EU REFERENCE LABORATORY FOR

EQUINE DISEASES

Report of the 12th Workshop of the National Reference Laboratories for equine diseases (Subject: Equine Arteritis virus and arthropod-borne encephalitis viruses (WNV, JEV, EEEV, WEEV and VEEV)

15 -16 April 2021
ANSES, Maisons-Alfort, France

Introduction:

The programmes and all the presentations of these two workshops are available on the website **https://eurl-equinediseases.anses.fr/.**

Nota bene: a preliminary inscription is necessary before having access to the private part of the website. The modality of this inscription is described in annex1.

Due to Covid crisis, the workshop was organised online. 113 participants coming from NRLs or third country (Argentina, Tunisia, Switzerland, USA) were registered to attend these two workshops. The agenda of these workshops is in annex 2 of this report

Workshop on EVA.

SESSION 1: EVA epidemiology and research program

1. Overview of EVA epidemiological survey in Europe (2017-2020) (JC. Valle-Casuso, ANSES, France)

Dr. José Carlos Valle-Casuso presented an overview of the EAV situation in Europe from data sent by 21 NRLs belonging to the EU and one Third Country. The data collected from the different NRL and summarised in this presentation highlighted that EAV is present in Europe. Indeed, between 2016 and 2020, 14 countries reported seropositive stallions, and at least 11 countries reported positives semen EAV samples. Those stallions are not located in a particular region of Europe since EAV was detected in stallions from Spain, France, Belgium, Germany, Italy, UK, Sweden, Estonia, Czechia, Hungary, and Poland. EAV establishes their reservoir in some infected stallions; the viral reservoir is a key component of EAV epidemiology and transmission since they can disseminate the virus in the horse population during breeding seasons, mainly when contaminated semen is used for artificial insemination.

Comments of participants:

Falko Steinback (UK) gave a comment about the increasing number of outbreaks during the last 4 years. He shared their vision about how the increase of horses tested could be an important variable related to the increase of the outbreaks reported by the NRL and we should be attentive to the number of underneath cases of EVA that we have in our countries.

Patricia Koenig (DE) gave an exhaustive explanation about the EVA outbreaks reported by the German NRL. The large number of outbreaks reported in 2020 (28) were most of them related to large structure for Semen Collection & Embryo Transfer Station. Probably an EVA-infected horse that enters this structure infects by the respiratory way other's horses. Those horses do not show clinical signs before returning to their original holdings, where those signs were identified.

Alf-Eckbert Füssel (EU Commission) gave a comment remembering the AVE policy of the EU commission. We have to certify that imported stallions to the EU countries and those held in semen collection centers should be clean for this disease. The situation identified in 2020 in Germany invites us to be more attentive in controlling the health status of the stallions held by structures for Semen Collection & Embryo Transfer Stations and the horses that enter in those structures. We certify these biological materials to be trade in the EU, so we need to identify if the problem came from the analysis done by the laboratories or by the management of the stallions held in this kind of structure.

Please see: Overview of EVA epidemiological survey in Europe (2017-2020) (Part: PT/workshops/Workshop presentations)

2. Overview of EVA epidemiological survey in Italy (2017-2020) (M.T Scicluna, IZS, Italy)

Dr. M.T Scicluna presented the Italian control program for EVA. She explained the importance of controlling the shedder stallions before the breeding season and the improvements of their organization to trace the tested equids. Indeed, she presented an overview of the EAV situation in Italy between 2017-2020, where they concluded that the number of serologically positive equidae is quite constant over these years.

Please see: EVA epidemiological survey in Italy (2017-2020) (Part: PT/workshops/Workshop presentations)

3. EVA 2018 outbreak in France (D. Gaudaire, ANSES, France)

In October 2018, a laboratory belonging to the National French Network for the virological EAV detection requested help from the NRL to confirm EVA positive results. The positive samples were obtained from several tissues collected during the autopsy of an aborted foetus. Then those samples were analysed by the NRL that confirmed the presence of EAV genome. To better understand the situation, the owner kindly collaborates with the NRL. He allowed a serological survey and shared their samples to facilitate the isolation of this new EAV strain. This stud farm has around 200 mares and five stallions. For the serological survey, an EAV Neutralizing Antibodies detection assay (EAV NA) was performed using the VNT between D0 and D92. At D0, blood samples were collected from 202 horses, and according to the VNT results, 91 sera were negative, and 111 were positive since and exhibited a VNA titre ranged from 4 to 768. At D30, among the 207 collected blood samples, 82 blood samples (39.6%) were negative, 122 blood samples (58.9%) exhibited antibodies anti-EAV, and 3 blood samples were cytotoxic. At the end of this serological surveillance (D92), out of the 151 collected blood samples, 70 (46.7%) were negative, and 81 (53.3%) exhibited antibodies anti-EAV. From these samples, we identified 81 sera, which exhibited EAV NA. From these 81 sera, we identified that 69 equids exhibited a stable VN EAV antibodies titre, and 8 equids showed a rising VN EAV titre (4-fold or greater). We also identified 4 seroconversions that showed the expected humoral immune response peak at two to four months after the exposure to the EAV. To complement the serological survey and better characterize this AVE outbreak, a molecular biology diagnosis was performed to identify the viral strain. The tissue samples collected were analysed by RT-qPCR targeting a portion of the ORF7, and the presence of the EAV genome was detected in the lung, liver, and kidney tissues. Then we characterize the ORF 5 of the EAV isolated strain of this outbreak. According to the phylogenetic analysis, the isolated strain of this EAV outbreak is closed to the EAV sequences belonging to the European subgroup two. As a final

action, we try to do a viral isolation assay from AVE-positive tissues, but unfortunately, we failed to amplify this strain in our cell culture.

Comments of participants:

Falko Steinback (UK) gave a comment pointing that the data shows that after 93 days, we still have a little viral circulation in the structure. We agree with this comment, but as reassuring data, we can complete this outbreak analysis data with an additional one year after the outbreak that shows no circulation of the virus in this structure.

Please see: 3. EVA 2018 outbreak in France (Part: PT/workshops/Workshop presentations)

4. EVA 2019 outbreaks in England (F. Steinback, APHA, United Kingdom)

Falko. Steinbach presented two outbreaks of EVA that occurred in the UK in 2019. The phylogenetic analysis of the two outbreaks showed that the isolated viral strains belong to the same genotype (D), but they were unrelated. He remained that continuous monitoring of the breeding stallions is highly advisable since EVA is not an exotic disease to Europe.

Comments of participants:

Stephan Zientara (FR) asked about the origin of these two cases of AVE in UK. Falko Steinbach explains that the origin was not clear, but all of these horses were show horses that travel a lot and could become infected by the respiratory way in one of these shows.

Please see: EVA 2018 outbreak in England (Part: PT/workshops/Workshop presentations)

5. Development of an HTS assay for EVA drugs Screening (JC. Valle-Casuso, ANSES, France)

Dr. José Carlos Valle-Casuso presented the development of an in vitro assay suitable for testing candidate antiviral molecules on equine dermal cells infected by EAV. Using this assay, they identified three molecules that effectively suppressed the cytopathic effects associated to EAV infection, and strongly inhibited viral replication and production of infectious particles.

Comments of participants

Alf-Eckbert Füssel (EU Commission) gave a positive comment highlighting the importance of the research projects that could help manage this disease. Indeed, at this moment, a delegated act is being discussed in the EU about the authorized products that will be available to control the listed diseases and is an excellent moment to discuss about the AVE vaccines, the GnRH vaccines, and other new therapeutic approaches to highlight the best strategies to develop to fight this disease.

Please see: Development of an HTS assay for EVA drugs Screening (Part: PT/workshops/Workshop presentations)

SESSION 2: EVA proficiency tests

6. Outcome of Proficiency test on EVA serological diagnostic (D. Gaudaire, ANSES, France)

A PT for "Equine Viral Arteritis (EAV) diagnosis using the Virus Neutralisation Test (VNT)" was organized in 2020 within the framework of the activities of the EURL for equine diseases other than African horse sickness as prescribed by the European Commission's Directorate General for Health and Food Safety (DG SANTÉ). This PT aimed to evaluate the performance of each participant in detecting neutralizing antibodies to EAV in serum samples as described in chapter 3.5.10 of the OIE manual.

Twenty four laboratories registered to participate in this PT, 22 participants were acknowledged as National Reference Laboratories for EVA in their respective countries. EAV VNT ILPT participants were allowed 6 weeks to analyse 22 serum samples of this PT as requested and were to upload their results on LEILA dashboard portals by October the 30th.

To evaluate the VNT performance of the participants, the acceptance criteria for qualitative analysis were: all negative sera (9) should be found as such, all positive sera (13) with an expected anti-EAV antibody titre above or equal to four should be found positive and only one error was allowed for 22 samples, that means to obtain at least 92.3% of satisfactory qualitative results. For quantitative analysis, the serum titre of positive sera should be found within a range of plus or minus one dilution compares to expected titres and only one serum titre error was allowed for 13 positive samples, which means to obtain at least 87% of satisfactory quantitative results.

According to the qualitative result analysis, for the negative samples, 100% of results were satisfactory and for positive samples, 19 labs obtained 100% of satisfactory results, 3 labs obtained 92.3% of satisfactory results and 2 labs obtained less than 92.3% of satisfactory results. Regarding the quantitative result analysis of this PT, nine out of the 24 participants found the VNA titre to EAV in the range of plus or minus one dilution compared to the expected titer. Quantitative results were satisfactory for 37.5 percent of labs. The quantitative analysis of labs providing unsatisfactory results for this PT can be divided into two parts: in the first, unsatisfactory results are systematically with a too low value - this concerns 7 labs out of the 24, in other words, 29.2 % of participants. In the second part, we have grouped together labs that obtained more than one unsatisfactory result and always with a too high value. 8 participants (33.3%) obtained more than one titre out of 13 with a too high titre compared to the expected result.

By comparing the qualitative results obtained in the PT organized in 2016 and in 2020, 86.4% of labs in 2016 obtained a specificity of 100%; in 2020 specificity percentage was 100% for all participants. Furthermore, in 2016, 21 out of the 22 results obtained a sensitivity of 100%, in other words, they detected all the expected positive samples in the PT. In 2020, 86.4% of participants obtained a sensitivity of 100%. Regarding the quantitative analysis of both PTs organized in 2016 and in 2020, for the positive samples, VN antibody titres to EAV are more homogeneous between all participants in the PT organized in 2020 and no drift was observed between the PTs.

To conclude on the analysis of the results of the PT organized last year, specificity was very good for all participants. Sensitivity was also very good since 95.5% of positive samples were detected by the NRL for EAV in the European Union, 97.5% for all participants of this PT. According to the analysis of the quantitative results, in 2020 62.7 % and 67 % of the obtained titre were found within the expected range among the NRL and among all participants, respectively.

Please see : Outcome of PT on EVA serological diagnostic (D. Gaudaire, ANSES, France) (Part: PT/workshops/Workshop presentations)

7. Outcome of Proficiency test on EVA virological diagnostic (D. Gaudaire, ANSES, France)

A PT "Equine Viral Arteritis (EAV) diagnosis using Equine Viral Arteritis (EAV) diagnosis using virus isolation and RT-PCR (VI) as described in the OIE manual chapter 3.5.1" was organized in 2020 within the framework of the activities of the EURL for equine diseases other than African horse sickness as prescribed by the European Commission's Directorate General for Health and Food Safety (DG SANTÉ).

The aim of this PT was to evaluate the performance of each participant in detecting the presence of EAV in semen samples, as described in chapter 3.5.10 of the OIE manual. Two methods were asked in this PT scheme: Detection of EAV genome using RT-PCR method following the OIE manual Chapter 3.5.10. and detection of EAV infectious particles using Virus Isolation on cell culture following the OIE manual Chapter 3.5.10.

Twenty-five laboratories registered to participate in this PT, twenty-two participants were acknowledged as National Reference Laboratories for EVA in their respective countries. Labs were allowed 6 weeks to analyse the 18 semen samples of this PT as requested and were to upload their results on LEILA dashboard portals.

In this PT, each participant was required to find three negative samples and twelve positive samples using the RT-QPCR, one negative semen and two positive semen using virus isolation on cell culture.

To evaluate the performance of the participants of this PT, the acceptance criteria were: all negative semen should be found negative and all positive semen should be found positive. Only one mistake out of 15 samples is allowed for the RT-PCR that means at least 93,3% of satisfactory results.

According to the RT-PCR method results, for the negative samples, 96% of results were satisfactory 24 out of the 25 participants obtained 100% of satisfactory results. For positive samples, 19 labs obtained 100% of satisfactory results, five labs obtained 91.6% of satisfactory results and one lab obtained 75% of satisfactory results. The specificity and the sensitivity of this IL-PT according to the RT-qPCR result analysis was 99.7% and 97.3%, respectively.

By analysing the results of the 21 participants that performed the virus isolation on cell culture method, 14 labs obtained 100% of satisfactory results, 4 labs 50% of satisfactory results and 3 labs lab out of the 21 did not detect any expected positive sample. According to the result obtained by each participant when they performed the virus isolation on cell culture, the specificity and the sensitivity of this method in this PT were 95.2% and 76.2%, respectively.

By comparing the RT-PCR qualitative results obtained by the participants of the both PT organized in 2016 and in 2020, a 100% specificity has been obtained by 88.9% and 100%, respectively. In 2016 and in 2020, 88.9% and 83.3% of the participants respectively obtained a sensitivity of 100%.

To conclude on the analysis of the results of this PT organized in 2020, specificities of both requested methods were very good for all participants as it was 98.5% and 93.8% according to the RT-PCR and virus isolation, respectively. Sensitivity was also very good since 98.9% of positive samples were detected by the NRL for EAV in the European Union using RT-PCR. The performance of the NRL in EU to detect EAV using virus isolation on cell culture is less good as the specificity and sensitivity were 93.8% and 68.6%, respectively. According to the acceptance criteria of this PT organized in 2020, 95.5% of the NRL in EU obtained satisfactory results using the RT-PCR and 61.1% using the virus isolation. In this PT, all NRL (22)

for EAV in EU performed the RT-PCR detection and only 18 out of the 22 performed the Virus Isolation test. As in 2016, RT-PCR results are better and much more harmonized rather than virus isolation results.

Please see: Outcome of PT on EVA virological diagnostic (D. Gaudaire, ANSES, France) (Part: PT/workshops/Workshop presentations)

Workshop on arthropod-borne encephalitis viruses (WNV, JEV, EEEV, WEEV and VEEV)

SESSION 3: WNV and USUV epidemiology

In introduction, Dr Alf-Eckbert Füssel, from the European Commission SANTE/G2 informed formerly that Eva Camara will be the new desk officer for EURL for equine disease from 1rst of July 2021. He emphasized the importance for NRLs to participate in PT test organized by the EURL.

1 Animal Health Law and Equine Diseases - state of play (Alf-Eckbert Füssel, EC)

Dr Alf-Eckbert Füssel reminded the listing and categorization of equine diseases. (AHL) and presented the Animal Health legal framework. This new AHL compiles several different delegated and implementing acts into a single legal framework on animal health for the EU animal health policy. There are three levels of legislation: first, the law, second the delegate acts and third the implementing acts set up in the countries.

Some Delegate acts are currently in preparation like for example: "Use of Vet Medicinal products for disease control". Some delegate acts are adopted like for example "horse movement conditions in the EU (OJ L 174, 3.6.2020, p. 140); the approval of germinal product establishments and the traceability and animal health requirements for movements within the Union of germinal products of certain kept terrestrial animals (OJ L 174, 3.6.2020, p. 1)"

Some implementing acts are already adopted: animal health certificates and model animal health/official certificates used for the entry into the Union and movements between Member States of consignments of certain categories of terrestrial animals and germinal (OJ L 113, 31.3.2021, p. 1). In these model certificates, the tests needed for entrance of animals from third countries in EU are described:

VEE: haemaglutination inhibition test and PCR ; EIA: ELISA or AGID ; Glanders: CFT ; Dourine: CFT and Surra; card agglutination test for trypanosomosis (CATT)

For testing of donors stallions (EVA and CEM on germinal products), the tests are not changed

The source of information of these tests can be found in the EURL websites (AHS and EURL for equine diseases) and is open access

Comments of the participants

Cécile Beck (FR): Is it an obligation for the EURL to implement haemaglutination inhibition test (HIT) for VEEV serology testing? For the moment, PRNT/MNT are the methods used by the EURL. Answer: for the third countries: we have to agree with the manual test OIE terrestrial manual, which is the international standard. The HIT for VEEV is required in the certificate. The EURL has to carry out the same test as required in the certificate if the status of an animal has to be verified.

Stéphan Zientara (FR): Is it necessary to put in place a surveillance system with organization of PT for this new set of diseases (Surra and VEEV for example). Answer: Yes, they are in the list of notifiable disease that carry out necessary surveillance. We have to manage at the same level like dourine disease. The new Certificates will be applicable from around 15 of October 2021.

Please see: Animal Health Law and Equine diseases- state of play (Alf-Ecbert Füssel) (Part: PT/workshops/Workshop presentations)

2 Overview of WNV epidemiological survey in Europe (2017-2020) and France (G. Gonzalez, ANSES, France)

Dr. Gaëlle Gonzalez presented an overview of WNV circulation in Europe from 2017 to 2020 in equids, birds and mosquitoes. A "one health" surveillance approach for WNV surveillance is implemented in Europe. Indeed, WNV infection is notifiable in Humans and equids in the European Union. The European centre for disease control (eCDC) reports WNV positive cases in humans since 2010, in equids since 2017 and in birds (on a voluntary basis) since 2020.

Several countries implement an active surveillance system on equids, birds and mosquitoes as Spain, Italy, Denmark and Serbia ; on equids and birds (Poland and Cyprus) and on equids only (Greece, Romania and Czech Republic). The other EU members are doing a passive surveillance. In order to confirm WNV cases, NRLs practise both indirect diagnostics by serology (ELISA and VNT) and direct diagnostic by qRT-PCR and virus isolation. From 2017 to 2020, EU members reported WNV outbreaks in equids in Mediterranean (high number of cases in Spain, Italy, and Greece) and Central (Germany and Hungary). European countries. Positive bird cases were also reported in countries where equids outbreaks occurred. This four years period was marked by an important increase of WNV outbreaks in equids in 2018, a drop in 2019 and an increase in 2020. Positive bird cases were detected in each country that reported equid outbreaks. In 2018, Germany reported its first's equid and bird WNV outbreaks and the introduction of WNV lineage 2 was demonstrated. In 2019, the number of cases increased with an endemic seasonal circulation of WNV lineage 2 through resident birds. Another important point highlighted was the dramatic increase of WNV positive horse and bird cases in Hungary in 2018. France notified its first outbreak in avifauna in 2018 with the identification of WNV lineage 2. Focusing on human surveillance, Europe faced in 2018 its largest outbreak (> 7,2 higher than in 2017).

In 2020, Spain faced a dramatic increase in WNV positive cases among horses in the Southern provinces of Seville, Cádiz and Badajoz (WNV lineage 1 was identified). The Spanish NRL also identified WNV lineage 2 in 3 birds in Catalonia (as in 2017). The Netherlands reported its first WNV outbreaks among birds in 2020.

In conclusion, 2018 was an important year for the "One health" approach for WNV surveillance. A 30% increase of WNV equid outbreaks was noticed in 2018 with an expansion of WNV lineage 2 circulation in Northern (Germany and The Netherlands) and further West in the Mediterranean regions (South of France and North of Spain). This highlight the need to strengthen national preparedness for seasonal WNV outbreaks (integrated animal-human WNV surveillance.

Please see: Overview of WNV epidemiological survey in Europe (2017-2020) (Part: PT/workshops/Workshop presentations)

3 WNV 2018-2020 outbreaks in NRLs: Italy (G. Savini, IZS), Greece (K. Tasioudi, Veterinary center), Netherlands (H. Graham, WBR)

Dr Giovanni Savini presented the National Integrated monitoring plan for WNV and USUV in Italy and WNV circulation in Italy from 2017 to 2020. To date, 16 out of 20 Italian regions are considered endemic and WNV circulation caused by genetically divergent isolates has been recorded every year.

Please see –WNV 2018-2020 outbreaks in Italy (Part: PT/workshops/Workshop presentations)

Dr Heather Graham presented the WNV 2020 outbreaks in Netherlands: In August 2020, there was the first detection of WNV in birds (3 birds) and mosquitoes (2 pools) in the Netherlands. In October 2020, there was the first detection of autochthonous human WNV infections (6 human cases). Patients were presumed to have been infected with WNV between July and August 2020 and all of them resided in regions where WNV was detected in birds and mosquitoes.

Comments of the participants

Stéphan Zientara (FR): Do you have a specific surveillance system for horses? Answer: No we have not.

Please see –WNV 2018-2020 outbreaks in Netherlands (Part: PT/workshops/Workshop presentations)

Dr Konstantia Tasioudi presented the WNV surveillance programme with passive and active surveillance on horses and wild birds and WNV 2018-2020 outbreaks in Greece. She also described a case of WNF in dogs in 2018. The genome of distemper diseases and WNV viruses were detected by rtRTPCR in this dog.

Comments of the participants

Giovani Savinni (IT): do you know the Ct value of the positive dog and if yes do you think dogs have titres enough to infect mosquitoes ?

Answer: Ct around 14 – 17 in different organs and for distemper disease, ct among 20 and 30. Regarding the second question, it is a difficult question. I do not know.

Sylvie Lecollinet (FR): did you obtain results from the brain? Could you identify WNV in the brain and do you know if there was a suspicion of comorbidity and/ or immunosuppression in this dog?

Answer: The brain was also positive for this dog.

Tamas Bakonyi (SE): we can find analogy cases of immunosuppression in 2003 with Geese co-infected with WNV lineage 1 and with a circovirus

Please see WNV 2018-2020 outbreaks in Greece; WNV 2018-2020 outbreaks in Netherlands (Part: PT/workshops/Workshop presentations)

4 EU-wide surveillance of WNV infections: public health challenges (C. Gossner, ECDC)

Dr Celine Gossner presented the European wide Surveillance and Early warning WNV infection surveillance systems in humans in the EU/EEA. The first objective of this real time surveillance is to ensure blood safety.

Epidemiological situation between 2015- 2020 was presented with 2839 human cases that were reported in the EU. The year 2018 was a very special year with highest number of cases, highest number of countries that has reported cases, human cases for the first time in two countries (Czech Republic and Slovenia), first bird and equid cases in Germany. In 2020 was also a special year with birds and human first cases in Netherlands and large equine outbreaks in Spain with lineage 1.

Challenges : Some countries declare positive asymptomatic blood donors and others don't, some countries declare probable and confirm cases while others only report confirm cases, some others declare only WNND etc…Comparison between countries should be made carefully. Timeliness: due to cycle transmission of reporting (detection and confirmation at national level) data are obtained very late at the European level

Another big issue is that blood safety measures for the travellers are only based on human cases in the region despite maps with horses and bird cases are provided:

ECDC actions in 2021: enhanced the surveillance, systematic review to define the reliability of urine testing to detect flavivirus, provide laboratory support to member states (EVD LabNet), and develop a guidance for the surveillance prevention and risk assessment of WNV and USUV

Comments of the participants

Tamas Bakonyi (SE): 2019 was also the second highest number of human cases reported (400 human cases) and for 2020, you have to keep in mind that this year was influenced by a bias due to Covid crisis.

Please see: EU-wide surveillance of WNV infections: public health challenges (Part: PT/workshops/Workshop presentations)

5 Current state of USUV circulation in Europe (G. Gonzalez, ANSES, France)

Dr. Gaëlle Gonzalez presented the circulation of Usutu virus (USUV), another flavivirus closely related to WNV transmitted in the same enzootic cycle. USUV infection affects mostly wild and captive birds (93 species infected) and is highly pathogenic to blackbirds, grey owls and house sparrows. It induces a massive mortality of wild and captive avifauna. The EU members implemented a passive surveillance for USUV detection on birds and mosquitoes. There is a genetic diversity of USUV strains circulating in Europe. The majority of USUV strains detected in humans, bids, mosquitoes and bats belong to the European lineages with sometimes the incursion of African lineages. As for WNV outbreaks, 2018 was marked by an important increase of USUV positive birds reported in Austria, Belgium, France, Italy, Switzerland and The Netherlands. In 2020, the United Kingdom reported the emergence of USUV Africa 3 in wild birds. In 2017 and 2018, Germany reported the circulation of four different lineages of USUV with the first occurrence of USUV Europa 2 in 2018 and a massive spread toward North in 2018. France reported an expansion of USUV circulation across the territory and the identification of USUV

Africa 3. USUV infection in humans remain mostly asymptomatic. There is since 2018, an increase of human cases with neurological signs (identification of USUV Europa 2).

In conclusion, there is the circulation of European and African USUV lineages. We could notice the occurrence of numerous and sustained outbreaks in avifauna that represent a serious warning signal of zoonotic risk for USUV.
Another important point is the expansion of USUV Europa 2 that induces neurological symptoms in humans.
As USUV is closely related to WNV and transmitted by the same vector (*Culex spp*.), what do we know about the co-circulation, the co-infection of birds and mosquitoes? All these questions will be explored during research projects.

Please see Current state of USUV circulation in Europe (Part: PT/workshops/Workshop presentations)

6 Co-Infections: Simultaneous Detections of West Nile Virus and Usutu Virus in Birds from Germany (U. Ziegler, FLI, Germany)

Dr Ute Ziegler presented the distribution of USUV positive birds in Germany in 2018 with 1183 laboratory confirmed cases and a massive distribution to all federal states. The first introduction of WNV in Germany was observed in 2018 with bird and equine cases and by an extension of the circulation area in 2019. In this framework, co-infections with WNV and USUV were detected in six dead birds collected in 2018 and 2019. Genomic sequencing and phylogenetic analyses classified the detected WNV strains as lineage 2 and the USUV strains as lineages Africa 2 (n = 2), Africa 3 (n = 3) and Europe 2 (n = 1). Further evidence for WNV-USUV co-infection was obtained by sampling live birds in four zoological gardens with confirmed WNV cases. Three snowy owls had high neutralizing antibody titres against both WNV and USUV, of which two were also positive for USUV-RNA. Further monitoring in live captive birds from different zoological garden in 2020 showed massive antibodies against WNV or USUV on pink flamingos (unpublished data). Several birds were also positive, with high neutralizing antibody titres, against both viruses. In conclusion, further reports of coinfections in animals as well as in humans are expected in the future, particularly in areas where both viruses are present in the vector population

Comments of the participants

Cécile Beck (FR): How can you assert that there was co-infection or sequential infection because of the serological cross-reactions between WNV and USUV?
Answer: In lower titres, we cannot conclude. In this context, you have very high titres against both viruses and we know that WNV and USUV circulated in this area in the same year.

Please see: Co-Infections of WNV and USUV in Birds from Germany

SESSION 4: Arthropod borne encephalitis diagnostic and Proficiency tests

1 The encephalitic alphaviruses: Ecology and Epidemiology of Human and Equine Diseases (S C. Weaver, UTMB, USA)

Dr Scott Weaver presented the epidemiology of three main alphaviruses: VEEV, EEEV and WEEV.

*VEEV remains a major threat for both human and equine disease and is overdue for epizootic emergence in South America. The 1993 Venezuelan outbreak (at least 26 equine cases and 5 virus isolation positive human cases) was due to a single E2 mutation that transformed the Enzootic ID equine Phenotype into the Epizootic (IC) Phenotype. The Signs and symptoms of VEE are nonspecific in humans and can be easily confused for dengue and other infectious diseases. The mortality rate in equids are 50-70% with neurological sequelae in many survivors. The number of endemic VEE cases annually is estimated around tens-of thousands. For example, 20-60% human seroprevalence was observed in Chiapas State, Mexico in coastal town.

*EEEV remains a threat for both human and equine disease, with recent evidence of human disease in Latin America and increasing cases in North America.2019 saw the largest epidemic in many decades.

*WEEV has virtually disappeared as a human and equine disease in North America, probably due to ecologic factors that have reduced enzootic circulation, but remains a threat in Latin America

Comments of the participants

Markus Keller (DE): do you think we can have recombination across alphaviruses (EEEV and WEEV for example) with higher zoonotic potential ?

Answer: I think it's very unlikely that an alphavirus will recombine between another group of RNA viruses alphavirus. Alphaviruses are very inefficient to recombine among themselves.

Stéphan Zientara (FR): there is no EEEV vaccine in human: is it because the market is very small or is it link to special biology?

Answer: EEEV is a very potent biological weapon. US army has been developing vaccine to protect military personal for a long time. A phase one clinical trial was done a few years ago with DNA vaccines and that probably the closest we have gotten to have a vaccine for human. I suspect we will eventually have one, just because the military are so concerned that it could be used as a bioweapon. If that happens, it would be a very good thing because we know where high-risk locations are, where people would need one. We don't need to vaccinate the entire population and it could be done in a very targeted manner. So, If the military payed to have a vaccine, which they have some interest in doing. it would be very good for the resident population. They is a little bit more interest in VEEV in the military because it was more developed, and weaponized during the cold war.

Stephan zientara (Fr): Western equine encephalitis. You have shown that there is no case anymore in North America since 98 and that it is probably linked to ecological factors, do you have an idea of which factors could be involved to explain this situation?

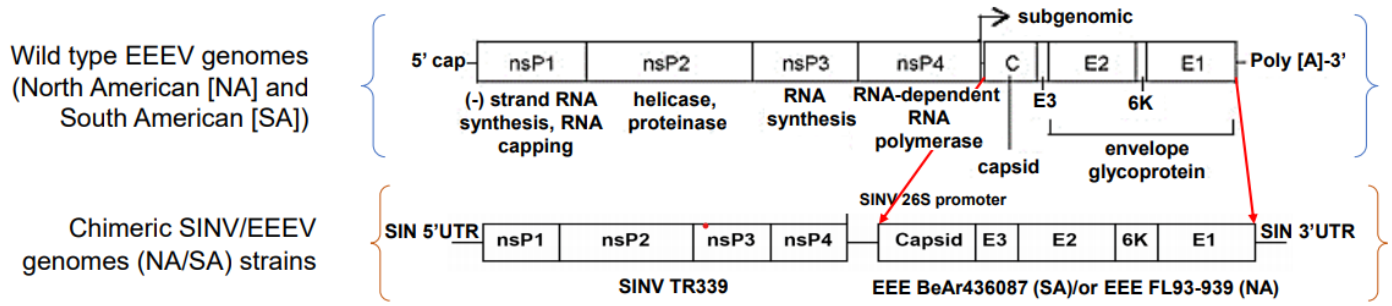
Answer: I suspect is has something to do with avian populations and changes in agricultural practices. In western areas of the US, water is in very short supply and irrigation of farmland is much more efficient now than in the past: less standing water

on the field at the end of irrigation. Therefore, the population of the *Culex* vector is probably lower than it was several decades ago. But this is my best educated guess but we really don't have a complete understanding over what has happened.

Please see: the encephalitic alphaviruses: Ecology and Epidemiology of Human and Equine Diseases (S C. Weaver (Part: PT/workshops/Workshop presentations)

2 Equine encephalitis testing (R. Garrison, USDA, USA)

Dr Robert Garrison presented the advantages of a Sindbis virus (SINV)/EEEV recombinant virus in diagnostic test. Eastern equine encephalitis virus (EEEV) is a highly virulent, mosquito-borne alphavirus that causes severe and often fatal neurological disease in humans and horses in eastern North American (USDA, 2019: 184 equine cases). EEEV infection is diagnosed serologically by anti-EEEV-specific IgM detection, plaque reduction neutralization test (PRNT), hemagglutination inhibition (HI) or complement fixation (CF). Live virus is used in the PRNT procedure, which currently requires biosafety level 3 (BSL-3) conditions. Chimeric SINV/EEE virus grows in both Vero81 cells and C6/36 insect cells, is completely inactivated using 0.2% of beta-propiolactone allowing this use in BSL-2 conditions. Chimera can replace wild type (WT) antigen in ELISA and HI and is under evaluation to replace WT EEE in PRNT assays. It can be used as a positive control in molecular diagnostics. In conclusion, this chimeric SINV/EEE has comparable performance characteristic to WT EEEV in every diagnostic assay tested in the Diagnostic virology laboratory



Considering their benefits in increased safety and reduced regulatory requirements, these chimeric viruses should be highly useful in diagnostic laboratories throughout the Americas.

Comments of the participants

Stéphan Zientara (FR): do you use horses as surveillance tools for human cases of EEEV in the US?

Answer: They are not used in an active surveillance sense. The short answer is no we do not use horses for sentinel purposes.

Stéphan Zientara (FR): How many labs in the US can do a diagnostic of EEEV?

Answer: there are a number of state laboratories. Almost every state has a diagnostic laboratory. Not all labs utilize EEEV in their assays but several do: Florida, Cornell, Michigan state university. Wherever there is a consistent footprint of EEEV, the state have the facilities to utilize the EEEV.

Stéphan Zientara (FR): Do you organize proficiency test?
Not at this time

Gaelle Gonzalez: Can you detect the virus in the birds before the cases in equine and human happen?
Answer: It's an interesting question. Vector biology is to some extant a mystery. We know there is an ongoing enzootic cycle.We would like to know more about the enzootic EEEV bird cycle: which bird are involved etc. There is a lot surveillance on mosquitoes work done in US. For birds, It's done in limit basis it can be done but not done widely.

Gaelle Gonzales: What do you know about the migration of these birds?
Answer: The question of bird migration as a mean to transporting the virus is very valid. My suspicion is that it is not a major factor. It is possible but not a major factor for moving the virus from one state to another, unlike influenza for example.

Céline Gossner (EU CDC): what would be the likelihood, or the pathway of introduction of these viruses, and the likelihood of spread within the EU? Do you think the member states would be capable of detecting a case?
Cécile Beck: we have RT PCR to detect the virus, and we can used VNT test to detect antibodies. At the EURL level, we have some capabilities.

Gaelle Gonzalez: Is it possible develop an IgM Elisa test with chimeric virus for VEEV?
Answer: For VEEV, we don't have enough samples to validate it properly but it should be a similar test like EEEV

3 Outcome of Proficiency test on arthropod-borne encephalitis viruses serology (C. Beck /S Lecollinet, ANSES and CIRAD, France)

Dr Cécile Beck presented the outcome on arthropod born encephalitis viruses serology Proficiency tests, which included 31 participants (25 NRLs from EU member states + 3 NRLs from neighbouring countries (Switzerland, Serbia and Tunisia), as well as 3 external participants (institutes involved in WNV surveillance, kit producers). For the first time, NRLs were proposed also to test their serological tools for the diagnosis of EEEV, VEEV and WEEV infections in horses. The PT allowed Anses to estimate the number of NRLs that could perform such analyses and to compare the serological tools available. Conclusion: Competition ELISA tests are well established with 25 NRLs performing the test in 2020 (1 more NRLs compared to 2016). Satisfactory results for 23/25 participating NRLs. MAC ELISAs are now also well established, with 19 NRLs performing the test in 2020 (3 more NRLs compared to 2016). Satisfactory results for every participating NRLs . We encourage the NRLs to implement this assay in their lab. There is a low establishment of WNV or JEV VNT in NRLs. The sensitivity of WNV VNTs could be slightly improved on low positive samples in 4 out of 6 NRLs .The development of alternative tests to flavivirus VNT (that could be performed with reduced constraints and in particular under BSL1/2) should be promoted. Concerning EEEV, WEEV and VEEV serological diagnostics: only 3 NRLs developed serological tests against alphaviruses. There is a need to improve EEEV, WEEV and VEEV diagnostic methods but but it is very difficult to find well-characterized IgG or IgM positive seraThe development of

alternative tests to alphavirus VNT should be promoted too (cf new VEEV DE status in the animal health law, Regulation (EU) 2018/1882)

Please see: Outcome of PT on arthropod-borne encephalitis viruses serology (C.Beck) (Part: PT/workshops/Workshop presentations)

4 Outcome of Proficiency test on arthropod-borne encephalitis viruses molecular diagnostics (C. Beck/S. Lecollinet, ANSES and CIRAD, France)

Dr Cécile Beck presented the outcome on arthropod born encephalitis viruses molecular diagnostics Proficiency tests, which included 30 participants (24 NRLs from EU member states + 2 NRLs from neighbouring countries (Switzerland and Serbia), as well as 4 external participants (institutes involved in WNV surveillance, kit producers). For the first time, NRLs were proposed also to test their molecular tools for the diagnosis of EEEV, VEEV and WEEV infections in horses. Conclusion: WNV rtRTPCR diagnostic tests well established in NRLs (24 NRLs in 2020 versus 22 in 2016). Good results for the EU NRLs network. Sensitivity and/or specificity should be improved for 3 NRLs. Keep in mind the lack of specificity (for USUV and JEV) of some methods (Eiden et al 2010-NS2A and LSI Vet Max) for WNV molecular detection. Keep in mind the lack of sensitivity of some rtRTPCR methods (Linke et al 2007, Del Amo 2013, Jimenez Clavero (2006) for WNV lineage 3 detection only. Panflavirus RT-PCR is implemented in 13 NRLs with higher sensitivity of Scaramozino and/or Vina-Rodriguez assays in comparison with the protocol developed by Weissenbock et al. The development of multipex assays for the differentiation of WNV isolates or of diverse flaviviruses should be pursued. Concerning EEEV, WEEV and VEEV molecular diagnostics, 8/24 NRLs implemented JEV rtRTPCR with 100% of satisfactory results, 9/24 NRLs implemented EEEV, WEEV and VEEV rtRTPCR with 100% of satisfactory results. More NRLs are encouraged to implement these methods (cf new VEEV DE status in the animal health law, Regulation (EU) 2018/1882)

Please see: WNV 2020 PT test on molecular diagnostic tools (C. Beck) (Part: PT/workshops/Workshop presentations)

Sandra Revilla-Fernandez (AT) asked if there is a panflavivirus rtRT-PCR to replace the Scaramozino method which is long and subject to a high risk of contamination?
Answer: two participants used a panflavivirus rtRTPCR from the article of Elizalde (2020) with very good results. The EURL would like to validate this method and share the results to the NRLs.

SESSION 5: research program relying on the WNV activities

1 Molecular determinants governing WNV tropism, host range and virulence in France and Europe" (G. Gonzalez, ANSES, France)

Dr Gaëlle Gonzalez presented a research project on the molecular determinants governing WNV and USUV tropism, host range and virulence. She would like to characterize the molecular mechanisms underlying such differences in virulence and pathogenicity between the two viruses and between different strains/ lineages. She is employing interactomic approaches

in order to establish of a robust inventory of common and specific cellular proteins and critical viral determinants implicated in pathogenesis and/or virulence of USUV/WNV in a specific host (equines vs avian vs mosquito's vs humans)

Please see: Molecular determinants governing WNV tropism, host range and virulence (Part: PT/workshops/Workshop presentations)

2 Development of a platform for the identification of antiviral drugs for the treatment of West Nile virus-induced encephalitis (M. Coulpier, INRA France)

Dr Muriel Coulpier presented the development of *in vitro* models of infections by WNV, using equine primary-like brain cells to perform screens (by image and impedancemetry-based screens) to identify antiviral drugs against WNV. These models allowed the identification of molecules from Prestwick and Molport collections that modulate WNV replication.

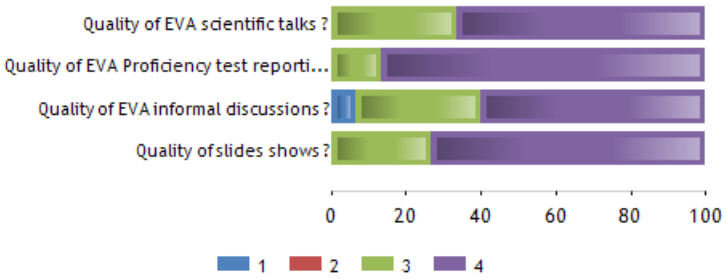
Please see: Development of a platform for the identification of antiviral drugs (Part: PT/workshops/Workshop presentations)

EU RL workshop 2021 - Analysis of questionnaires

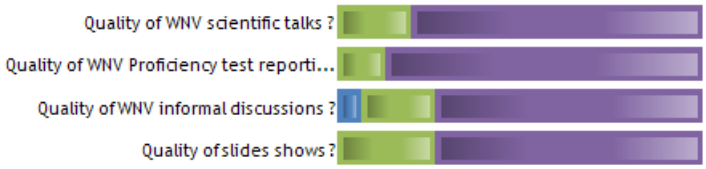
A questionnaire and global evaluation of the online EVA and WNV workshops were sent on 03 of October to know the feedbacks of NRLs. 15 answers for these workshops were received from participant, anonymously or not.

The evaluation of each item followed this graduation scale: **1: poor; 2: average; 3: satisfactory; 4: very satisfactory**

Content: EVA workshop



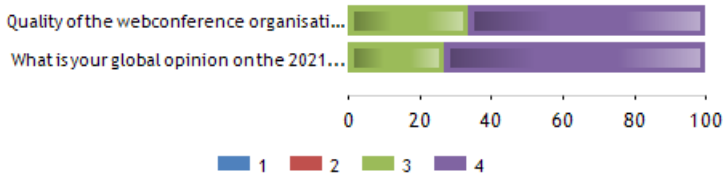
Content: arthropod-borne encephalitis viruses workshop



- **General comments or proposals on topics for the next arthropod-borne encephalitis viruses workshop**

	Participant's comments	EU RL response
1	It would be interesting to discuss how to deal with those countries where monitoring investigations is not planned, but research work shows that the disease is relevant in the country.	

Organisation and global assessment of the 2021 workshops



- Have you any other comments or proposal concerning the organisation of the workshop?

	Participant's comments	EU RL response
1	Informal discussions were not really possible due to the nature of the workshop (Teams, online), but there is no easy solution for this.	Yes. We really hope to be able to organize a face-to face seminar in 2022

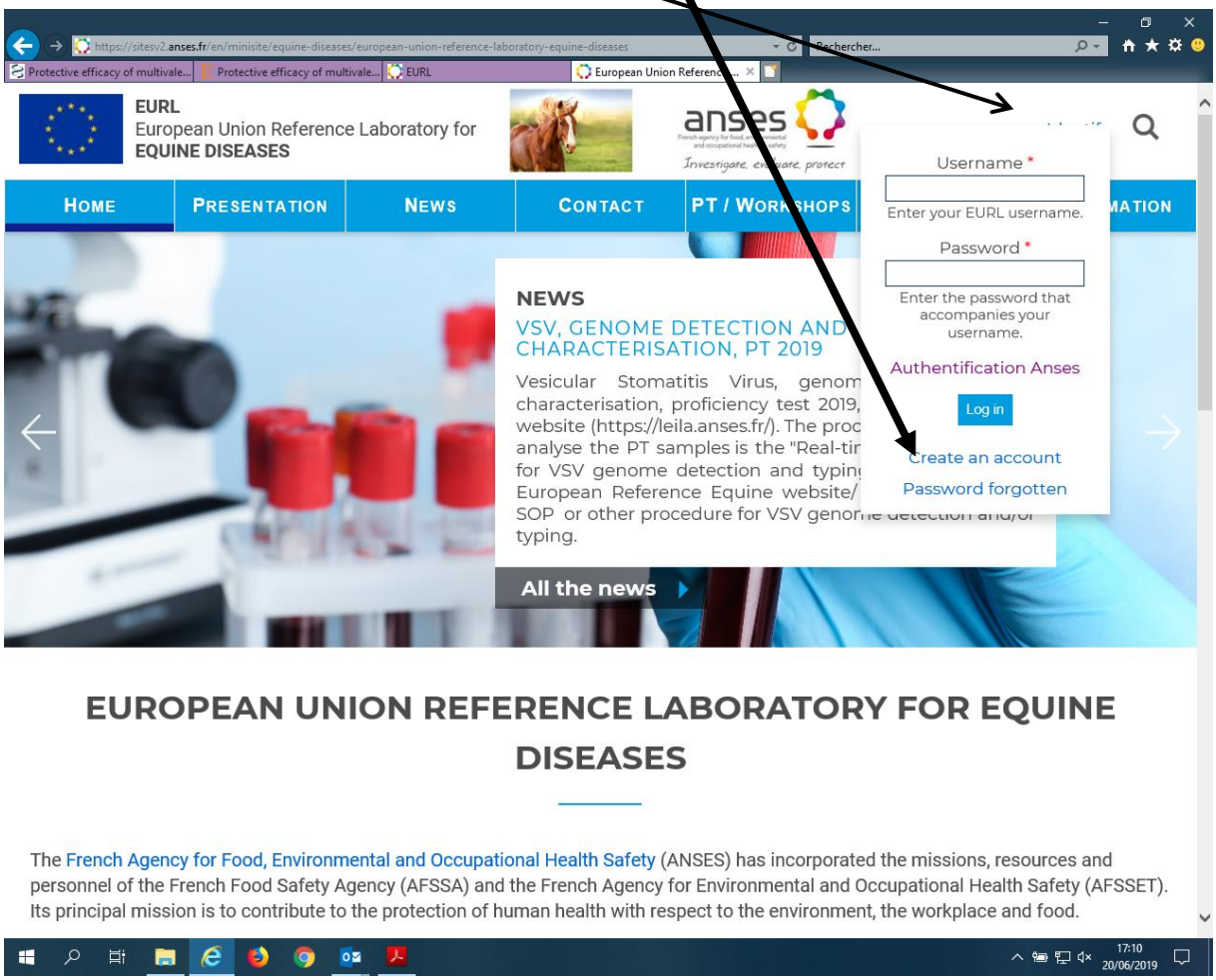
Annex 1

To create an account

Home and presentation are in open access ; the others links (news, contacts ….) are in private access.

The URL is: **https://eurl-equinediseases.anses.fr**

To go to the private access you have to identify and create an account Chose Microft Edge, chrome or Firefox



You can choose (and remember !!) your username and password

The validation Key is : EQUINE2014

When you have submitted your registration you will receive a confirmation of your submission by email .

After validation of your inscription by the webmaster, you will receive by email a confirmation of your inscription and you'll be able to connect to the private access.

Workshops of the European Reference Laboratories for EVA and arthropod-borne encephalitis viruses (WNV, JEV, EEEV, WEEV and VEEV).

Agenda

15 – 16 April 2021

Webconference, France

Workshop of the European Reference Laboratories for EVA

Thursday, April 15, 2021

SESSION 1: EVA epidemiology and research program

9:20 Opening Session

9:30 Overview of EVA epidemiological survey in Europe (2017-2020) (JC. Valle-Casuso, ANSES, France)
Overview of EVA epidemiological survey in Italy (2017-2020) (M.T Scicluna, IZS, Italy)

10:00 EVA 2018 outbreak in France (D. Gaudaire, ANSES, France)

10:15 EVA 2019 outbreaks in England (F. Steinback, APHA, United Kingdom)

10:30 Development of an HTS assay for EVA drugs Screening (JC. Valle-Casuso, ANSES, France)

SESSION 2: EVA proficiency tests

10:45 Outcome of Proficiency test on EVA serological diagnostic (D. Gaudaire, ANSES, France)

11:15 Outcome of Proficiency test on EVA virological diagnostic (D. Gaudaire, ANSES, France)

11:45 Discussion and conclusion of the EVA session.

Workshop of the European Reference Laboratories for arthropod-borne encephalitis viruses (WNV, JEV, EEEV, WEEV and VEEV)

Thursday, April 15, 2021

SESSION 3: WNV and USUV epidemiology

13:40 Opening session

13:45 Animal Health Law and Equine Diseases - state of play (Alf-Eckbert Füssel, EC)

14:00 Overview of WNV epidemiological survey in Europe (2017-2020) (G. Gonzalez, ANSES, France)

14:30 WNV 2018-2020 outbreaks in NRLs: France (G. Gonzalez, ANSES), Italy (G. Savini, IZS), Germany (U. Ziegler, FLI), Greece (K. Tasioudi, Veterinary center), Netherlands (H. Graham, WBR)

15:30 EU-wide surveillance of WNV infections: public health challenges (C. Gossner, ECDC)

15:45 Break

16:00 Current state of USUV circulation in Europe (G. Gonzalez, ANSES, France)

16:15 Co-Infections: Simultaneous Detections of West Nile Virus and Usutu Virus in Birds from Germany (U. Ziegler, FLI, Germany)

16:30 Discussion and conclusion of the first day

Friday, April 16, 2021

SESSION 4: Arthropod borne encephalitis diagnostic and Proficiency tests

13:40 Opening session

13:45 The encephalitic alphaviruses: Ecology and Epidemiology of Human and Equine Diseases (S C. Weaver, UTMB, USA)

14:15 Equine encephalitis testing (R. Garrison, USDA, USA)

14:45 Outcome of Proficiency test on arthropod-borne encephalitis viruses serology (C. Beck /S Lecollinet, ANSES and CIRAD, France)

15:15 Outcome of Proficiency test on arthropod-borne encephalitis viruses molecular diagnostics (C. Beck/S. Lecollinet, ANSES and CIRAD, France)

15:45 Break

SESSION 5: research program relying on the WNV activities

16:00 Molecular determinants governing WNV tropism, host range and virulence in France and Europe" (G. Gonzalez, ANSES, France)

16:15 Development of a platform for the identification of antiviral drugs for the treatment of West Nile virus-induced encephalitis (M. Coulpier, INRA France)

16:30 Discussion and conclusion of the second day