals and virological molecular testing by real-time RT-PCR was performed on EDTA whole blood (live or dead animals) or on pooled samples of target organs (brain, heart, spleen and kidney) (dead animals). All samples were analysed by a TaqMan[®] One-Step RT-PCR protocol distinctive for WNV Lineage 1 and Lineage 2 with WN-LCV-F1 and WN-LCV-R1 primers [44] and a TaqMan[®] One-Step RT-PCR protocol for USUV with USU F and USU R primers [45].

Active surveillance activities have been updated according to the epidemiologic scenario of the previous year in each region.

3.1.4.1. Piemonte region

In 2009, the WNV National Surveillance plan introduced serological screening of horses in a small wetland area in southeast Piemonte Region. The area named 'Garzaia di Marengo' was classified as at risk for WNV introduction due to the presence of waterfowls, including species of migratory birds.

A fixed number of seronegative unvaccinated equines sentinel (28 animals: 10% of prevalence, 95% confidence level) was selected in order to uniformly cover the study area. Horses were sampled three times during the transmission season: immediately before (month of May), at seasonal peak (last week of August) and at the end of the transmission season (last week of September), with the aim to monitor the raising of WNV-specific IgM antibodies that are related to recent infection by commercial competitive ELISA. Positive samples were confirmed by seroneutralization assay (SN) and IgM ELISA at the CESME. Seroconversion was confirmed if SN titre was at least 1:10 and there was evidence of IgM antibodies [49].

Since 2014, the sentinel horses system in 'Garzaia di Marengo' was replaced by a one-shot random sampling of horses in order to detect IgM antibodies suggestive of WNV early infection. The number of animals to be tested for each Province during the transmission season was assigned based on the provincial equine census, as recorded in National Livestock Database [50].

Since 2015, after the detection of WNV circulation in mosquito pools and in horses in Piemonte Region, veterinary surveillance procedures were revised, and the Region has been considered as WNV Endemic Area by the National Surveillance programme.

Serological testing on horses was suspended while active virological surveillance on synanthropic non-migratory birds was implemented. From April to November, synanthropic birds (magpies—*Pica pica* and hooded crows—*Corvus cornix*), in the framework of the Regional control programme against pest birds for cultivated land protection, were weekly captured by Larsen traps and then killed or directly shot by trained hunters. Samples of organs (brain, heart and kidney) of each bird were pooled and tested for WNV and USUV.

All samples were analysed by a TaqMan® One-Step RT-PCR protocol distinctive for WNV Lineage 1 and Lineage 2 with WN-LCV-F1 and WN-LCV-R1 primers [44] and a TaqMan® One-Step RT-PCR protocol for USUV with USU F and USU R primers [45].

In 2017, the control programme against synanthropic birds was not activated in Torino Province. Then, as alternatively recommended by the National Surveillance Plan, a specific serological monitoring activity was enforced on rural poultry, with the aim to detect IgG antibodies by commercial ELISA test.

3.1.4.2. Liguria region

Since 2014, Liguria Region was included in WNV National Surveillance plan by serological random sampling of horses in order to detect IgM antibodies suggestive of WNV early infection. The number of animals to be tested for each Province during the transmission season was assigned based on the provincial equine census, as recorded in National Livestock Database [50].

3.2. Human surveillance West Nile virus and others MBDs in Piemonte region and Liguria region

Human surveillance activities against MBDs in Piemonte Region are regulated by both the National Surveillance Plan of imported and autochthonous MBDs (CHIKV, DENV and ZIKV), annually published by MoH and by National Integrated Surveillance Programme against WNV and USUV.

Local Health Authorities must establish an active surveillance system against human MBDs during the transmission season. Furthermore, passive surveillance has to be set up during the whole year, requesting physicians to report all possible, probable and confirmed cases according to national case definition [32]. Surveillance against Zika virus infection includes investigation on neurological disorders such as Guillain-Barré syndrome in adults and microcephaly in infants.

Every case confirmed by the Regional and National Reference Laboratories has to be subjected to epidemiological investigation with the aim to establish any local viral circulation. Moreover, during the transmission season, measures for vector control have to be implemented promptly.

According to the blood directive, if veterinary or human cases of WNV are detected, immediate WNV NAT screening of all blood and haematopoietic stem cell donations of solid organ donations is introduced in affected areas (provinces). Every suspected case of Chikungunya or Zika virus has to be temporarily deferred from donations for 28 days starting to recovery [51].

In addition to surveillance actions, another important item of the MBDs surveillance is the information activity to raise awareness on population on the links between mosquitoes and the diseases they can transmit.

Information campaigns focused on personal protection measures against mosquito bites, especially for pregnant women, and mosquito fighting were conducted in Piemonte and Liguria since 2014 due to WNV spread in Northern Italy and in 2016 due the Zika virus emergency in South America.

Training courses have been organized for public health physicians and veterinarians in order to early detect any MBDs clinical suspected case. Advertising as poster and leaflets was shared in hospitals, offices of health authorities and travel medicine centres.

4. Results

4.1. Entomological and virological surveillance on mosquitoes

4.1.1. Piemonte region

Since 2011, a total of 111.676 adult mosquitoes, divided in 4620 pools, were identified and analysed.

The most abundant species detected was *C. pipiens*, mainly collected by CDC traps followed by *Ochlerotatus caspius*, *Anopheles maculipennis* s.l. and *A. albopictus*.

Details of collected species for years are shown in **Table 1**.

USUV was reported since 2011 [52]. After the first detection, it was found every year in field-collected mosquitoes (*Cx. pipiens*), confirming the establishment of the virus in the Region.

WNV Lin2 was detected for the first time in 2014, in Alessandria Province, in two *C. pipiens* pools (August 27, 2014; September 10, 2015).

| Mosquito species | Total | |
|------------------------------|---------|------|
| | N | Pool |
| <i>Ae. albopictus</i> | 8692 | 875 |
| <i>An. maculipennis s.l.</i> | 12,126 | 502 |
| <i>An. plumbeus</i> | 74 | 38 |
| <i>Ae. vexans</i> | 4074 | 288 |
| <i>Ae cinereus</i> | 1 | 1 |
| <i>Cx. hortensis</i> | 4 | 2 |
| <i>Cx. pipiens</i> | 50,660 | 1672 |
| <i>Cx. territans</i> | 16 | 2 |
| <i>Cx. theileri</i> | 34 | 10 |
| <i>Cs.annulata</i> | 6 | 3 |
| <i>Cx.modestus</i> | 1621 | 100 |
| <i>Cs.subochrea</i> | 14 | 6 |
| <i>Cs. longiareolata</i> | 5 | 4 |
| <i>Culiseta sp</i> | 2 | 2 |
| <i>Oc.caspis</i> | 34,085 | 1053 |
| <i>Oc.geniculatus</i> | 198 | 51 |
| <i>Oc.cantans</i> | 56 | 8 |
| <i>nd</i> | 8 | 3 |
| Total | 111,676 | 4620 |

Table 1. Total amount of mosquitoes collected and tested in Piemonte region since 2011, sorted by species and number.

In 2015, WNV Lin 2 was detected in six pools of *Cx. pipiens*. They were collected with CDC dry ice-baited traps from July in four provinces: Novara (July 29, 2015, and August 26, 2015), Alessandria (August 6, 2015), Vercelli (August 21/26, 2015) and Torino (September 23, 2015). In 2016 and 2017 in Alessandria and Novara provinces, respectively, positive *Cx. pipiens* pools were found. No other *flavivirus* of medical interest was detected [53, 54].

4.1.2. Liguria region

Since 2011, a total of 33,244 adult mosquitoes were collected.

Virological investigations were performed since 2013 on 23,050 adult mosquitoes, split in 1255 pools (**Table 2**).

The most abundant species detected was *C. pipiens*, mainly collected by gravid traps followed by *A. albopictus* collected in Bg-Sentinel. Since 2015, in the city of Genova, adult female *A. koreicus* specimens were detected [55]. Pool collected in 2015 and 2016 was

analysed with bio-molecular assays to confirm the species identity. Since 2017, considering the most abundant number of adults trapped, *A. koreicus* specimens were also screened for flavivirus.

In 2014, WNV and USUV were detected for the first time in the area. A WNV-positive pool was collected by gravid traps at Genova airport on September 29. Actually, this is the only report of WNV circulation in the Region.

USUV was detected in a *C. pipiens* pool in La Spezia Province by gravid traps on September 23. Unlike WNV, in the following years, USUV continued to circulate in the Region, and it was annually reported in mosquitoes. No other flavivirus of medical interest was detected [5].

| Mosquito species | Total | |
|------------------------------|--------|------|
| | N | Pool |
| <i>Ae. albopictus</i> | 9054 | 493 |
| <i>Ae. koreicus</i> | 26 | 15 |
| <i>Aedes spp</i> | 92 | 11 |
| <i>An. claviger</i> | 4 | 4 |
| <i>An. maculipennis s.l.</i> | 3 | 3 |
| <i>An. plumbeus</i> | 10 | 8 |
| <i>Anopheles. spp</i> | 1 | 1 |
| <i>Cq. richiardi</i> | 19 | 3 |
| <i>Cx. hortensis</i> | 5 | 5 |
| <i>Cx. impudicus</i> | 21 | 4 |
| <i>Cx. mimeticus</i> | 1 | 1 |
| <i>Cx. pipiens</i> | 13,191 | 536 |
| <i>Culex spp</i> | 184 | 34 |
| <i>Cx. territans</i> | 2 | 2 |
| <i>Cx. theileri</i> | 3 | 3 |
| <i>Cs. annulata</i> | 9 | 9 |
| <i>Cs. longiareolata</i> | 381 | 99 |
| <i>Culiseta sp.</i> | 2 | 2 |
| <i>Oc. caspius</i> | 35 | 15 |
| <i>Oc. geniculatus</i> | 1 | 1 |
| <i>Oc. mariae</i> | 2 | 2 |
| <i>Ochlerotatus spp</i> | 4 | 4 |
| Total | 23,050 | 1255 |

Table 2. Total amount of mosquitoes collected and tested in Liguria region since 2013, sorted by species and number.

4.2. Veterinary surveillance

4.2.1. Piemonte region

Since 2009, in the framework of passive surveillance, 35 suspected equine clinical cases were notified: three in 2014, 24 in 2015, six in 2016 and two in 2017.

Nine of the suspected cases were confirmed: six in 2015 (Alessandria Province), one in 2016 (Vercelli province) and two in 2017 (Asti and Cuneo Provinces).

Within the passive surveillance on wild birds that were dead, from 2009 no WNV infection cases were detected.

The active surveillance from 2009 to 2013 on 28 sentinel horses selected in the risk area sited in 'Garzaia di Marengo', annually tested three times during the transmission season, did not detect any seroconversion. In 2014 and 2015, a total of 1819 equines sentinel were screened by ELISA IgM serological test. The only seropositive horse detected (2014) lived in Alessandria Province, within 4 km of radius from the first WNV-positive mosquito pool.

Since 2016, the Surveillance Plan was selectively targeted on the active surveillance of wild, synanthropic and rural birds, and then sentinel horses system was dropped out.

A total of 2451 wild birds were tested by RT-PCR for WNV, mainly corvids species: 14 corvids were confirmed WNV infected, in 3 out of 8 Piemonte Provinces (Alessandria, Torino and Vercelli).

In 2017, the serological monitoring activity on rural poultry in Torino Province allowed to detect 17 WNV positive hens; 4 animals out 17 tested SN positive both for WNV and for USUV by the CESME, providing demonstration of co-circulation of both viruses in the same areas.

4.2.2. Liguria region

Since 2009, in the framework of passive surveillance, seven suspected clinical cases were notified: none was confirmed by laboratory investigation.

A total of 97 wild birds found dead were virologically tested by RT-PCR for WNV. In 2013, the necropsy of an Eurasian hobby (*Falco subbuteo*), a long-range migratory species, found dead at the end of spring in Imperia Province, accidentally allowed to identify WNV Lin2. This report has not been considered a significant proof of virus circulation, because the infection was probably contracted in Africa. This is to date the only WNV virological positivity in birds in Liguria region.

By active surveillance, since 2014, 611 horses were screened by ELISA IgM serological test: no positivity was detected.

4.3. Human surveillance of West Nile virus and others MBDs in Piemonte and Liguria region

Every WNV or MBDs suspect case was promptly reported to the Regional Public Health Authority. For Piemonte Region, biological samples were transmitted to the Laboratory of Microbiology and Virology, within the Regional Reference Centre for Infectious Diseases of

the Amedeo di Savoia Hospital, Torino. From 2015 to date, four WNND autochthonous cases were detected: one fatal case in 2015 in Torino Province, one fatal case in 2016 in Novara Province and two fully recovered cases in 2017 in Asti province [56].

For Liguria Region, the Regional Reference Centre for MBDs diagnosis is located at the Institute of Hygiene of the Policlinico San Martino Hospital, Genova.

Notified human cases of CHIKV, DENV and ZIKV since 2011 in Piemonte and Liguria Regions are displayed in **Table 3** (all imported cases).

As WNV surveillance involved both Veterinary and Human Public Health Regional Authorities, specific flow charts were prepared and shared among working group with the aim to coordinate the notification of viral circulation triggers and the implementation of control measures. Hierarchy of actions is shown in **Figure 5**.

| Arbovirosis human surveillance | | Piemonte | Liguria |
|--------------------------------|------|----------|---------|
| 2011 | CHIK | 0 | 0 |
| | DEN | 1 | 0 |
| | ZIK | 0 | 0 |
| 2012 | CHIK | 0 | 0 |
| | DEN | 5 | 0 |
| | ZIK | 0 | 0 |
| 2013 | CHIK | 0 | 0 |
| | DEN | 12 | 0 |
| | ZIK | 0 | 0 |
| 2014 | CHIK | 1 | 0 |
| | DEN | 5 | 1 |
| | ZIK | 0 | 0 |
| 2015 | CHIK | 1 | 0 |
| | DEN | 12 | 0 |
| | ZIK | 0 | 0 |
| 2016 | CHIK | 2 | 0 |
| | DEN | 14 | 0 |
| | ZIK | 14 | 1 |
| 2017 | CHIK | 1 | * |
| | DEN | 18 | * |
| | ZIK | 2 | * |

*data not available.

Table 3. Notification of Chikungunya (CHIK), dengue (DEN) and ZIKA (ZIK) human cases in Piemonte and Liguria regions from 2011 to 2017 (all imported cases).

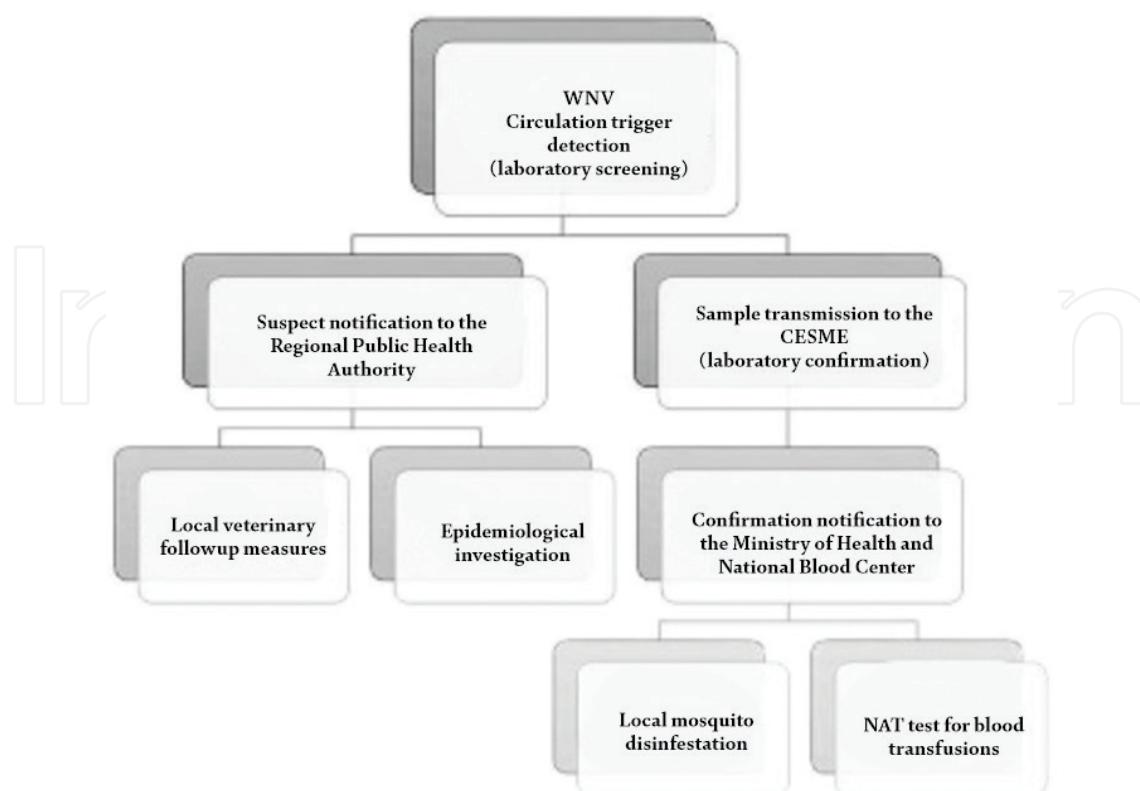


Figure 5. Flow chart for the management of WNV viral circulation trigger event in Piemonte and Liguria regions, since 2015.

During transmission season, following the detection of WNV viral trigger by the national Integrated Surveillance Plan, the NAT test was progressively introduced throughout affected Provinces of Piemonte Region. According to National legislation, it was stopped on November 30.

In 2014 and 2015, only Alessandria province was involved in blood screening.

In 2016, four (Alessandria, Novara, Torino and Vercelli) of the eight Provinces in the Piemonte Region were involved in NAT testing of blood donors.

In 2017, blood screening was enforced in Novara, Vercelli, Cuneo and Asti Provinces.

Since 2014, a total of 99,882 blood bags were screened by NAT (5172 in 2014; 38,623 in 2015; 35,812 in 2016 and 20,275 in 2017). No infected blood bags were detected.

5. Conclusions

MBDs have complicated transmission cycles, involving different reservoirs, competent vectors and environmental and climatic features that influence their epidemiology. Therefore, surveillance methods have to be planned according to these aspects.

In Italy, WNV and USUV are considered endemic in several Regions, whereas CHIKV, DENV and ZIKV are essentially related to infected travellers returning from endemic countries [57].

Two National Surveillance Plans regulate MDBs management in Italy: the first regarding WNV and USUV, which integrates human and veterinary surveillance and the second regarding MBDs specifically transmitted by *Aedes* mosquitoes, mainly CHIKV, DENV and ZIKV, based on the surveillance of both imported and autochthonous human cases.

In Piemonte and Liguria Regions (Northwestern Italy), a WNV multisectoral task force, including representatives of Public and Animal Health and Vector Control, was established in 2011 and then strengthened annually according to epidemiological findings. Since 2013, the working group cooperates with experts of four neighbouring Regions, sharing integrated WNV and USUV surveillance guidelines (human, entomologic and veterinary) throughout the whole Po river valley area. The main goal of this standardization has been the reduction of the transmission risk through blood transfusions, quickly implementing local preventive measures when the virus (in animals, vectors or humans) is detected in a specific Province. This allows to definitely decrease the risk of human transmission and consequently results in a significant reduction of health-care costs [58].

The active virological surveillance on mosquitoes and birds is considered an important tool to early detect virus circulation in a specific area, since it has been shown that the virus in mosquitoes and birds appears much earlier than the occurrence of clinical cases in dead-end hosts (humans and equines).

Passive surveillance of clinical cases in horses also can be considered a useful tool for the detection of WNV activity, but it will be less sensitive, and a positivity regarding the proof that the cycle of the virus is ended must be considered. Being dead-end hosts, the probability of infection is similar for horses and humans; then, an early detection tool especially in an endemic area cannot be considered. Furthermore, considering that in affected countries, vaccinations of horses are progressively adopted, it is estimated that surveillance in equidae will gradually become irrelevant [59].

The entomological surveillance can be a valuable additional tool also for the surveillance of MBDs caused by non-endemic viruses (CHIKV, DENV and ZIKV) in which humans are the reservoirs. Even if the syndromic surveillance on human cases is considered the most adequate approach to detect the introduction of these viruses, the presence of risk factors, as competent vectors and suitable climatic conditions, can significantly increase the potential risk for the local transmission in vectors, as happened in Emilia Romagna (2007) and Lazio (2017).

Indeed, the selection of the most suitable mosquito-trapping method and the identification of areas with major risks of introduction of exotic mosquitoes and pathogens are crucial, and surveillance should be planned in response to a recognized risk and carried out to support subsequent actions. In Piemonte and Liguria, the entomological surveillance in such sites (ports, international airports, international connection points and hospitals) revealed the presence of *A. albopictus*, competent vector of CHIKV, DENV and ZIKV, but no positive mosquito was found. In Liguria region, Genova city, this risk-based surveillance allowed to detect in 2015 the introduction of *A. koreicus*, an Asiatic mosquito which has become invasive in Europe in the recent years, proven to be an effective transmitter of Japanese encephalitis virus, *Dirofilaria immitis* and CHIKV [60]. While the origin of these specimens remains unknown, the presence of *A. koreicus* in such an important commercial and tourism hub is worrisome, as it might strongly accelerate the species' spread in Italy and in the rest of Europe, as already happened with *A. albopictus*.

Given the long-distance passive transfer of infected mosquito by trades, or movements of infected tourist and business travellers, particularly during the vectors season and the favourable transmission period, the potential risk of introduction and spread of emerging MBDs exists [61].

In conclusion, the creation of a regional working group composed by public and animal health authorities, together with the authorities in charge of vector surveillance and control that regularly share information, is a crucial point towards the achievement of an integrated surveillance. The approach adopted in Northwestern Italy has been demonstrated a key point to promptly implement control measures and save resources, reducing the risk of MBDs human transmission.

The periodical evaluation of planned actions and their updating according to the evolving epidemiological scenario is of paramount importance for the prevention of the diseases and the maintenance of both human and animal health.

Further cost–benefit evaluations, including an accurate estimation of indirect costs, are needed to improve the knowledge of the economic context of MBDs and its mitigation, allowing to better target the Public Health response.

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Conflict of interest

The authors declare that no competing financial interests exist.

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