EU REFERENCE LABORATORY
FOR

Report of the 10th Workshops of the National Reference Laboratories for equine diseases (Subject: EIA and dourine)

12 & 13 September 2018
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.

Workshop on EIA

46 participants coming from 22 NRLs (Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Estonia, France, Finland, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Netherland, Poland, Romania, Slovakia, Slovenia, Spain and United Kingdom) and two third countries (Argentina and Serbia) attended the EIA workshop which took place at ANSES Maisons-Alfort. Alf-Eckbert Füssel was representing of the European Commission.

1. Opening and participant introduction (S. Zientara and C. Beck, ANSES Maisons-Alfort - France)

All the participants introduced themselves during the round table. Cécile Beck, deputy head of the equine diseases EU-RL, presented the website (webmaster: Cécile Beck) and the convivial event.

2. Equine Diseases and Animal Health Law – listing and categorisation (Alf-Eckbert Füssel, EC)

Alf-Eckbert Füssel presented the new Animal Health Law (AHL) regulation that will be applicable starting april 1st 2021. Five diseases (Foot and Mouth Disease, Classical Swine Fever, African Swine Fever, Highly Pathogenic Avian Influenza, African Horse Sickness) are mentioned in this new regulation. A new classification was made for all the diseases based on different assessment criteria. The assessment of diseases was performed with the support of EFSA and EU reference laboratories and classified in 5 categories A, B, C, D and E. A diseases are diseases considered exotic with immediate eradication for EU; B diseases = compulsory eradication (tuberculosis, brucellosis…); C disease = voluntary free status or programmes of eradication or not (Bluetongue, Bovine Leukosis…); D diseases= trade measures (EIA, Dourine…); E diseases = monitoring and notifiable diseases. A, B, C and D diseases are also and always E diseases.

Regarding equine diseases the classification is as follow:

A diseases = AHS and Glanders

D diseases = EIA, Dourine, surra, VEE, anthrax, rabies and EVA, CEM for germinal products only.

E diseases = JE, WN, EEE, WEE only on monitoring surveillance due to zoonotic aspect related to human case as WNV.

Please see: Equine Diseases and Animal Health Law – listing and categorisation (part workshop_ private access)

Comments of participants:

- Participant question: Which trade measures can be implemented for diseases D? Do we ask to trade country or a part of the country to be free for the disease or just the holding?

- Answer: It will be like the OIE code. The country is free for 3 years or otherwise recognised to carry out a control/eradication programme since 6 months; the holding is considered free along the lines described by the OIE code.

Participant question: stallions are tested for EAV and can move around if they are positive or only the semen is tested?

- Answer: according to legislation, shedders stallions can move inside EU without control but EU asked to third countries that only non-shedders stallions can enter in EU.

- EURL participant question: about equine exotic encephalitis (eastern, western, venezuelan and japanese equine encephalitis) do we have to organise a PT for those diseases as for WNV?

- Answer: those diseases are in the EU-RL. The main question is more about who will confirm a case? It is the EU-RL so. EU-RL needs to have the material to perform analysis.

- Participant question: for which diseases, horses need to be tested before importation in EU?

- Answer: Sanitary rules for importation of horses inside EU are existing, depending the country of origin.

3. **Overview of Equine infectious anaemia in Europe (A. Hans, ANSES, France)**

Aymeric Hans presented an overview of EIA in Europe between 2014 and 2018. Data presented were sent by NRLs. In north of Europe (Norway, Sweden, Lithuania, Denmark, Latvia, Estonia, Finland). Between 2014 and 2018, around 30,000 EIA analyses have been performed and only 2 outbreaks with 2 positive horses have been declared. In the western european countries (Belgium, Netherlands, Spain, France, Ireland, UK) 9 outbreaks were declared with 9 positive horses from 2014 to 2018. In central european countries (Germany, Poland, Slovakia, Czech Republic, Hungary, Austria), 60 EIA outbreaks were confirmed with 79 EIA positive horses. In Italy and the balkan countries (Croatia, Romania, Slovenia, Serbia, Bulgaria), 1896 EIA outbreaks were registered between 2014 and 2018 with more than 2200 EIA positive horses. In conclusion, during the last 4 years EIA positive cases have been declared in 15 european countries indicating that EIA is not an exotic disease for EU and the majority of EU countries have to face this problem.

Please see: Overview of Equine infectious anaemia in Europe (A. Hans, ANSES, France)

Comments of participants:

- Participant question: which countries present the real prevalence of EIA?

- Answer: we do not have the prevalence since positive horses have to be euthanised in the majority of EU countries. So it is very difficult to have a voluntary testing from owners

- Participant comment: 7000 to 8000 thoroughbred horses tested per year are negative in Ireland.

4. **EIA 2017 outbreaks in NRls : Spain: (M.V. Castro Cuello, Central Veterinary Laboratory) ; Netherlands (E. Weesendorp, WUR)**

Victoria Castro, from the Spanish NRL, presented the outbreaks that occurred in Spain in 2017. First EIA outbreak recorded in 1976 in Spain was followed by several outbreaks in 1978 affecting racehorses with more than 900 horses at risk. Before 2017, the last EIA outbreak was declared in 1983. In 2017, the first outbreak was located in Candeleda in Castilla Léon with one asymptomatic positive case out of 32 animals. Notification to the OIE was carried out on July 18th 2017. Samples of spleen and lymphnodes were sent to the EU-RL in September 2017. A second outbreak was declared in Placencia in a group of 8 horses; one was positive without clinical sign. The spanish NRL implemented some PCR protocols (Nagarajan et al. 2001; Dong et al. 2012 as well as the Gaudaire et al. 2017) to detect the virus in different organs of the positive horse for EIA. They were able to detect the virus in lymphocytes and in organs (spleen and lymphnodes) of the three positive horses. Virus sequences obtained indicated that the 3 horses were infected with the same virus.

Eefke Weesendorp, from the NRL of Netherlands, presented the outbreak that occurred in the polo ponies in the Netherlands during 2017. Historically, the Netherlands was free from EIAV. On Friday 23rd of June 2017, one serum sample out of 62 received for EIA AGID testing, was positive without clinical signs. Those horses, from a polo club, were tested due to EIA cases declared in Germany earlier during the spring. This case was officially declared to OIE on July 4th. The epidemiological survey indicated that this horse was held in Germany between 2001 and 2004 and was previously imported from Uruguay and sold to Netherlands in 2004. Samples were sent to the FLI and to ANSES (EU-RL) for sequencing and the strain seems to come from South America.

Please see:

- EIA 2017 outbreaks in Spain (part workshop_ private access)
- EIA 2017 outbreaks in Netherlands (part workshop_ private access)

Comments of participants:

- Participant question: molecular characterisation showed a link between the two 2017 spanish outbreaks but how the virus disseminate between these two outbreaks?

- Answer: no idea, but results presented here are preliminary data that need to be confirmed by using other primers in order to have more sequences of the virus to compare.

- Participant question: those horses were mix races and were resident horses in Netherlands, but one of them had a German passport.

- Answer: horses from the Polo club were collected on a private initiative of the Polo Club, not on government initiative.

- Participant question: is any indication of needle transmission in the outbreak that occurred in the polo ponies?

- Answer: there is a strong indication for the spread of the virus by needle sharing.

5. **Molecular characterisation of EIAV french strains (A. Hans, ANSES France)**

Aymeric Hans presented epidemiology and molecular characterisation of EIA viruses isolated in France during the last 4 years. In 2014, 2 cases were declared in south-east of France of 2 horses positive separated by 3 km; the index case was an asymptomatic “Frison” stallion of 6 years old. This horse was coming from Netherlands at 6 month old. The second case was a mare in correlation with the index case. In 2017, in December and January, we had an outbreak with 2 premises in the south-east of France. The two cases were related. The latest one was still in the southeast of France with 3 horses positives with the index case having exhibited clinical signs. During the last 4 years, the vast majority of EIA cases declared in France were in the southeast region of the country. Phylogenetic analysis of french strains isolated between 2008 and 2018 were performed. Interestingly in 2012, we had a premise with 3 positives horses infected by 3 different viruses which is very uncommon, meaning three independent events of infection in this farm.

Please see: Molecular characterisation of EIAV french strains (part workshop_ private access)

Comments of participants:

- Participant question: does EU-RL has SOP established for PCR and real-time PCR for EIA?
- Answer: there is no PCR available that is able to detect all EIA strains. That is why, to date, PCR and Real-time PCR are not suitable for EIA diagnosis and should not be used for such purpose. It is recommended to sequence the full gag gene sequence to perform phylogenetic analysis in order to compare data between EU countries.

6. **EIA whole-genome Sequencing by SureSelect Target Enrichment (A. Deshière, ANSES, France)**

Alexandre Deshière from the EU-RL presented his research project regarding the development of a new innovative molecular diagnostic tool for EIA diagnosis using the SureSelect Target Enrichment system. This system is independent of primers and conventional PCR. Up to now, there are only four EIA strains that have been fully sequenced and they belong from 4 monophyletic groups. PCR diagnosis is not suitable even in the gag region since EIA virus has a high mutation rate that is why we decided to develop a new system independent of primers and PCR and based on NGS. The goal was to determine EIA phylogeny of outbreaks based on full genome sequence of the virus and not only on gag gene sequence.

Please see: EIA whole-genome sequencing by Sure Select Target Enrichment (part workshop_ private access)

Comments of participants:

- Participant question: normal NGS without target enrichment is it working?.
- Answer: No
.**- Participant question**: how close are you to have this protocol validated for diagnosis purposes?

- Answer: in term of research project we can have something validated by the end of 2019. For diagnosis purpose it can takes years before OIE approval.

7. EIA surveillance programme in Italy 2014-2018 (M. T. Scicluna , IZSRLT, Italy)

Maria Teresa Scicluna, from the NRL of Italy presented the Risk based surveillance (RBS) programme implemented in Latium region of Italy between 2013-2015. This RBS focused all working equidae; all mule-holding premises; animals previously not tested; slaughtered equids and equids within a 3-km radius from outbreaks for EIA testing. Targeted surveillance reduces the intensity of control but still allows detection of at least the same absolute numbers of cases/outbreaks expected when examining the whole population. As a result, RBS was estimated between 44% to 60% more sensitive in outbreak detection and was clearly more cost effective (reduction of 80% of annual cost surveillance in comparison with 2012)
On this basis, a new surveillance programme based on high risk and low risk region was applied in Italy. In high risk region, all horses older than 12 months are tested every year whereas horses from low risk are tested every three years only. In addition to these low and high risk regions, control of horses considered as high risk continue to be tested too such as working horses, slaughtered equids, mules and all holdings with mules. Then Maria Scicluna presented the development and the use of the WEB-based geographic information system (WEBGIS) tool for the control of EIA in Italy. The main goals are to support Regional RBS programme, ensuring real time tracking of outbreaks, triggering immediate alerts on new cases and outbreaks, providing management tools for outbreak management, allowing "on-field" risk evaluation and management, and providing immediate and updated information to health authorities.

Please see: EIA surveillance programme in Italy 2014-2018 (part workshop_ private access)

Comments of participants:

- Participant question: 17% of samples were positive in ELISA but negative in AGID how do you manage these horses? -- **Answer:** these animals are suspect animals and have to be tested every 6 months until obtaining AGID positive test. Moreover all ELISA positive horses are tested in immunoblot (IB). IB positive is a band for p26 and have gp45 or gp 90 in addition to p26 protein band.

8. EIA surveillance in NRLs: Romania (I. Olvedi, IDAH) and Germany (P. Koenig, FLI)

Irina Olvedi from the NRL of Romania, presented data regarding EIA in Romania during the last 4 years. EIA surveillance programme is implemented according to the strategic programme, approved by the Governmental decision and NSVFSA President order No 35/2016. Equids older than 6 month old are all tested as follows: Equids from backyards are tested once a year, equids from specialised structures are tested every 6 months and stallions are tested 3 times per year.

Patricia Koenig, from the NRL of Germany presented data regarding EIA outbreaks in Germany. Low number of clinically diseased horses and relatively low number of seropositive horses in total compared to the number of outbreaks were notified.

Several peaks of EIA cases were recorded:1/ in 2010, 27 EIA cases mainly from Romania 2/ in 2012, 12 cases due to infected blood donor.3/ in 2014, 2 outbreaks in the Saxony region4/ in 2015,4 outbreaks in Bavaria region and 1 in Saxony regions, 5/ in 2017,2 outbreaks in Bavaria, 10in the polo horse "story" and 2 in Baden-Württemberg regions and 6/in 2018, 1 outbreak declared in the Mecklenburg-Western Pomerania region. Since then, a negative test result is now required for participation in sport events and introduction of new horses in the polo structures.

Please see: EIA surveillance in Romania (part workshop_ private access)

Comments of participants:

- Participant comment: figures about horses slaughtered in Romania would help to see the effort of romanian authorities.
- Speaker comment: no use of ELISA anymore in Romania, because ELISA should be confirmed by Coggins test and thousands of samples were positive for EIA in ELISA and cost was too important.
- Participant comment: need a collaboration between NRLs to have a repository of sequences in Europe in order to compare EIA sequences between the different countries and follow the viruses around Europe.

9. Development and validation of the EU-RL EIA reference sera (D. Gaudaire – ANSES France)

Delphine Gaudaire presented the production of EIA reference sera by the EU-RL. In March 2017, two horses entered in the animal facility. One week later horses were experimentally infected. The first one W601 was infected with plasma collected from positive horse declared in France in 2012. The second one, W670, was infected with the supernatant of Equine Derm cells infected with the EIA Wyoming strain. During the experimental infection, blood was collected from Day 0 to Day 91 post-infection. Antibodies anti-EIA were detectable from days 21 post-inoculation in horse W601 and D 17 post-inoculation in horse W670. No clinical sign was seen in both horses apart a brief hyperthermia. From those animals two batches of sera have been prepared. One weak, 600 vials, and one medium, 610 vials, positive samples were prepared in vials of 500 µl. On those two batches, homogeneity, reproducibility and stability have to be perform before release sera to NRL upon demand.

Please see: Development and validation of the EU-RL EIA reference sera (part workshop_ private access)

Comments of participants:

- Participant comment: it is quite surprising to have higher antibody titer with AGID compare to ELISA, how to explain?
- Answer: it may also depends on the strain for the p26 antigen used by manufacturers (VMRD and ID-Vet) in their kit, which is not known.

10. Outcome of proficiency test on EIA serology (D. Gaudaire, ANSES France)

Delphine Gaudaire presented the proficiency test results of the European NRLs. This year, 2 PTs were organized one using AGID test and one using ELISA test. Lab codification was randomly defined for each PT and the same codification was done for samples for each PT. Samples were lyophilised before sending. For AGID PT 32 laboratories participated and 26 were NRLs in Europe,.24 serum samples were sent to participants with 12 negative sera for EIA and 12 positive for EIA. The 29 participants exhibited a very good specificity and 29 out of 32 were able to find all the positive samples present in the panel. For ELISA PT, 23 laboratories participated to this PT. 24 samples were sent, with 8 negatives and 16 positives for EIA. Each laboratory obtained very good specificity and 21 out of 23 laboratories were able to find all the positive samples. Training session will be proposed to laboratories that obtain unsatisfactory results to those 2 PTs on EIA diagnosis.

Please see: Outcome of proficiency test on EIA serology (part workshop_ private access)

Comments of participants:

- Participant question: how you can be sure that participants do not use ELISA test for AGID PT. Indeed, some samples seemed to be weakly positive in AGID?

- Answer: laboratories should perform PTs as they perform analysis routinely in their laboratory and should not use ELISA to test samples for AGID PT.

Workshop on dourine

46 participants representing 20 NRLs (Austria, Croatia, Cyprus, Czech Republic, Estonia, France, Finland, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Netherlands, Poland, Romania, Slovakia, Slovenia, Spain and United Kingdom) and two third countries (Argentina and Serbia) attended the dourine workshop. Four scientists: François Diaz from OIE, Amber Gustafson from USDA in USA, Teótimo Becú from Clinica Equina in Argentina and Professor Philippe Büscher, ITM Antwerp, Belgium were also invited to participate to this workshop as guest speakers. Dr Alf-Eckbert Füssel was the representing of the European Commission.

1. Opening and participant introduction (S. Zientara and C. Beck, ANSES Maisons-Alfort - France)

All the participants introduced themselves during the round table. Cécile Beck, deputy head of the equine diseases EU-RL, presented the website (webmaster: Cécile Beck). Stéphan Zientara presented the agenda.

2. Equine Diseases and Animal Health Law – listing and categorisation(Alf-Eckbert Füssel, EC)

Alf-Eckbert Füssel, described the Animal Health law adopted in 2016 (Regulation (EU) 2016/429) by the European Commission and the set out of procedures to establish listing and categorisation of animal diseases. For each disease, the control and prevention measures were classified in 5 categories: A exotic/immediate eradication, B compulsory eradication, C voluntary free status or programmes, D trade measures and E surveillance, notifiability. The assessment of each disease has been completed (with support by EFSA and EU-RLs) and there is currently a public concertation on these diseases. When the concertation will be finished, the diseases list will be adopted by the Commission. The current classification of the diseases was presented and it was noted that A and D diseases are also E-diseases (E-diseases = notifiable diseases).

Please see: Equine Diseases and Animal Health Law – listing and categorisation (part workshop_ private access)

Comments of participants:

- Participant question: what are the reasons for classifying rabies in category D?
- Answer: there is an eradication programme for rabies in foxes but for the rest, we have no particular rules concerning rabies in UE legislation except vaccination of dogs.
- Participant question: vesicular stomatitis (VS) is in annex 2 but it is for horses or for all the animals?
- Answer: annex 2 is based on financial analysis of the disease and it has to be reviewed by the Commission and that's why there is now delegated and implementing acts in order to replace annexe 2. VS will most probably be removed. This regulation will only be applied in April 2021. An important emphasis will be put on traceability and surveillance of horses under animal health law (identification of horses, registration of location where animal are regularly kept and a record of horse movement).

3. Activity report on dourine: scientific news, reagents production (Laurent Hébert, ANSES, France)

Laurent Hébert, head of the French Dourine NRL and scientist in charge of dourine activities at ANSES Dozulé laboratory for equine diseases, presented:

an overview of the epidemiology of dourine (according to Wahid database),

- the recent isolation of new *T. equiperdum* strains
- the recent advance on the characterisation of the genetic relationships between the members of the *Trypanozoon* subgenus
- the recommendation of the OIE Terrestrial Manual for dourine and surra case definition
- a description of the OIE Non Tsetse Transmitted Animal Trypanosomoses Network
- the biological reagents provided by the EU RL

Please see: Activity report on dourine: scientific news, reagents production (part workshop_ private access)

Comments of participants:

- Participant question: which strain will you use for the preparation of new positive sera?
- Answer: this point has indeed to be discussed before the setup of the sera production.
- Participant question: when there is a suspicion of dourine you say that you isolate the animal from insects vectors, but what about ceasing all breeding activities?
- Answer: indeed, the first step is to isolate the suspected animal from other animals, which thus include stop breeding.

4. Project of OIE Codes and Manual of Diagnostic Tests and Vaccines for Terrestrial Animals chapter on dourine modifications (François Diaz, OIE, France)

François Diaz presented the activities of the OIE in support of the surveillance and control of Trypanosomoses, in particular dourine. The two OIE standards were presented (1) the OIE Code = to ensure safety of international trade and protection of animal health, (2) the OIE Manual = list of relevant diagnosis methods and requirements for vaccines. It was presented two new draft chapters in discussion i) either a new chapter on *T. evansi* for multiple species and still the chapter 1.2.3. on dourine or ii) two new chapters on Trypanozoon in equids (*T. evansi, T. equiperdum* and *T. brucei*) + a chapter on Non-equine Surra. It was also presented that updated version of Chapter 2.5.3. on dourine (+ in Complement fixation test: Antigen preparation from in vitro propagated parasites) will be proposed for adoption in May 2019 to the World Assembly of Delegates. It was then presented, the role of the OIE Reference Laboratories, the OIE Collaborating Centre, the OIE Network of Reference Laboratories and the OIE Laboratory Twinning and the goal and objectives of the OIE NTTAT Network.

Please see: Project of OIE Codes and Manual of Diagnostic Tests and Vaccines for Terrestrial Animals chapter on dourine modifications (part workshop_ private access)

Comments of participants:

- Participant question: why do you think that for such a long time all the countries worldwide required negative dourine CFT for horses to be imported and didn't require surra to be tested while it is more worldwide represented?

- Philippe Büscher answer: for me surra is just a neglected disease in animals. It was considered since only a few years ago as a multispecies notifiable disease by the OIE; before it was just neglected. But indeed there is no reason why a horse should not be tested for *T. evansi* when it is tested for dourine.

- Participant questions: you say that the OIE is still thinking about these two chapters on surra and horse trypanosomosis. Do you have an idea of the deadline when the decision will be taken?

- What was the reaction of the members when it was proposed one chapter for trypanosomosis on equines?

- Answer: we received many comments regarding the draft chapter on trypanosomosis in equids, and that's why we organised another group and there are still discussions and decisions to be taken on the future organisation of both chapters. So, this is still on discussion.

- Alf-Eckbert Füssel comments: we also have to be careful about all this. We should have a risk assessment, since thousands of horses have been imported into the EU from the surra infected area.

In case of a global chapter, if we have to provide evidence of disease freedom on many species, the question is, how many horses will we move in the future? The health status of horses should not be determined by the status of few isolated animals. We have to be very careful until we have very good diagnostics and measures to be applied in case of outbreaks.

- Philippe Büscher comment: the idea to make a chapter in *Trypanosoma* in equids is that we have good diagnostics. The challenge in the diagnosis is that we cannot always make the difference between *T. evansi, T. equiperdum* and T. *brucei*, and probably because horses are screened for dourine, they are also tested for *T. evansi* because antigens cross react. That's probably the reason why *T. evansi* testing has not been so important in *T. evansi* endemic countries, because such horses will also be reacting for dourine.

- Participant comment: the USDA also indicated that they only test for dourine because it cross react with surra.

5. *T. equiperdum* reagent production and diagnostics at the National Veterinary Services Laboratory (Amber Gustafson, USDA, USA)

Amber Gustafson from the USDA, presented the reagent production and diagnostic activities at the National Veterinary Services Laboratory (NVSL). She described the processes of antiserum production, antigen production, the reagent evaluations for CFT (i.e. antigen titration and antiserum titres) and for IFAT (i.e. production of slides from infected blood, the evaluation using known positives and the standardisation of secondary antibody per lot with titration. Dourine reagent can be ordered following the presented procedure.

Please see: *T. equiperdum* reagent production and diagnostics at the NVSL (part workshop_ private access)

Comments of participants:

- Participant question: have you tested other buffer than the VBD?

- Answer: we have not tested other buffers because the VBD works well for us in the past.

- Participant question: is there any intention to become an OIE reference lab?

- Answer: we are in the works of preparing an application file.

- Participant question: do you make some research on *Trypanosoma*?

- Answer: being a diagnostic laboratory, we do not have a lot of founding for that but we will work on it to prepare an article.

6. Dourine serological diagnosis in Argentina and "Mal de Caderas-Surra" infection in horses (Teótimo Becu, Clinica Equina srl, Laboratorio Equino srl, Argentina)

Teótimo Becú presented the Clinica Equina activities and its relationship with the horse exportations. A focus on dourine serology tests controls for horses exportation from Argentina to all over the world was presented. Then the relationship between surra -Mal de Caderas (*Trypanosoma evansi*) and dourine (*Trypanosoma equiperdum*) was discussed. The presentation then focused on a study performed in North Argentina (Laguna Limpia farm). First, wild hosts and reservoirs of *Trypanosma evansi* (e.g. capybara, Coati…) and the potentials vectors of the disease: Tabanus, Stomoxys and vampire bats (*Desmodus rotundus*) were presented. Then Teótimo Becú presented the results of a sero-epidemiological study performed on 48 horses from a single farm by reporting: clinical signs, presence of ticks and the fresh bite of a vampire bat in the side of the neck of a horse. The results of the serological tests performed were: 43/51 positives to ELISA surra; 30/51 positives to CFT dourine, 51/51 positive to EIA AGID, 35/51 positives to CFT piroplasmosis (*Theileria equi*). It was concluded that this herd of horses suffers a considerable exposure to the Mal de Caderas-surra, EIA and equine piroplasmosis (*Theileria equi*).

Please see: Dourine and surra serological diagnosis in Argentina (part workshop_ private access)

Comments of participants:

- Participant question: were these animals tested for glanders?

- Answer: yes, they were all negatives by complement fixation for glanders.

- Participant question: were they tested for strangles?

- Answer: no, since they did not presented the typical symptoms.

- Participant question: were the animals tested for *B. caballi*?

- Answer: yes and Argentina is free from *B. caballi*.

- Participant question: do you have blood samples from wildlife?

- Answer: It would be possible but the problem is to catch the wildlife (coati or monkey).

- Participant question: Were the animals treated?

- Answer: 3 of them

- Participant question:: what was the outcome?

- Answer: they get better but is probably because in winter there is less acute infections.

- Participant question: did you try the monoclonal antibodies developed by Monzón?

- Answer: no, I use reagents from Philippe Büscher.

- Participant question: what difference do you observe between: dourine and surra? Because physiological misery has been reported in case of dourine. Do you observe such dramatic health condition?

- Answer: no, I have not been able to observe horses in such condition.

- Participant question: do we have to be worried about Chagas disease?

- Answer: Chagas affects human, I don't know if there is a cross reactivity with dourine or surra.

Philippe Büscher comment: there is one published *T. cruzi* case published. So if it happens it is very rare, and we don't have information about possible cross reactivity. For *T. vivax*, we know that there is possible cross reactivity but not for the antigens used by Teótimo Becú.

7. Dourine in Italy in 2011: epidemiological and clinical data, advances in laboratory diagnosis and preliminary genetic characterisation of the T.equiperdum isolate involved (Ilaria Pascucci, IZS Teramo, Italy)

Ilaria Pascucci, head of the dourine Italian NRL in Teramo presented information concerning the 2011/2012 dourine outbreak. The history of the outbreak and the surveillance plan were presented. with a focus on the definition of "suspected case of dourine" and "confirmed case of dourine". Ilaria Pascucci presented the most observed clinical signs, the clinical course, the laboratory diagnosis (Real time PCR, immunoblotting). This study allowed the identification of potential *T. equiperdum* biomarkers and the development of an Indirect ELISA for diagnosis and confirmation of dourine. The molecular characterisation of the isolated strain showed that the strain responsible for dourine outbreak in Italy in 2011 is not genetically related to any *T. equiperdum* strain reported in the literature.

Please see: Dourine in Italy (part workshop_ private access)

Comments of participants:

- Participant question: what was the source of the infection?

- Answer: we were not able to identify the exact source. We identified a potential index case but due to problem of identification of horses it was not possible to trace further the disease.

- Participant question: have you try to adapt directly the strain to in vitro culture?

- Answer: we tried with different media but not with the soft agar since it was not published yet.

- Participant question: you said that you have identified 37 proteins unique for *T. equiperdum*. So can you be able to set up a test that do not cross react with surra?

- Answer: it is a critical point. This study was prelaminar. They did this study comparing the sequences present on database and not on all the strains. You can contact my colleagues for more information.

Philippe Büscher comment: from the according publication, they did not test the immunoblotting from *T. evansi* or *T. brucei* infected animals.

- Answer: indeed, now, they need to compare with serum from animals infected by other trypanosomes.

- Participant question: our kazakhstan colleagues proceed to intratesticular infection of rabbits, and they did not detect parasites in blood.

- Answer: we observed the presence of the trypanosome in the scrotal sac not inside the testicules.

8. **Melarsomine hydrochloride (Cymelarsan®) failed to eliminate T. equiperdum from the CSF of experimentally infected horses (L. Hébert, ANSES, France)**

This study showed that 6 horses presenting *T. equiperdum* OVI parasites in their cerebrospinal fluids (CSFs) were not permanently cured from the infection by the daily administration of 0.5 mg/kg of Cymelarsan® over 7 consecutive days. Therefore, this outcome discourages the use of Cymelarsan® to manage a horse trypanosomosis outbreak, especially in a context when it is not technically feasible to proceed to CSF sampling in order to assess whether the parasites are already present in the CSF or central nervous system (CNS).

Please see: Melarsomine hydrochloride (Cymelarsan®) failed to eliminate T. e (part workshop_ private access)

Comments of participants:

- Participant question how often did you collect the spinal fluid? In addition, how did it work?

- Answer: when you have the material and the competent persons, it is not so complicated. You can proceed to CSF sampling about every 5 days.

Philippe Büscher comment: it was decided not to sample before 5 days to avoid introduction of the trypanosomes in the CSF by the manipulation itself. In addition, at the first samples some horses were already positives.

Alf-Eckbert Füssel comment: about the question of surra and dourine treatments, since now, surra is a listed disease. During EC discussion, it was included the option of treatment of surra affected animals, and the member states immediately said that they will not encourage treatment for surra. However, for the moment, it is just for information.

- Participant question: what do you think of prolonging the treatment?

- Answer: it is technically difficult due to the nature of the drug (arsenical compound) to increase the number of injection during a treatment phase.

Philippe Büscher comment: in fact, the treatment failure was predicted by the nature of the treatment. The results obtained by Hagos was quite surprising.

Laurent Hébert answer: we however observed a slight decrease in the number of parasites in the CSF of treated animals.

Philippe Büscher comment: indeed, these treatments can kill trypanosomes in the CSF in some cases but when the parasites reach the parenchyma of the brain, there is no way to kill them with these drugs.

9. **Trypanozoon infections in horses: diagnostic challenges (Philippe Buscher, ITM Antwerp, Belgium)**

During its presentation, Philippe Büscher analysed the challenge represented by the diagnostic of *Trypanozoon* infection in horses. He first described the relationship between the different trypanosomes strains and their related diseases. The transmission routes and the geographical distribution of these diseases were also discussed. He then presented the limits of the different control measures that could be envisaged (vaccines, interruption of transmission, diagnosis and treatment). Philippe Büscher then detailed the different methods allowing: clinical, parasitological, serological and molecular diagnosis.

Please see: Trypanozoon infections in horses: diagnostic challenges (part workshop_ private access)

Comments of participants:

- Participant question: if I use your antigen (Rotat 1.2) to test a commercial positive serum for dourine, will it be negative?
- Answer: not fully, it depends on where you put the cut off. It is a purified antigen very specific for *T. evansi* but it remains a complex protein with many epitopes shared with *brucei* and *equiperdum*.
- Participant question: which would be the ideal test for the control of horse trypanosomosis in a context of international movement of horses?
- Answer: in principle, you have to follow the OIE manual recommendation and use CFT but there is a lot of discussion. To replace CFT by ELISA, somebody (I hope soon) has to convince OIE that it works. So that is one of the thing to do in the OIE NTTAT network: organise comparative testing between different methods to convince OIE to change the officially recommended method. Personally, I would avoid the setup of a taxon specific serological test diagnosis, since for me it is sufficient to have a test specific of "*Trypanozoon*". Knowing that in any case (*equiperdum*, *evansi* or *brucei*), neurological stage is not treatable. So when you have a positive animal, you have to isolate it or you have to kill it. A positive animal should not travel anyway.
Alf-Eckbert Füssel question*:* for the moment, we consider dourine as a holding disease. If this is now a wild picture we have to look on the holding and the surrounding because vectors can fly and the question is how far shall we look? If we don't want to say that the country has to be free then we have to have an idea of the area. Ex: 200 meters for equine infectious anemia. But what is the distance for surra? How can we regulate this?
- Answer: the answer is not easy. For horses that travel, what you should recommend is a testing before the travel for *Trypanozoon*, preferably by an antibody detection since all infections generate antibodies and if you have a suspected case, the animal should not travel. If animals are negatives but you know that there is surra around with a risk of infection, in that case you can just repeat the tests 2 weeks later. In any case, when you want an animal to travel from an endemic region to a non-endemic region you have to test animal so you can confirm that the animal is negative.
However, for me, dourine should not be considered as a disease that is only caused by *T. equiperdum*, but as a disease that can be caused by any Trypanozoon.

- Participant question: why, the recovery period for dourine is 6 month, if you can detect rather quickly? Why not 2 months? In addition, do we have to test the whole holding, with, as an example, camels for surra, since both diseases are listed? In addition, should be preferred CATT/T. evansi or dourine CFT?

- Answer: this is the issue, that we have now different tests and we know that some are cross reacting. So the issue is to organise a comparative evaluation of the different tests with the different sera from the different infections. We have many sera (from Dozulé, Italia, Mongolia, *brucei* form Gambia). So it is a question of putting person together and to encourage them to share their biological material and to see what tests and serum are available.

For me, the recovery period of 6 month is a non-sense, if an animal is positive serologically after 14 days or even 1 month, so why waiting for 6 month?

- Participant question: about the distance of disease transmission by tabanides, the work on EIA shows that those flies won't go further than 200 m before they go back to the same horse. So it seems that the recommended distance should be 200 m as for EIA.

- Aymeric Hans answer: in US, it was shown that it was 200 yards and it has been then transformed in 200 meters. However, you have to add the effect of the wind and it is not easy to setup a precise distance such as 200 m.

- Alf-Eckbert Füsse comment: As a reference laboratory, you have to look at to these things and to advise the commission on which test is preferred as another.

10. Outcome of the proficiency test about serology of Dourine (L. Hébert, ANSES, France)

Laurent Hébert presented the outcome of dourine serology proficiency tests, which included 26 participants (21 NRLs from EU member states + 1 participant from neighboring countries (Switzerland), as well as 4 external participants (national or private institutes involved in dourine surveillance): Argentina, Chile, Indonesia and USA.

Please see: Outcome of the proficiency test about serology of Dourine (part workshop_ private access)

Comments of participants:

- Participant question: based on which criteria do you accept one mistake. Because one mistake can led to dramatically consequences.

- Answer: it is and artificial limit due to the organisation on PT based on samples prepared from immunised horses. In addition, training session will be organised for laboratories that presented a limit of sensitivity and specificity.

11. Set-up of ELISA for dourine (A. Madeline, ANSES, France)

Anthony Madeline presented the optimisation of an ELISA for the serodiagnosis of dourine. This study shows the comparison of sensitivity and sensibility of the ELISA protocol described by the OIE and of an optimized ELISA protocol bringing significate benefits (faster, cheaper, and easy to perform). Similar results were obtained with both ELISA protocols thus validating the optimised protocol.

Please see: Set-up of ELISA for dourine (part workshop_ private access)

Comments of participants:

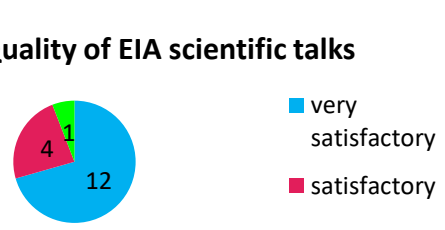
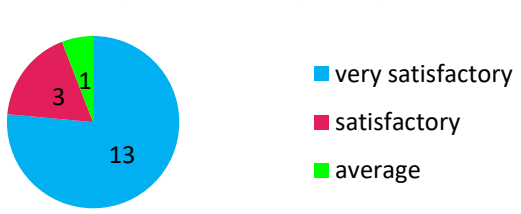
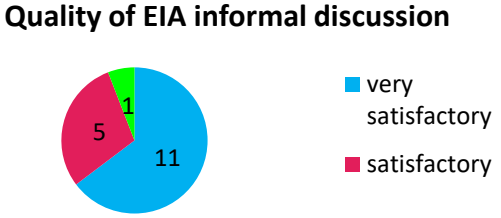
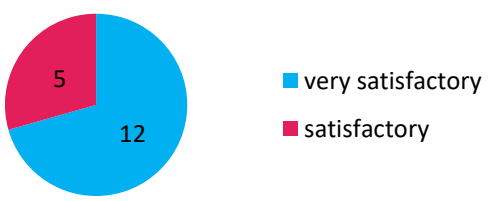
- Participant question: what kind of conjugate did you use? Was it a monoclonal or a polyclonal conjugate?
Anthony Madeline: Polyclonal Horse radish peroxidase conjugate providing by sigma.
- Philippe Büscher comment and question: I would not recommend you to use water for the washing step because that will destroy or at least change the conformation of the Ig that are already bind. But we saw that by changing from ABTS to TMB we had to dilute the sera more. Do you use the same dilution here?
- Answer: Yes:
- Philippe Büscher comment: I am not sure that TMB is cheaper than ABTS, and I would recommend you to dilute your sera (e.g. 1:200) and to make a dilution of your positive sera to confirm your results.
Alf-Eckbert Füssel question: I have a question about the suggestion to change from CFT to ELISA because in many cases, member states complain about using ELISA because if you have not a lot of samples, you have to open a plate only for one or two samples and then you have to discard the plate and they feel that it is expensive. So that's why they prefer to perform individual tests. What is your opinion about this concern?
- Answer: you can have only one strip to test one or two samples. In addition, if you have many samples you can have automates to test many samples by ELISA but it is not the case for CFT.
- Philippe Büscher comment: if you want to perform individual testing, you can think about Immunochromatography that should be produce on commercial bases, but I don't know any single company willing to investigate that because of market failure. I know that there are two companies working on ELISA for *T. evansi*, they would maybe be interested by working on dourine serodiagnosis.
- Answer: If any laboratory wants to develop serological test for dourine, we can share biological material from experimentally infected horses.
- Philippe Büscher comment: as long as you work with native antigens there will always be a problem of reproducibility and I would recommend you to try to develop such test on the based on recombinant antigens and good recombinant antigens exist.

EU RL EIA and dourine workshops 2018 - Analysis of questionnaires

A questionnaire on EU RL EIA, dourine and global evaluation of the workshops were sent on 17 of September to know the feedbacks of NRLs. 17 answers for EIA and dourine and 23 answers for the global evaluation of the workshops were received from participants, anonymously or not.

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

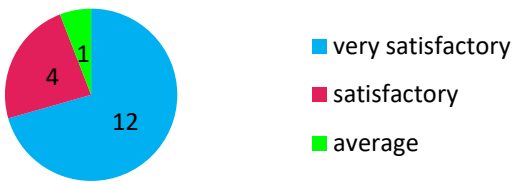
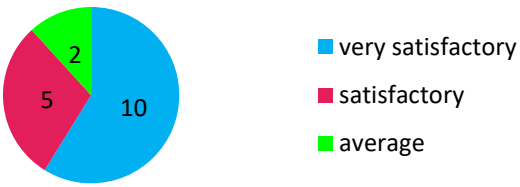
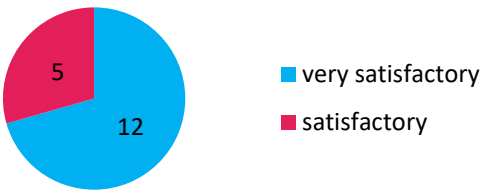
- Content: EIA workshop

Quality of scientific talks: m=3.6	
- Quality of PT tests reporting: m=3.7	
Quality of informal discussions m=3.6	
Quality of EIA slides shows : m=3.7	

- **General comments or proposals on topics for the next EIA workshop**

	Participant's comments	EU RL response
1	I think that it is important to compare reagent batch numbers, especially if there are some deviations on expected results.	**We agree with this statement, that is why the batch number of the kit used is asked to each PT participants**
2	If possible, in the future we would like to fill the PT results in the Excel forms.	**In the future, participants will have to complete their results directly on a web page with a personal and confidential code.**
3	We need more scientific and practical discussions about the lab diagnosis and all those topics relative to this disease that affect the international movement of horses. I strongly believe that a satisfactory proficiency test must have no mistakes, not even one. This is true also for dourine.	For the next workshop, EU-RL will ask to MS NRLs whether they have specific needs for the WS.

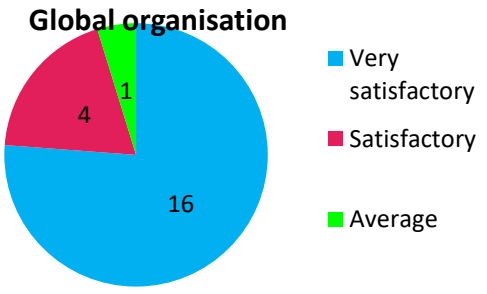
Content: dourine workshop

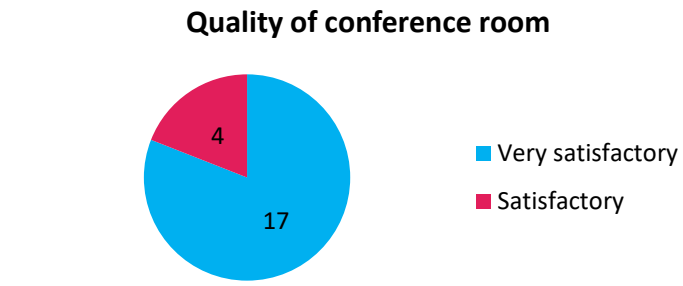
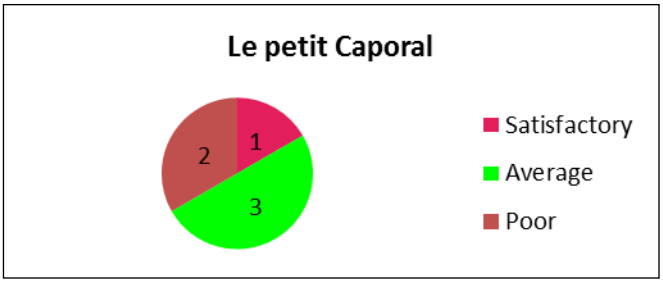
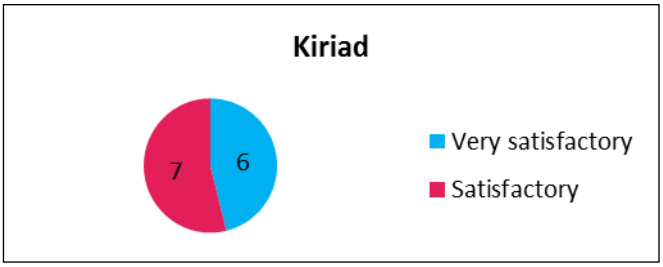
Quality of scientific talks m=3.7	
- Quality of dourine PT tests reporting m=3.6	
Quality of dourine informal discussions m=3.4	
Quality of dourine slides shows m=3.7	

- General comments or proposals on topics for the next dourine workshop

	Participant's comments	EU RL response
1	For the next workshop, it could be interesting to implement focus groups to improve the level of involvement and participation of each participant	Thank you for your comment, the implementation of working groups will be considered during the preparation of the next dourine workshop.
2	May organise a proficiency test only for Elisa in order to validate your protocol.	Thank you for your comment. The optimisation of our Elisa still remains in progress and the organisation of a PT will be taken into account for its validation.
3	If possible, in the future we would like to fill the PT results in the Excel forms.	**In the future, participants will have to complete their results directly on a web page with a personal and confidential code.**
4	We need more scientific and practical discussions about the lab diagnosis and all those topics relative to this disease that affect the international movement of horses	To improve the scientific and practical discussions, we will consider to implement working groups during the preparation of the next dourine workshop.
5	Dourine PT reporting got less points as the final report was not finished and will be presented later on.	Try to follow these comments for the next workshop. Final reports for dourine serological diagnosis has been sent to all participants and is available on our website.

Organisation

Global organisation (welcome, breaks, lunches, dinner) **M=3.7**	

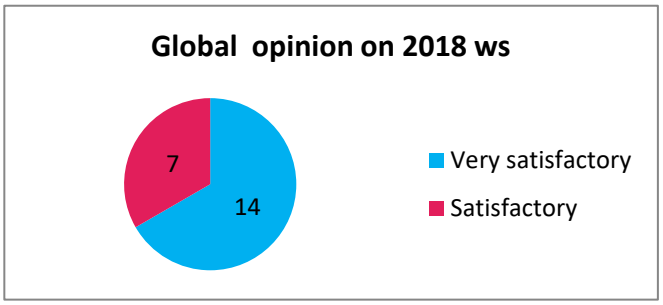
Quality of the conference room, m=3.8	
Hotel quality **Petit Caporal : m=1.8 !** **Kiriad Hotel m=3.5**	 

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

	Participant's comments	EU RL response
1	I have no claims, everything is organized perfectly.	Thanks for this positive feedback!
2	Everything was fine	Thanks for this positive feedback!

Global assessment

- What is your global opinion on the 2018 EIA and Dourine workshops? M=3.7



- Do you have any general suggestions for future workshops?

	Participant's comments	EU RL response
1	My suggestion - the presentations on Proficiency Test results could be planned in agenda earlier (not as one of the last presentations), especially at the second day, when usually participants should leave the workshop earlier because they have to manage be in time at the airport. i think that presentations on PT results are interesting for all participants.	Try to follow these comments for the next workshop
2	A free time could be left between the afternoon activity and the dinner.	Try to follow these comments for the next workshop
3	More scientific and practical discussions	Try to follow these comments for the next workshop

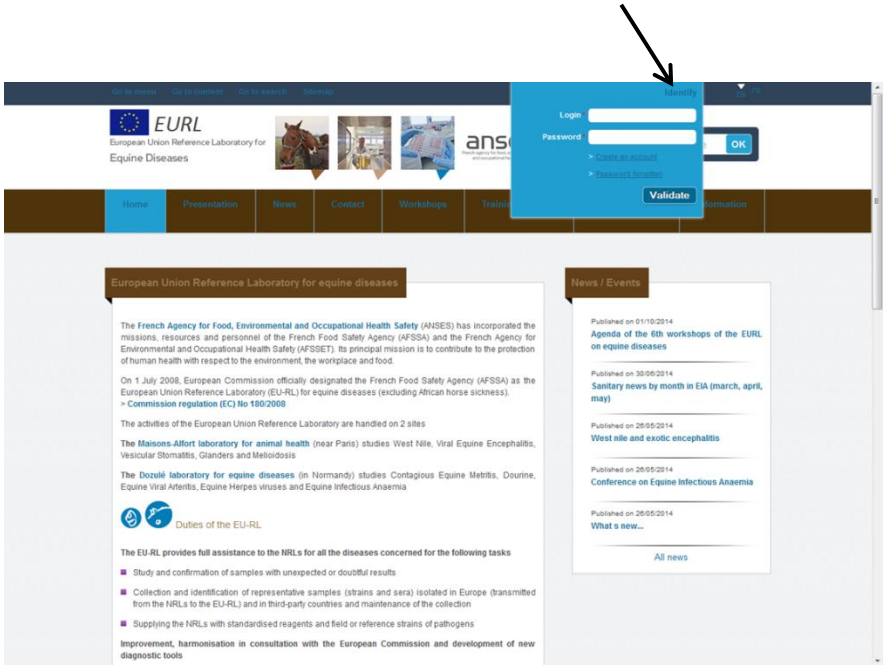
Annex 1

To create an account

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

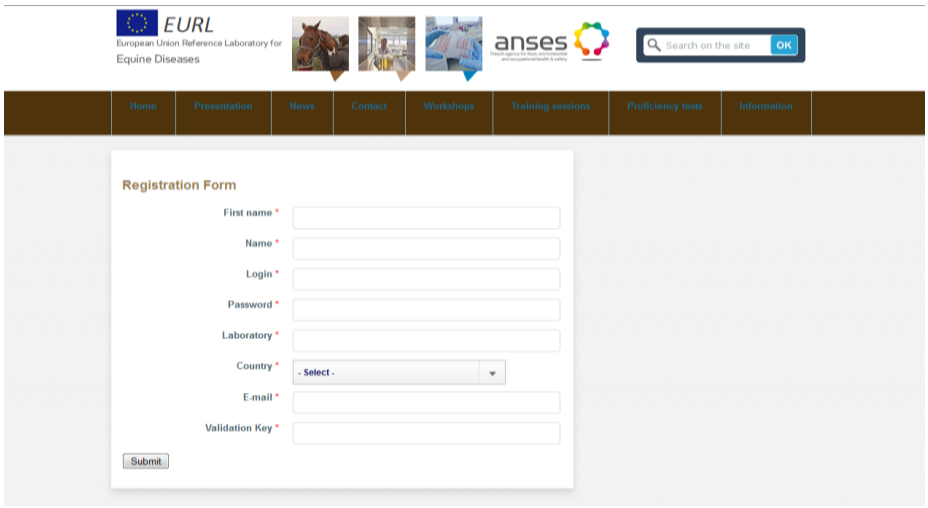
The link: https://sites.anses.fr/en/minisite/equine-diseases/european-union-reference-laboratory-equine-diseases

To go to the private access you have to identify you:



Firstly you have to create an account

You can choose your login 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.