Site de
Maisons-Alfort
LABORATOIRE SANTE ANIMALE

Site de Dozulé
LABORATOIRE DE PATHOLOGIE EQUINE

EU REFERENCE LABORATORY
FOR
EQUINE DISEASES

2012 Technical report of the European Union Reference Laboratory for Equine Diseases

AVENUE DU GÉNÉRAL DE GAULLE
F-94706 MAISONS-ALFORT CEDEX
TÉLÉPHONE : + 33 (0)1 49 77 13 00
TÉLÉCOPIE : + 33 (0)1 43 68 97 62
www.anses.fr

RÉPUBLIQUE FRANÇAISE

Activity 1: Equine Viral Arteritis (EVA) / Equine Infectious Anemia (EIA) / Equine Herpes Viruses (EHV)

➢ Outputs achieved

✓ Equine viral arteritis (EVA)

To develop and validate a molecular tool for diagnosis

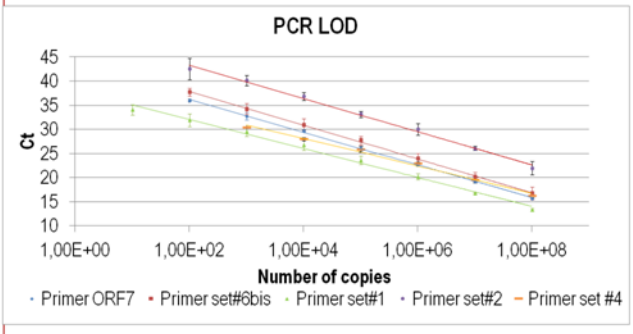
This project is a collaborative work with our colleagues from CVI (Netherlands), AHVLA (UK) and SVA (Sweden) funded by the CoVetLab organization. The main objective of this research program is to develop and validate a new qRT-PCR for the EVA diagnosis. The development of this new molecular tool should help to harmonize even more the diagnostic tool used in European NRLs. A meeting has been organized between partners on September 27th and 28th, 2012 in the Anses laboratory for equine diseases in Normandy, France.

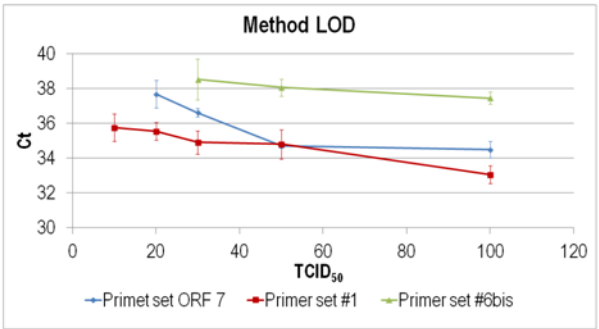
EAV infection of horses is endemic in much of the world and results in occasional outbreaks of respiratory disease in adult horses, abortion in pregnant mares, and interstitial pneumonia in young foals. Clinical signs of EAV infection may vary between outbreaks but most of them are subclinical. Many stallions become persistently infected carriers following EAV infection and continue to shed the virus in their semen for variable periods of time and act as a natural reservoir. In this study, we have developed a new qRT-PCR to detect the virus in semen of infected stallions. Indeed, a qRT-PCR targeting the Open Reading Frame 7 (ORF7) of the virus has been developed in 2002 by Balasuriya *et al.* At that time full genome sequences of EVA were not available. During the last 10 years, evolution in knowledge regarding EVA genome sequences enables us to develop a qRT-PCR targeting specifically the most conserved region of the genome, ORF1. We have designed seven primers set and probes targeting the most conserved regions, among 10 field isolates sequenced in the laboratory and 29 sequences on GenBank database, along the ORF1 sequence using Primer3 software (http://frodo.wi.mit.edu/). Primer sets #1, #2, #3, #4 target non structural protein 1 (nsp1), Primer set #5 targets nsp 8 and sets #6 and #6bis on nsp10. Validation of PCR protocol has been performed following the AFNOR XP U47-600 standard in order to define the PCR limit of detection (LOD) and the method LOD for each of the 7 primers set and probes designed in comparison of the reference method published by Balasuriya *et al.* in 2002. Primer set #6bis and probe associated exhibited the same sensitivity compared to the reference method whereas the primer set #1 and probe associated has a much better sensitivity compared to the reference method. In conclusion, we were able to developed and validate a new qRT-PCR, targeting ORF1, for EVA diagnosis that can be used in addition or/and in place of the reference one.

PCR LOD and METHOD LOD IN ACCORDANCE WITH STANDARD AFNOR XP U47-600

Bucyrus RNA, *in vitro* transcribed, range from 10^{-2} to 10^{8} copies. PCR LOD is determined after 3 independent sessions with 8 replicates of each dilution. PCR LOD is the number of copy where 95% (23 out of 24) of repetitions gave a positive result.

Serial dilution, range from 100 to 0 $TCID_{50}$, of Bucyrus virus strain in horse semen has been used. Method LOD is determined after 2 independent sessions with 4 replicates of each dilution. Method LOD is the smallest amount of doses where 100% (8 out of 8) of repetitions gave a positive result.





	Primer ORF 7	Primer # 6bis	Primer #1	Primer #2	Primer #4
PCR LOD	10^2 copies	10^2 copies	10^1 copies	10^2 copies	10^3 copies
Ct of PCR LOD	35.99	37.74	34.04	42.54	30.49
Method LOD	20 doses	30 doses	10 doses	-	-
Ct of Method LOD	35.7	38.54	35.76	-	-

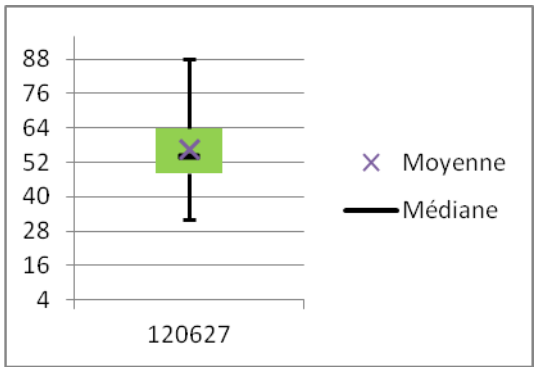
To develop and validate reference sera

Positive and negative reference sera for EVA have been developed by the EU-RL. Different batches have been lyophilized and tested for their stability and homogeneity. Two different sera with two different titers have been developed. One serum has a titer of 24 and another one has a titer of 64, batch # 120627 and 120711 respectively. Sera will be presented during the next workshop in 2013. Two other sera are still under titer evaluation and will be done in 2013 in collaboration with OIE reference experts and laboratories based at the Gluck Equine Research Center, Lexington, KY, USA and at the AHVLA, UK. Sera with a titer at 24 and 64 are available to EU NRLs on request. Titer evaluation and homogeneity of each batch is described below.

Batch # 120627 :

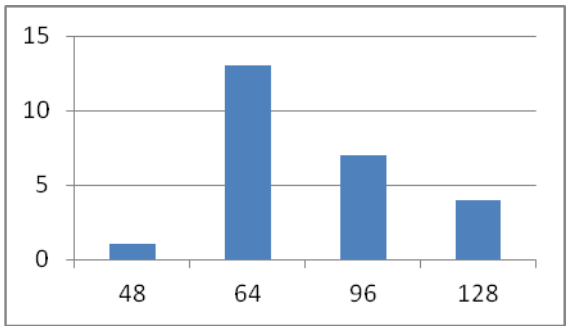
- **Titer evaluation:**

Mean of titer : **64.5**
Median titer obtained : **64**
Minimum titer obtained : **45.3**
Maximum titer obtained: **128**



- ***Homogeneity test on 25 lyophilized tubes :***

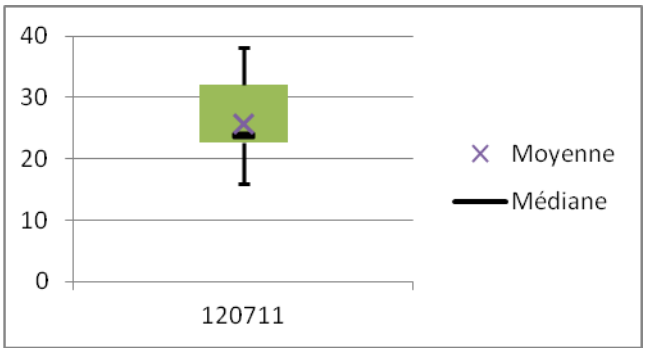
Ab titer	Percentage (%)
48	4
64	52
96	28
128	16



Batch # 120711 :

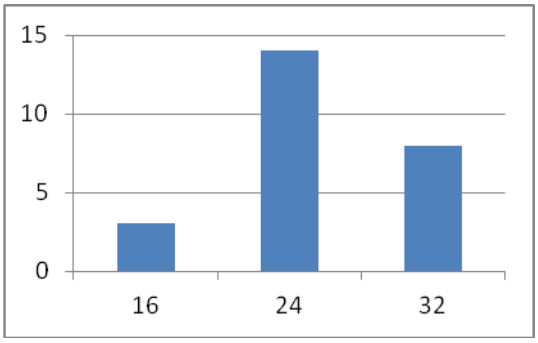
- **Titer evaluation :**

Moyenne du titre : **26.2**
Médiane du titre obtenu : **24**
Titre mini obtenu : **16**
Titre maxi obtenu : **48**



- ***Homogeneity test on 25 lyophilized tubes :***

Ab titer	Percentage (%)
16	12
24	56
32	32



Confirmation of cases by the EU-RL

The EU-RL has confirmed the presence of EVA in the semen of stallions located in Netherlands. Those semen samples have been sent by the Central Veterinary Institute in Lelystad (Netherlands) in a collaborative way. Virus characterization has been undergone and the phylogenetic analysis (see below) shows that viruses sent by the NRL from Netherlands belong for 5 of them (#12D734, 735, 737, 739 and 743) to the European Subgroup 2 and one of them to the European subgroup 1 (12D732). Sequences have been done on the ORF2 –ORF7 regions.

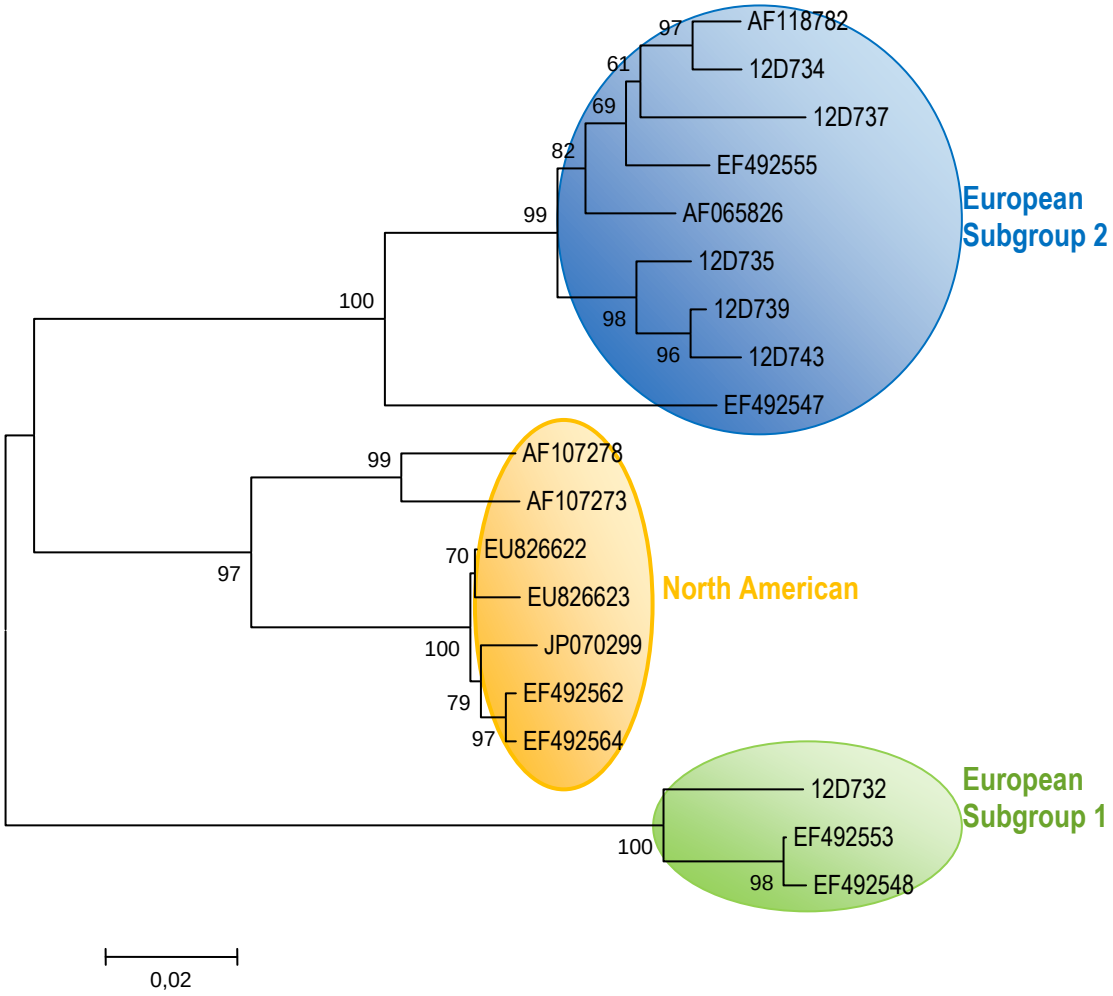


Figure 1: **Molecular Phylogenetic anaylsis by Maximum Likelihood method.** The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-4929.1282) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1385 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

Support to EU NRLs

- ✓ The EU-RL has sent the Bucyrus viral strain as well as RK-13 cells used in the virus neutralization test (VNT) as described in the OIE manual Chapter 2.5.10 to the NRL from Lithuania and Poland.
- ✓ The EU-RL has sent strong and medium positive sera for EVA to NRL from Finland and Ireland,

Valorization

Publication in peer reviewed journal:

Miszczak F., Legrand L., Balasuriya U. B., Ferry-Abitbol B., Zhang J., Hans A., Fortier G., Pronost S., Vabret A. Emergence of novel equine arteritis virus (EAV) variants during persistent infection in the stallion: Origin of the 2007 French EAV outbreak was linked to an EAV strain present in the semen of a persistently infected carrier stallion. **Virology** 2012 Feb 20;423(2):165-74.

International conference:

Miszczak F., Legrand L., Balasuriya U. B., Ferry-Abitbol B., Hans A., Fortier G., Pronost S., Vabret A. Evolution et émergence de variants de l'artérite virale équine(AVE) chez un étalon porteur chronique asymptomatique suspecté d'être à l'origine de l'épizootie française d'AVE en 2007. **In proceedings of XIVème Journées Francophones de Virologie**, **Virologie** 2012 (16) : S31.

C. van Maanen, R. Buter, Th Dijkstra, M. van der Lei, D. Gaudaire and A. Hans. Selection and validation of a real-time rt-PCR assay for the detection of equine arteritis virus and preliminary investigations into the prevalence of carrier stallions in the Netherlands. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S60

A. Hans, D. Gaudaire, S. Pronost, B. Ferry-Abitbol, C. Laugier and S. Zientara. Molecular epidemiology of Equine Arteritis Virus in France following the 2007 outbreak. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S89

French Publication:

Aymeric Hans, Loïc Legrand, Fanny Lecouturier, Cécile Beck, Stéphan Zientara. L'artérite virale équine en France et en Europe. **Bulletin épidémiologique santé animale-alimentation**, Spécial Equidés, 2012, 49 : 39-41

- ✓ Equine Infectious Anemia (EIA)

  - ➢ **Outputs achieved**

To develop and validate a molecular tool for diagnosis

This project is a collaborative work with our colleagues from CVI (Netherlands), AHVLA (UK) and SVA (Sweden) funded by the CoVetLab organization. The main objective of this research program is to develop and validate a qRT-PCR for the EIA diagnosis from blood sample and/or from tissues. The development of this new molecular tool should allow a quicker diagnostic especially in case of a first infection where antibodies titer will rise and will be detected in Agar Gel Immuno Diffusion test (AGID) after a minimum of 30 days of infection.

Confirmation of cases by the EU-RL

In 2012, two unrelated outbreaks of EIA have been declared in France. The first one, with 4 positive horses, was confirmed in January 2012 and was located in the Vaucluse county near the city of Avignon (South-east of France). The second one, with 4 positive horses, was confirmed in September 2012 and was located in the Gard county near the city of Nïmes (South-east of France). Horses were euthanized, blood and tissues samples have been collected before death for genetic analysis of the viral strains.
In addition, the EU-RL has received from the NRL of Romania samples collected from EIA positives horses. Sera, plasma, lung, spleen and liver have been collected from seven positive horses, held in Romania, slaughtered in 2011. These samples will be included in a project to study the epidemiology of EIA in Europe and decipher the origins of viruses isolated in Europe during the last few years.

Support to EU NRLs

The EU-RL has sent some OIE reference sera for EIA to the NRLs from Ireland and the Netherlands
The EU-RL has sent 24 encoded serum samples to the NRL from the Netherlands for their quality insurance purposes
The EU-RL has sent to the NRL from Ireland some positive and negative RNA and DNA for EIA in order to implement the PCR method in their laboratory.

The EU-RL has supervised a training session on EIA diagnostic tests with 25 participants involved mainly from the Balkan region; some EU NRLs, with scientists from Bulgaria, Hungary, Croatia, Macedonia, Boznia-Herzegovinia joined the training session. This training session was organized by the IAEA on October 16-18th in Izmir, Turkey (IAEA TC PROJECT RER/5/016) (see the program in annex).

Valorization

Publication in peer reviewed journal:

A. Hans, N. Ponçon et S. Zientara. Situation épidémiologique de l'anémie infectieuse des équidés en France et en Europe de 1994 à 2011. **Bull. Acad. Vét. France** — 2012 - Tome 165 - n°1 p27-34

International conference:

A. Hans, D. Gaudaire, E. Morilland, C. Laugier, S. Zientara and C. Leroux. Molecular epidemiology of Equine Infectious Anaemia Virus in France from 2007-2009. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S75

A. Hans, D. Gaudaire, E. Morilland, F. Lecouturier, C. Laugier, N. Ponçon, and S. Zientara. Report of the surveillance of Equine Infectious Anaemia in France in 2010. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S88

French Publication:

Gaudaire D., Morilland E., Lecouturier F., Cadore J.L., Zientara S., Leroux C., Hans A. *Epidémiologie de l'Anémie Infectieuse des équidés.* **Equ'idée**, p 14-16 n°78 Printemps 2012

Aymeric Hans, François Moutou, Delphine Gaudaire, Lucie Napolitan, Emmanuel Le Guyader, Nicolas Ponçon. L'anémie infectieuse des équidés en France et en Europe. **Bulletin épidémiologique santé animale-alimentation**, Spécial Equidés, 2012, 49 : 35-38

- ✓ Equine Herpes Viruses (EHV)

  - ➢ **Outputs achieved**

Support to EU NRLs

The EU-RL has sent EHV-1 and EHV-4 viruses as well as ED cells (Equine Dermis) used for *in vitro* EHV isolation and amplification to the NRL from Lithuania. In addition to those biological materials the EU-RL provided protocols too.

Valorization

Publication in peer reviewed journal:

F. Laabassil, B. Mamache, D. Gaudaire, G. Amelot, A.M. Nasri, S. Pronost, L. Legrand, A. Hans. Enquête séro-épidémiologique sur la grippe équine en Algérie. **Revue de Médecine Vétérinaire** - Année 2012, Volume 5, p227-234

International conference:

A. Hans, F. Laabassi, G. Amelot, L. Legrand, D. Gaudaire, C. Laugier, S. Pronost, and S. Zientara. Serological evidence of circulation of Equine H3N8 Influenza Virus in Algeria and its molecular Characterization. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S11.

J. Tapprest, B. Bouyer, M. Pannequin, C. Sévin, J. Cauchard, J.L. Le Net, F. Duquesne, S. Petry, A. Lefleche-Mateos, A. Hans, C. Laugier and F. Moutou. Major outbreak of rhodococcosis in adult equidae. **9th International Conference on Equine Infectious Diseases (ICEID) Journal of Equine Veterinary Science**, Lexington, KY (USA), 2012, 32(10): S15

F. LAABASSI, B. MAMACHE, D. GAUDAIRE, G. AMELOT, A. M. NASRI, S. PRONOST, L. LEGRAND, A. HANS. La Grippe Équine En Algérie (2011) : Caractérisation, Séro-épidémiologie, Analyse phylogénétique du gène HA. **29ème Congrès Vétérinaire Maghrébin** Alger : 07 -08 avril 2012

Amelot G., Laabassi F., Legrand L., Gaudaire D., Laugier C., Pronost S., Zientara S., Hans A. Mise en évidence de la circulation de virus de la grippe équine en Algérie. **In proceedings of XIVème Journées Francophones de Virologie**, **Virologie** 2012 (16) : S47.

F. Laabassi, D. Gaudaire, G. Amelot, L. Legrand, S. Pronost, B. Mamache, A. Hans. Épizootie de Grippe Équine en Algérie (2011) : qRT-PCR et l'analyse phylogénétique du gène HA. **Association Vétérinaire Equine Française (AVEF) 2012,** 11-13 octobre 2012, Reims, France

French Publication:

Loïc Legrand, Aymeric Hans, Christelle Marcillaud-Pitel, Stephan Zientara, Stéphane Pronost**.** Prévalence des herpèsvirus équins en France au cours de l'année 2010. **Bulletin épidémiologique santé animale-alimentation**, Spécial Equidés, 2012, 49 : 21-25

Loïc Legrand, Aymeric Hans. Prévalence de la grippe équine en France de novembre 2005 à octobre 2010. **Bulletin épidémiologique santé animale-alimentation**, 2012, 51 : 2-4

Jackie Tapprest, Bertrand Bouyer, Jean-Loïc Le Net, Marion Pannequin, Fabienne Coroller, Julien. Cauchard, Sandrine Petry, Corinne Sévin, Aymeric Hans, Claire Laugier, François Moutou. Épisode de rhodoccocose à Mayotte. **Bulletin épidémiologique santé animale-alimentation**, 2012, 51 : Brève

Activity 2: West Nile /Others equine encephalomyelitis viruses (EEEV, WEEV, VEEV and JEV) and Vesicular stomatitis

- **Outputs achieved**

✓ West Nile virus (WNV)

Epidemiological surveillance and epidemiological research

Epidemiological investigations on domestic and wild birds in several African (Madagascar, Senegal) and Asian countries (Thailand) have been mostly completed (collaboration with V. Chevalier, CIRAD). Results are being processed and show a high seroprevalence of flavivirus antibodies (mainly WNV in Africa and Japanese Encephalitis in Thailand) in all the countries considered (Madagascar: Roger M et al, Manuscript submitted for publication). A study was also conducted in Morocco by the Epidemiology Unit at ANSES (B. Durand), showing that wider regions than the ones reporting WNV outbreaks are favorable to WNV circulation.

Epidemiological investigations on wild birds (magpies) in France allowed for the detection of West Nile virus neutralizing antibodies in one young bird in the Camargue region, demonstrating recent circulation of WNV even in the absence of WNV horse cases (Collaboration with Tour du Valat and INRA; Vittecoq M et al, Manuscript accepted in VBZD).

Wild birds (gulls) in Spain were also screened for flavivirus antibodies and the results suggest an endemic exposure to a flavivirus different from West Nile or Usutu viruses (Arnal A et al, manuscript in preparation).

Horses sampled in Austria for a serological survey on WNV lineage 2 turned out to be positive for TBEV neutralizing antibodies only. Even if Austria is known to be a TBEV-endemic area, the high seropositivity rate found in this study (40.4%) questions about recent TBEV exposure of equids and exceptional TBEV circulation patterns (Rushton et al, accepted in EID).

To investigate the role of amphibians in WNV transmission cycles, *Xenopus* cells and *Xenopus* frogs have been infected with a highly virulent lineage 1 West Nile virus strain (EDENnext program; 2011-2013). While *Xenopus* fibroblasts are very efficient at replicating this WNV strain, replication in frogs receiving a subcutaneous injection of WNV seems to be very quickly restricted (in particular, a low and short viremia was observed).

Vaccine development

Three different candidate WNV vaccines were evaluated for their efficacy at inducing WNV neutralizing antibodies and protection in horses (2 candidate vaccines) and in mice (for one candidate vaccine).

The recombinant canine adenovirus vectored vaccine proved to be very efficient at inducing humoral immunity and protection in a murine model of infection after intramuscular immunization, however failed to elicit a long-lasting and strong antibody responses in horses (collaboration with B. Klonjkowski, adenovirus team at the Virology Unit).

We demonstrated that a recombinant lentivirus vector could serve as an efficient vaccine against WNV in horses: induction of strong antibody responses, protection in mice afforded through the passive transfer of immunized horse sera (collaboration with P. Charneau, Institut Pasteur; Manuscript in preparation).

Naturally inactivated vaccines against West Nile, based on the use of recombinant fish rhabdoviruses expressing WNV envelop or part of WNV envelop at their surface, are being evaluated in a murine model. After a prime-boost protocol consisting of 3 subcutaneous injections of recombinant rhabdoviruses, anti-envelop antibodies could be visualized in every animals and neutralizing antibodies could be detected in a fraction of vaccinated animals (2/5 or 4/9 at most in 2 independent experiments); clinical protection after WNV challenge was generally obtained in animals with high WNV neutralizing antibodies (2/5 and 4/9 surviving animals respectively). Improvements to the vaccination protocol have been brought mainly through modifications in the recombinant viruses (for an enhanced expression of WNV antigens at their surface) in order to optimize the induction of WNV immunity (Collaboration with M. Brémont, INRA; PhD thesis: A. Nzonza).

Research activity: identification of molecular determinants of WNV pathogenicity

A molecular construct enabling the *in vitro* synthesis of WNV genome RNA (from the highly virulent Israel 1998 strain) has been constructed and a full validation of its derived virions has been completed in 2011 (Bahuon C et al., Manuscript published in Plos One). With this new molecular tool more adapted to WNV studies in the European context, we sought to evaluate the molecular determinants of the pathogenicity of European WNV strains. WNV recombinant viruses bearing point mutations (such as T249P in the NS3 protein) or equivalent fragments from less pathogenic European WNV strains have been generated (point mutations) or are being generated (chimeric viruses). The NS3 T249P mutant virus is being characterized in various *in vitro* and *in vivo* models.

Birds play a central role in transporting and amplifying WNV. Increasing numbers of WNV isolates are notified in Europe, and these genetically variable isolates are not well characterized for their virulence in birds. We therefore investigated whether European corvids (*Corvus corone* more particularly) were susceptible and sensitive to infection with European WNV strains and whether SPF chickens could be a valuable avian model for the pathotyping of WNV strains (Dridi et al, submitted). Inoculated crows were found to be sensitive to infections with the Israël98 and France2000 WNV strains and, as expected from the available epidemiological data, Is98 induced a higher mortality rate and a quicker fatal outcome, with higher viral RNA loads detected in the serum, oral swabs and feathers than in the Fr2000 group (Dridi et al, accepted in Vet Microbiol).

Support to NRLs

The EU-RL has sent West Nile virus RNA to NRLs from Ireland, Poland, Romania, Sweden and also Switzerland, RNA negative control to NRL from England, West Nile virus to the NRL from Romania and Greece, Vero cells for West Nile virus isolation and neutralization test to the NRLs from Poland, Romania, Greece and Sweden.
The EU-RL has sent a panel of 15 encoded serum samples to the NRL from Austria for their quality insurance purposes.
Reference sera positive for West Nile IgG or IgM have been provided to the NRLs from Poland, Romania and Sweden.

The EU-RL has supervised a training session on WNV diagnostic tests with 25 participants involved; some EU NRLs, with scientists for Bulgaria or Hungary, joined the training session. This training session was organized by the IAEA on October 8-12th at Izmir, Turkey (IAEA TC PROJECT RER/5/016) (see the program in annex).

Valorization

Bahuon C, Desprès P, Pardigon N, Panthier JJ, Cordonnier N, Lowenski S, Richardson J, Zientara S, Lecollinet S. 2012. IS-98-ST1 West Nile virus derived from an infectious cDNA clone retains neuroinvasiveness and neurovirulence properties of the original virus. PLoS One. 7(10):e47666.

Pradier S, Lecollinet S, Leblond A. 2012. West Nile virus epidemiology and factors triggering change in its distribution in Europe. Rev sci tech Off int Epiz. 31(3)

Dridi M, Vangeluwe D, Lecollinet S, van den Berg T, Lambrecht B. Experimental infection of Carrion crows (Corvus corone) with two European West Nile virus (WNV) strains. Vet Microbiol. Accepted

Vittecoq M, Lecollinet S, Jourdain E, Thomas F, Blanchon T, Arnal A, Lowenski S , Gauthier-Clerc M. Recent circulation of West Nile Virus and potentially other closely related flaviviruses in Southern France. VBZD. Accepted

Rushton JO, Lecollinet S, Hubálek Z, Svobodová P, Lussy H, Nowotny N. Tick-borne encephalitis virus in horses: higher prevalence of infection than anticipated. EID. Accepted

In French :
Lecollinet S., C. Beck, S. Zientara. 2012. Le virus West Nile : diagnostic, surveillance et évolution épidémiologique en Europe. Bull. Acad. Vét. France, 165 (1): 35-43.

Lecollinet, S., A. Leblond, B. Durand, S. Zientara, N. Ponçon. 2012. Le virus West Nile : bilan de la situation en Europe et point sur la surveillance en France. Bulletin épidémiologique Santé Animale Alimentation, spécial équidés, 49: 32-34

Lecollinet, S., F. Moutou. 2012. Recrudescence d'activité du virus West Nile dans les Balkans durant l'été 2012. Bulletin épidémiologique Santé Animale Alimentation, 55: 26.

✓ Exotic equine encephalitis

Improvement of ELISA tests for the diagnosis of exotic equine encephalitis based on recombinant proteins

Development of sensitive and specific ELISA tests with E2 glycoproteins from alphaviruses (Eastern, Western and Venezuelan equine encephalitis viruses; EEEV, WEEV and VEEV respectively) is in progress. E2 glycoproteins have been expressed in insect cells after cloning of the corresponding synthetic genes in a drosophila expression plasmid (collaboration with P. Desprès, Institut Pasteur). The first results in indirect IgG ELISA are encouraging with a good correlation between virus neutralization tests and these new ELISA tests. The difficulty is now to find enough positive horse alphavirus sera to validate the new ELISA methods.

Rabbit polyclonal sera have been produced against the Envelope domain III from Japanese Encephalitis (JE), Tick-Borne Encephalitis (TBE) and WN viruses and could be used for the establishment of competition ELISAs for the diagnosis of flavivirus infections in varied animal species. However, results obtained in competition ELISA with these polyclonal sera and well characterized horse sera were found to be inconclusive, most probably due to the presence of numerous antibodies directed against the SNAP tag (linked to the 3 recombinant DIII antigens) in the rabbit sera. Rabbit polyclonal antibodies should be used against non-SNAP tagged antigens.

Production of reference sera

Vaccination with inactivated vaccines against EEEV, WEEV and VEEV (Equiloid Innovator® and Triple E-T Innovator® from Fort Dodge) was carried out in equids in France in 2011 and horse sera were collected at 4 occasions after immunization. After having tested horse sera by SNT tests against the 3 above-mentioned viruses, three sera have been selected: one EEEV positive sera, one EEEV and WEEV positive sera and one EEEV, WEEV and VEEV positive sera.
For easier transport and longer storage, these 3 Alphavirus positive sera have been lyophilized in 2012 by IDEXX Company. Theses 3 batches have been tested for their stability; a decrease in neutralizing antibody titers has been evidenced for the 3 sera. Two lyophilized sera (one for VEEV and one for WEEV) are nevertheless available but with low titers of neutralizing antibodies (10 for VEEV and 30 for WEEV).

2361 pig sera originating from Vietnam farms and slaughterhouses (collaboration with CIRAD) have been tested with the WN ID Screen competition ELISA test (IDVet). Of these 2361 sera, 70% were found to be positive for flavivirus antibodies, 25 % negative and 5% doubtful. A panel of the positive sera will be tested by JE and WN virus neutralization tests to identify the flavivirus responsible for the infection. A positive reference serum for JE virus neutralization test (or other species-independent serological assays) will be prepared from JEV positive pig sera and will be available to NRLs on request by the end of 2013.

Improvement of real-time RT-PCR for the detection of equine exotic encephalitis viruses

Two synthetic calibrators (eg plasmids allowing for the synthesis of RNA transcripts), one for JEV and one for alphaviruses, have been generated to get a quantified standard for already published real-time RT-PCR protocols for JEV, EEEV, VEEV and WEEV and to calculate their limits of detection. These calibrators or RNA transcripts are now available to NRLs on request.

Valorization

French publications:
Beck C, Lecollinet S, Zientara S. Conduite à tenir en cas de suspicion d'encéphalites exotiques chez le cheval. Bulletin épidémiologique Santé animale-alimentation spécial équidé, Juin 2012

Risque d'introduction des encéphalites équines « exotiques » (encéphalite japonaise, encéphalite vénézuélienne, encéphalites américaines de l'Est et de l'Ouest) en France. Isabelle Dumas – veterinary thesis presented on January 2013

✓ Vesicular stomatitis

➢ **Outputs achieved**

- Organization of proficiency test (PT): A PT for detection of vesicular stomatitis virus (IND and NJ) by RT-PCR was organized. The objectives of this PT were on one hand to establish an European Laboratory Network for the diagnosis of vesicular stomatitis virus, on the other hand to establish an inventory of RT-PCR methods available for detection and characterization of VSV and to evaluate their performances in order to improve molecular diagnosis of VSV. Nine laboratories have participated to the PT. All laboratories results were consistent with those expected.
  See the PT tests results on : http://www.ansespro.fr/eurl-equinediseases/Documents/EQU-Pt-VSV-PCR.pdf

- Support to LNRs: RT-PCR protocols and positive RNA controls were provided to some laboratories before the PT in order to implement the RT-PCR in their laboratory

- Implementation and improvement of VS diagnosis: a one-step duplex real time RT-PCR method was developed and it is under validation.

- Research: The EU-RL conducts research to improve diagnosis of vesicular stomatitis. Two main projects are underway:
  - Development of multiplex serological diagnostic test based on luminex liquid array technology
  - Development of multiplex melecular diagnostic test based on luminex liquid array technology

Activity 3: Dourine

➢ **Outputs achieved**

Confirmation of cases by the EU-RL

We have been contacted by the Polish and Belgian NRLs in order to confirm or infirm one suspect case in each country. The cases were considered doubtful due to an anticomplementary effect and were found negative after resampling.

Training course in Italy

The EU-RL was invited in Teramo by the Istituto Zooprofilattico Sperimentale "G Caporale" in order to give a training course on Dourine. Pr Reto Brun from Switzerland and Dr Achim Schnauffer from Scotland were also invited as international experts. This two-day meeting was an exchange time of an outstanding interest giving the opportunity for discussions.
It was also the opportunity to build collaborations between the Italian NRL and the EU-RL. To date, serum from the Italian outbreak has been exchanged, and parasite DNA should soon be sent. Moreover, one oral communication in a French equine veterinary congress has been presented in October with French and Italian authors.

Actualization of the OIE terrestrial manual

Pr Noboru Inoue, OIE laboratory for Surra, has been designed in June by OIE administration to organize the actualization of the terrestrial manual chapter for dourine. Pr Inoue invited some experts including those of the EU-RL. The revision was submitted after active discussions at the OIE headquarters at the end of August. All of the EU-RL suggestions were validated except the replacement of the Veronal buffer (containing barbiturate) by PBS or HEPES for the complement fixation test. That point should be developed with laboratories working on diagnosis of other diseases based on CFT.

Ring trial

After updating the list of participants, blind samples have been sent to 19 NRLs and 3 third countries (Switzerland, South Africa and Dubai). Each participant received the samples in good conditions and the deadline for the answers was the 10th of August.
The samples were:

	Serum	Attended result	Description
1	Anses Negative	Negative	Batch 001
2	USDA Negative	Negative	Batch 1101
3	USDA low titer	Positive	Batch 9474
4	Italian horse	Positive	Serum from the 2011 Italian outbreak
5	USDA Medium titer dilution 1/2	Positive	Batch 9313
6	Mongolian Horse	Positive or negative or doubtful	12MPE098, Khorhon. Dourine was serologically suspected but never clinically confirmed
7	Ethiopian horse	Positive	Pool from batch 943-01 and 943-02, stallion artificially infected by strain Dodola
8	Ethiopian horse	Positive	
9	Mongolian horse Dilution 1/2	Positive or negative or doubtful	12MPE098, Khorkon
10	Mix Ethiopian Italian serum	Positive	50 µl from each of the Ethiopian and the Italian serum

Three sera were proposed for validation as candidate reference serum (samples 1, 7 and 10).

Globally for complement fixation test and imunofluorescence, there were no false positive, only false negatives (9.5%) and a major point is that all the participants gave a positive result for the Italian horse, corresponding to a real dourine outbreak case.

Among the samples that have been tested by the participants, the case of the Mongolian horse should not be considered as a real dourine. This horse was highly suspected for dourine, but the animal never showed clinical signs and the parasite could never be isolated. Having a positive or a negative result in this case confirms the fact that the results of the serological diagnostic have to be associated to the context as described in the OIE terrestrial code.

Complement fixation test
We observe a large variability of the titers, but the results are homogenous. The same participants returned globally lower or higher titers.
Except for one participant, there was a good repeatability with the serum of the Ethiopian horse and the titers obtained for the Mongolian horse clearly reflected the dilution of the serum.

Indirect fluorescent antibody test
Because IFAT is not the official test used for trade, and because of the difficulties to obtain optimal results with this method for dourine, only three laboratories performed the test. One participant performed the IFAT but not the CFT.
We also observed a large variability of the titers.

See the PT tests results on http://www.ansespro.fr/eurl-equinediseases/Documents/EQU-Pt-Dourine-2012.pdf

Workshop

To underline the fact that the EU-RL is located on two sites with the Maisons-Alfort laboratory for animal health and the Dozulé laboratory for equine diseases, the fourth workshop held in the regional council of Basse-Normandie (Low Normandy) on November 20th & 21st 2012 and focused on dourine disease.
The programmes and all the presentations of this workshop are available on the website:
http://www.ansespro.fr/eurl-equinediseases/

50 participants coming from 21 NRLs attended this workshop in the beautiful buildings of the Ladies' Abbey.
Each participant introduced himself. After this presentation, it could be observed that there was a very heterogeneous demand of dourine analysis in the countries. Some NRLs carry out a very few number of analyses each year although others perform up to 3,000 analyses each year. Concerning the research on dourine, Italian and German laboratories have a continuous activity.
The EuRL presented its activity and the results of the ring trial.
Dr Bjorn Bassarak, post-doctorant at the FLI from Jena in Germany, presented the preliminary results of his work on optimisation of the culture of *Trypanosoma equiperdum* on cells. The first data are promising and encouraging. It could be an alternative to the *in vivo* production and a solution to the ethical problem of animal experimentation.
One afternoon was dedicated to the 2011/2012 dourine outbreak in Italy. Two scientists, Ilaria Pascucci and Cesare Camma, from the Istituto G. Caporale Teramo presented the main information about this outbreak.
Pr Phillipe Büscher, from OIE reference Laboratory for surra in Antwerp – Belgium presented the up to date knowledge on surra epidemiology and diagnostic challenge. Only the RoTat 1.2 PCR is *evansi* specific and no single molecular test has been properly validated. Dog and camels surra import cases in Europe were also described.
Because differentiation between dourine and surra is a key point for the diagnosis and determine the possibility to treat or not the equids and the obligation to slaughter or not the sick animals, the second day was dedicated to this topic.
Pr Dave Barry, from the Wellcome trust in Scotland, presented the preliminary results of the sequencing of five strains, 1 *T. evansi* and 4 *T. equiperdum*. He showed that the Dodola/OVI and Teva1 strains are related to each other. He also demonstrated the presence of a large numbers of SNPs that could be studied to define *T. equiperdum*. The present results need to be supported by sequence data from other strains.
In this context, molecular definition of what is a *T. equiperdum* strain associated to the clinical data is required for the production of adapted reagents, antigens or serum.

Three sera validated by the proficiency test were proposed: the Italian serum from the 2011 outbreak, the Ethiopian serum was sampled during an artificial infection with a Dodola strain, and a mix of both sera. The Italian serum was sampled in a natural infected stallion but the strain is not yet totally characterized. The Ethiopian serum was produced using the Dodola strain. During his communication, Dave Barry could not certify that the Dodola strain is a real *T. equiperdum* strain.
After discussion, none of the three proposed sera was estimated to be totally adapted to be assigned as the reference serum. Nevertheless the Ethiopian serum will be proposed by the EU-RL as working serum awaiting the production of the reference serum.
No sequence information was available on the Italian strain and there was a doubt on the Dodola strain. Only the OVI strain could be certified as a real *T. equiperdum* strain. There was then a consensus on the facts that:
- OVI strain should be used for the in vivo production of antigens,
- Reference serum should be produced in horses by immunisation with antigens from the OVI strain,
- NRLs propose to validate the reagents produced.

Based on the discussion, there was also a consensus on the facts that:
- ELISA should be the method of choice for the development of an alternative to the CFT,
- A study should be performed to estimate the impact of the decomplementation temperature on the CFT results.

See the workshop report on: http://www.ansespro.fr/eurl-equinediseases/Documents/EQU-WS-Dourine_report-2012.pdf

Reagent production

- Antigens: a first batch of antigens has been produced before the workshop using the BoTat strain following the instructions of the OIE dourine chapter and the process was validated. Based on the workshop's consensus concerning the strain to be used for the production of the antigen, OVI, the large scale production began in December.
- Positive serum: based on the consensus of the workshop, the positive Ethiopian serum will be proposed as a working positive control. The production of a positive reference serum began in December by immunization of mares using the OVI strain. If each step of the process is validated, we plan to submit a file in order to get the "OIE" label for the serum. Having such a reference serum would allow us to work on the modification of the serology protocol, so that the controls of a test should always have a defined titer, like for Brucellosis.

Support to NRLs

- The EU-RL has organized two training courses (two days) on dourine, in March for two technicians from the Netherlands, and in November for the veterinarian in charge of the NRL from Portugal.
- Due to the difficulties to get antigens for the laboratories, the EU-RL has organized a turn-over of reagents, antigens or sera: the EU-RL send the reagents needed for free to the NRLs, and the NRLs send back the reagents due when they receive it from their supplier. With such a loop, we can supply antigen to all the NRLs up to the validation of our own production. Three NRLs (Romania, Netherland and Denmark) used the opportunity this year

Valorization

International conferences:

Cauchard J. (2012) European Union Reference Laboratory for equine diseases: focus on dourine diagnosis Training course on Dourine Istituto Zooprofilatico Sperimentale Teramo – Italie 18-19th April 2012.

Cauchard J., Madeline A. & Büscher P. (2012) Activity report of the European reference laboratory for dourine Meeting of the ad hoc group on Non Tsetse Transmitted Animal Trypanosomoses (NTTAT). Paris, 20th May 2012.

Cauchard S., Schlusselhuber M., Van Reet N., Bois C, Laugier C., Büscher P., Grötzinger J. & Cauchard J. (2012) Potential of antimicrobial peptides as treatment of equine trypanosomosis: preliminary results for eCATH-1 Meeting of the ad hoc group on Non Tsetse Transmitted Animal Trypanosomoses (NTTAT). Paris, 20th May 2012.

Schlusselhuber M., Sanguinetti, M., Jung S., Bruhn O., Cauchard S., Goux D., Leippe M., Laugier C., Leclercq R., Grötzinger J. & Cauchard J. (2012) Potential of equine antimicrobial peptides as alternative drugs in veterinary medicine, in vitro and ex-vivo studies of eCATH1. 3rd international symposium on Antimicrobial Peptides, Lille, France, 13th-15th of June 2012.

International poster:

Cauchard J., Madeline A., Petry S., Duquesne F., Claes F., Godeeris B. & Büscher P. (2012) Use of RAPD for the differencation of the agent of Dourine and Surra, *Trypanosoma equiperdum* and *Trypanosoma evansi*. Ninth International conference on equine infectious diseases, Lexington, USA, 21st -26th October 2012. In proceeding of J. Equine Vet. Science, 2012, 32(10):S82-S83

French publications:

Cauchard J. (2012) La dourine en Europe : état des lieux au 1er juillet 2011. Encadré dans le Bulletin Epidémiologique Santé animale – Alimentation. N°49, avril 2012 Spécial Equidés.

Cauchard J. & Büscher P. (2012) La dourine. Fiche maladie – A destination des sites internet RESPE, Syndicat des éleveurs de Pur-Sang et IFCE.

French conference:

Cauchard J., Madeline A. & Büscher P. La dourine en Europe : Impact des Foyers Italiens et activité du laboratoire communautaire de référence. 40èmes Journées annuelles AVEF, Reims, 11-13 octobre 2012.

Activity 4: Contagious equine metritis (CEM)

- **Outputs achieved**

Number of CEM cases

Since 2009 the EU-RL has made an inventory of the number of CEM cases per country as shown in the table below.

Although the European Union was not free from CEM, the number of CEM cases is decreasing (62 cases in 2009 to 36 cases in 2012).

Country	Number of CEM cases				CEM = notifiable disease
	2009	2010	2011	2012	
Austria	1	0	0	0	NO
Belgium	3	4	3	0	YES
Czech Republic	5	0	0	0	YES
Denmark	1	0	0	0	YES
Estonia	0	0	0	0	YES
Finland	1	0	0	0	YES
France	12 (7 outbreaks)	3	2	2	YES
Germany	10	5	12	14 (6 outbreaks)	YES
Hungary	0	0	0	0	NO
Ireland	0	0	0	2 (same premises)	YES
Italy	2	5	2	5 (3 outbreaks)	NO
Latvia	0	0	0	0	YES
Lithuania	0	0	0	0	YES
Poland	0	3	1	0	YES
Portugal	0	3 (1 outbreak)	0	0	NO
Slovakia	0	0	0	0	NO
Slovenia	4	1	1	0	YES
Spain	0	0	0	0	YES
Sweden	3	2	3	0	YES
The Netherlands	18	23	32	10	NO
United Kingdom	2	2	0	3 (1 outbreak)	YES

Support to NRLs and reference activity

Following the Workshop on November 24th 2011:

- ✓ The final report was made early January and published on the website on February 02nd, 2012;
- ✓ To improve the specificity of the CEM diagnostic, we need to differentiate both *Taylorella* species. For this, the EU-RL recommends to perform *Taylorella equigenitalis*- and *Taylorella asinigenitalis*-specific PCR on all isolates suspected of being *Taylorella equigenitalis*; all PCR methods currently published can be performed except Pleumink-Pluym's PCR unless it is combined with another PCR or sequencing of the PCR product;
- ✓ To improve the sensitivity of the CEM diagnostic, we need to limit the growth of genital flora. For this, the best method for doing this would be to develop a more selective medium using data from genomic sequences recently obtained. Therefore, the ANSES laboratory for equine diseases is currently seeking funding for a project on "Improvement of CEM bacteriological diagnosis and development of an infection model for the comparison of the pathogenicity of CEM organism and *Taylorella asinigenitalis*". Meanwhile, we can already optimize our practices by i) refrigerating swabs during transport, ii) using Timoney's medium or equivalent instead of chocolate agar with streptomycin, less attractive because most *Taylorella equigenitalis* isolates are streptomycin-sensitive, and iii) using PCR as a diagnostic tool alongside the culture method.

Training activity: no training session for the NRLs has yet been planed because no NRL has made the request.

Provision of biological elements: following a request of the NRL from Poland on February 13th, one *Taylorella asinigenitalis* reference strain and six field isolates of *Taylorella asinigenitalis* were sent February 21st by an authorized carrier.

Analysis activity: two French CEM-positive cases into two different outbreaks were confirmed in the first half of the year. Accordingly, four *Taylorella equigenitalis* strains were added to our *Taylorella* collection. A fifth strain from United Arab Emirates isolated in 2009 was also added to our *Taylorella* collection in 2012, bringing the number of *Taylorella equigenitalis* to 498. In addition, one French *Taylorella asinigenitalis* strain was isolated in 2012 and added to our *Taylorella* collection bringing the number of *Taylorella asinigenitalis* to 52.

The EU-RL participated in three proficiency tests for CEM, organized by the Veterinary Laboratory Agency, UK: two only for culture method in January and June and the third to compare culture and PCR methods in November.

Research activity

Development of a molecular typing tool for epidemiological studies:

The development of the MLST (multilocus sequence typing) tool to characterize the molecular diversity of *Taylorella* genus was finished in 2012. This tool is based on the sequencing of six to eight conserved DNA regions of approximately 500 bp each. In 2011, six DNA regions from six conserved genes were validated with 134 strains of the genus *Taylorella* (63% of *Taylorella equigenitalis*). The first half of 2012 was devoted to the selection of a seventh region of DNA and the addition of thirty *Taylorella equigenitalis* strains to increase the number of non-French strains and thus give more robustness to the tool. All DNA sequences were deposited in the GenBank database to obtain accession numbers. An international and specific MLST database was created to harbor all DNA sequences and all available epidemiological data on strains. This *Taylorella* MLST database will be available on the website http://pubmlst.org soon as this work will be published in a peer-reviewed journal with related impact factor. The second half of the year was devoted to analysis all results and the writing of a scientific publication.

The development of molecular typing tool was presented orally during the 9th International Conference on Equine Infectious Diseases (EID IX) in Kentucky (USA), and as a poster during a veterinary congress in France, in October 2012.

Study of the nutritional requirements of *Taylorella* genus to improve culture media used for the isolation and the characterization of the CEM agent:

The research funding has been underway since early 2012 for the project on "Improvement of CEM bacteriological diagnosis and development of an infection model for the comparison of the pathogenicity of CEM organism and *T. asinigenitalis*". Funding requests were made to the Regional Council of Lower Normandy (accepted in June 2012) and the European Regional Development Fund (accepted in November 2012). The recruitment of a junior scientist was effective in January 2013 for a period of two years. A partnership with AES chemunex, manufacturer of culture medium, has been finalized in June 2012.

Valorization

Publications in a peer-reviewed journal with related impact factor:

- Hébert L., Moumen B., Pons N., Duquesne F., Breuil M.F., Goux D., Batto J.M., Laugier C., Renault P. & Petry S. (2012) Genomic characterization of the *Taylorella* genus. PLoS One. 7(1):e29953

- Tazumi A., Hayashi K., Tasaki E., Petry S., Moore J.E., Millar B.C. & Matsuda M. (2012) Demonstration of the absence of intervening sequences (IVSs) within 16S rRNA genes in the genus *Taylorella*. Res. Vet. Sci. 92(3): 435-437

- Tazumi A., Petry S., Hayashi K., Moore J.E., Millar B.C. & Matsuda M. (2012) Molecular identification and characterization of the intervening sequences (IVSs) within 23S ribosomal RNA (rRNA) genes of *Taylorella asinigenitalis* isolated in France. Res. Vet. Sci. 92(1):45-52

French publications:

- Petry S. & Marcé C. (2012) Etat des lieux de la métrite contagieuse équine (MCE) en France en 2011 : deux cas sur des femelles trotteurs français. Bulletin Epidémiologique Santé animale – Alimentation. N°54, déc. 2012 Spécial MRE – Bilan 2011

- Hébert L., Duquesne F., Laugier C., Renault P. & Petry S. (2012) Séquençage et comparaison des génomes complets de *Taylorella equigenitalis* et *Taylorella asinigenitalis*. Equ'idée. N°79, été 2012

- Petry S. (2012) La métrite contagieuse équine en France. Bulletin Epidémiologique Santé animale – Alimentation. N°49, Avril 2012 Spécial Equidés

- Beck C., Zientara S. & Petry S. (2012) Métrite contagieuse équine : une chute constante du nombre de cas en Europe. La Dépêche Vétérinaire. N° 1153 du 21 au 27 janvier 2012

International conference:

Duquesne F., Hébert L., Breuil M.F., Laugier C. & Petry S. (speaker) Development of a multilocus sequence typing method for analysis of *Taylorella* genus. 9th International conference on equine infectious diseases schedule, Lexington, USA, 21st - 26th October 2012. In proceeding of J. Equine Vet. Science, 2012, 32(10):S70

French poster:

- Duquesne F., Hébert L., Breuil M.F., Laugier C. & Petry S. Development of a multilocus sequence typing method for analysis of *Taylorella* genus. 40èmes Journées annuelles AVEF, Reims, 11-13 octobre 2012

Activity 5: Glanders

- **Outputs achieved**

Following the Workshop on November 24th 2011:

- ✓ The final report was made early January and published on the website on February 02nd, 2012.

Following the Proficiency test (PT) for the serological and PCR diagnosis organised in September 2011:

- ✓ The final report was sent to the DG SANCO regarding PT results and is available on the EU-RL website.

Support to NRLs

- ✓ A CFT training session was organized on December 13-14th, 2012. Initially, 2 NLRs should attend this session (Greece and Poland). Unfortunately, the Greek NLR could not attend the training session.
- ✓ Sera from different NRLs (Irish and Greece) were received by the EU-RL for complementary serological analysis.
- ✓ Advices were given on request to NRLs (about reactive for Greece, about CFT technical points for Sweden).

Serology

- ✓ The *in vivo* diagnosis of Glanders relies on the complement fixation test (CFT).The CFT is the only officially recognized serological test for international trade of horses and other Equidae in non endemic areas or areas with low Glanders prevalence. Due to the absence of an internationally standardised technique, the EU-RL has performed several preliminary tests aiming at standardizing the complement fixation test for Glanders serological diagnosis, applying the general principles described in the OIE manual. A detailed protocol is available on the EU-RL website since October 18th, 2012.
- ✓ A commercial competitive ELISA kit for Glanders has been tested by the EU-RL. Preliminary testing being not satisfactory, contacts have been taken with the company. An updated version of this kit is available since February 2013. This new version will be soon tested.

Research activity

Serology

- ✓ CFT is a routinely used and internationally recognized test to screen equine sera for glanders. Supplementary confirmatory tests are needed. A Western blot assay based on partly purified lipopolysaccaride (LPS) containing antigen of three *Burkholderia mallei* strains was previously published (Elschner *et al*., 2011). *B. mallei* ATCC 15310 and *B. pseudomallei* ATCC 23343 strains were cultivated and LPS-enriched fractions were prepared in order to implement the Western blot testing in the EU-RL laboratory for suspicious sera testing. The potential of these LPS-enriched fractions for ELISA will be soon evaluated with positive commercially available sera and with sera from experimentally immunized horses. Contacts were made with Brazil, India and Pakistan in order to include sera from naturally infected horses.

Valorization

French publications:

Beck C, Zientara S, Derzelle S, Garin-Bastuji B. 2012. Morve : Les laboratoires doivent harmoniser leurs méthodes. La Dépêche vétérinaire. 1160: 20-21.

Activity 6: Website / EURL communication

- ➢ **Outputs achieved :** documents added on the website on 2012 :

Part "News"

- Equine diseases news on 2012

- Summary by weeks of equine diseases notifications in European countries

Part "training sessions"

- Detection and titration of WNV specific antibodies using VN methods

- Extraction by Qiagens columns of WNV RNA genome

- Detection of WNV RNA genome by WNV rt RTPCR (adapted from Linke and al)

- Detection of flavivirus RNA genome by panflavivirus RT PCR

- Diagnosis of IgM antibodies specific for WNV in horses by capture ELISA – ID Screen WN IgM Capture, IDVET

- SOPs of JEV, EEV and WEEV rtRTPCR

- SOP of complement fixation test for Glanders

Part "Workshop"

- Report of the third workshops of the NRLs for equine diseases (23&24 November 2011)

- All the presentations on the Dourine workshop (20&21 November 2012)

Part "Proficiency test "

- Outcome of the 2011 Proficiency test about Glanders CFT and PCR methods

- Outcome of the 2012 Proficiency test about Vesicular Stomatitis by rtPCR methods

Part "documentation"

- The 2011 technical report of the EU-RL for equine diseases

- All the presentations on the IZS EIA conference (Roma, 1 October 2012)

- ➢ Valorization

- Number of view of the website on **2012** : **1461** with **1059** distinct visitors

French publication

Beck C, Bakkali L, Boireau P, Hans A, Lecollinet S, Laugier C, Madani N, Petry S, Zientara S. Le Laboratoire de référence de l'Union européenne pour les maladies équines (Autres que la peste équine). Bulletin épidémiologique Santé animale-alimentation

Annex 1 :

IAEA TC PROJECT RER/5/016:
SUPPORTING COORDINATED CONTROL OF TRANSBOUNDARY ANIMAL DISEASES WITH SOCIOECONOMIC IMPACT AND THAT AFFECT HUMAN HEALTH

"Regional Training Course on Early and Rapid Nuclear and Nuclear-Related Diagnostic and Tracing Technologies for West Nile Fever, Hepatitis E and Equine Infectious Anaemia"

Bornova Veterinary Control and Research Institute;
Erzene mah. Ankara cad. No:172/155; 35010 Bornova; Turkey

08-19 October 2012

DRAFT PROGRAMME

Monday, 08 October 2012

Time	Activity
09:00-11:00	Administration formalities
11:00-12:00	Welcome address, presentation of the host institution and the tasks of the project RER/5/016 by the host
12:00-13:00	Lunch break
13:00-14:00	**Lecture 1 (Dr LECOLLINET, Sylvie):** General review of the West Nile Fever and current trends of the disease, especially in the European region
14:00-14:30	**Lecture 2 (Dr WIDÉN, Frederik):** General review of the Hepatitis E and current trends of the disease, especially in the European region
14:30-14:45	**Break**
14:45-15:45	**Lecture 3 (Dr LECOLLINET, Sylvie):** Diagnostic methods for West Nile Fever (current status and future challenges)
15:45-16:15	**Lecture 4 (Dr WIDÉN, Frederik):** Diagnostic methods for Hepatitis E (current status and future challenges)
16:15-18:00	**Reception**

Tuesday, 09 October 2012

Time	Activity
09:00-10:00	**Practical work 1a (in groups):** IgG ELISA for West Nile Fever-Preparation of the buffers and adding samples into the ELISA plates
	Practical work 1c (in groups): ELISA for Hepatitis E- Preparation of the buffers and adding samples into the ELISA plates
10:00-10:45	-Incubation of the ELISA plates -Discussions with the lecturers on the scope of application and limitations (shortcomings) of the tests.
10:45-11:00	**Break** (after or during the incubation step from 1a-1c)
11:00-12:00	**Practical work 1d (in groups):** IgG ELISA for West Nile Fever- Washing ELISA plates and adding the conjugate solution
	Practical work 1f (in groups): ELISA for Hepatitis E- Washing ELISA plates and adding the conjugate solution
12:00-13:00	**Lunch break**
13:00-14:00	**Practical work 1g (in groups):** IgG ELISA for West Nile Fever- Washing ELISA plates and adding chromogen/substrate and stopping solution
	Practical work 1i (in groups): ELISA for Hepatitis E- Washing ELISA plates and adding chromogen/substrate and stopping solution
14:00-15:00	**Practical work 1j (in groups):** Reading the ELISA plates and interpretation of the results
15:00-16:30	Discussion dedicated on technical and epidemiological interpretation of the results, with emphasis on the context of OIE and EU regulations

-For the Tuesday training (09 October 2012), the participants should be divided in groups of 2 or 3 (for example 7 groups of 3 and 2 groups of 2). They should work together in a dedicated workspace. In order to become familiar with all three ELISA tests, after finalizing individual ELISA phases, the groups should move to another workspace (without moving the reagents)!
-Breaks are not foreseen, as there are many long incubation steps in the procedure.

Wednesday, 10 October 2012

Time	Activity
09:00-10:00	**Practical work 2a (in groups):** Extraction of RNA for PCR (For West Nile Fever, RT phase of the 2 step RT PCR).
10:00-11:00	**Practical work 2b (in groups):** Preparation of the PCR master mix for conventional PCR, adding of the samples, start the thermo-cycling. *Please include serial dilutions of 1 quantified (or strong positive) RNA extract for each disease (if available) in 5, 10 fold dilutions for testing. This should be performed by local staff members.*
11:00-12:00	**Lunch break**
11:00-14:00	**Practical work 2c (in groups):** Thermo-cycling
13:00-14:00	**Practical work 2d (in groups):** Preparation of the buffers, and the gel for gel-electrophoresis
14:00-16:00	**Practical work 2e (in groups):** a) Preparation of the master mix for the nested PCR (Hepatitis E). The gels for Hepatitis E to loaded during the next day b) Application of the samples on the gel and running the gel-electrophoresis
16:00-17:00	**Practical work 2f (in groups):** Reading the gels and photo-documentation of the results
17:00-18:00	**Practical work 2g (in groups):** Discussion of the results with the lecturers and advice on gaps and lessons learned, as well as definition of improvement targets. *Determination of analytical sensitivity of the method using the quantified (strong positive) sample.*

-For the Wednesday training (10 October 2012), the same "group" work should be organized as described for the day before, with necessary precautions to minimize the risk of between contamination.
-Breaks are not foreseen, as there are many long incubation steps in the procedure.
-Please keep the extracted RNA for the day after (Thursday, 11 October 2012)

Thursday, 11 October 2012

Time	Activity
09:00-10:00	**Practical work 3a (in groups):** Prepare the RNA extracts from the day before
10:00-11:00	**Practical work 3b (in groups):** Preparation of the PCR master mix for real time PCR, adding of the samples, start thermo-cycling. *Please include serial dilutions of 1 quantified (or strong positive) RNA extract for each disease in 5, 10 fold dilutions for testing. This should be performed by local staff members.*
11:00-12:00	**Lunch break**
11:00-14:00	**Practical work 3c (in groups):** a) Thermo-cycling b) Application of the samples for Hepatitis E on the gel and running the gel-electrophoresis (see activity 2e from the previous day)
14:00-16:00	**Practical work 3d (in groups):** Reading and interpretation of the results
16:00 ~18:00	**Practical work 3e (in groups):** Discussion of the results with the lecturers and advice on gaps and lessons learned, as well as definition of improvement targets. *Determination of the analytical sensitivity of the PCR method using quantified (strong positive) RNA.* *Demonstration of the correlation curve for quantification (Ct value vs concentration)* *Advice on production of local quantified standards related to appropriate OIE or EU referent laboratory*

-For the Thursday training (11 October 2012), the same "group" work should be organized as described for the day before, with necessary precautions to minimize the risk of between contamination.
-Breaks are not foreseen, as there are many long incubation steps in the procedure.

Friday, 12 October 2012

Time	Activity
09:00-10:00	**Lecture 5 (Dr LECOLLINET, Sylvie):** Current strategies for control of West Nile Fever (current and future considerations)
10:00-11:00	**Lecture 6 (Dr WIDÉN, Frederik):** Current strategies for control of Hepatitis E virus (current and future considerations)
11:00-11:15	Break
11:15-11:45	**Lecture 7 (Dr LECOLLINET, Sylvie):** The role of reference laboratories in standardization and harmonization of diagnostic techniques, in light of the OIE regulations and the EU directive 882/2004.
11:45-12:00	**Lunch break**
12:00-14:00	General discussion

Monday, 15 October 2012

Time	Activity
09:00-10:00	**Practical work 4a:** Titration of 1 strong positive serum for West Nile Fever in 8 2 fold dilutions (each group 1 titration) using ELISA test
10:00-11:00	**Practical work 4b:** Incubation of the ELSA plates
11:00-12:00	**Practical work 4c:** Washing ELISA plates and adding conjugate solution, incubation
11:30-12:30	Lunch break
12:30-13:00	**Practical work 4d:** Washing ELISA plates and adding chromogen/substrate solution
13:00-14:00	**Practical work 4e:** Reading and interpretation of the results
14:00-14:15	Break
14:15-15:15	**Practical work 4f:** Draw the titration curves on a paper or using Microsoft Excel (each group individually), in percentage of positivity (PP-values)
15:15-16:15	**Practical work 4g:** Draw a common chart of all the titration curves in Microsoft Excel, in percentage of positivity (PP-values)
16:15-16:30	Break
16:30-17:30	**Practical work 4g:** Produce a single serum in dilution corresponding to the first dilution after the dilution curve falls beyond the plateau value (strong positive signal). **Practical work 4h:** Produce a single serum corresponding to the half of the dilution where the dilution curve is crossing the cut-off value (weak positive) **Practical work 4i:** These sera should be in sufficient amount for all groups of participants (I assume 1 ml should be enough, depending on initial dilution) Keep these sera in a refrigerator until the exercise next Friday!

Tuesday, 16 October 2012

Time	**Activity**
09:00-10:00	**Lecture 8 (Dr. HANS, Aymeric):** General review of the Equine Infectious Anaemia and current trends of the disease, especially in the European region
10:00-11:00	**Lecture 9 (Dr. HANS, Aymeric):** Diagnostic methods for Equine Infectious Anaemia (current status and future challenges)
11:00-11:15	Break
11:15-12:15	**Practical work 5a:** Agar Gel Immuno-diffusion (AGID): preparation of the Agar plate, punching the wells and sample preparation.
12:15-13:15	**Lunch break**
13:15-15:15	**Practical work 5b:** Load the Agar plates with EIA antigen, control sera and testing samples
15:15-16:15	**Lecture 10 (Dr. HANS, Aymeric):** Current strategies for control of Equine Infectious Anaemia (current and future considerations)
16:15-17:15	General discussion

Wednesday, 17 October 2012

Time	**Activity**
09:00-09:30	**Practical work 6a (in groups):** Principle of the Synbiotics ViraCHEK®/EIA
09:30-10:30	**Practical work 6b (in groups):** Synbiotics ViraCHEK®/EIA - Preparation of the buffers and adding samples into the ELISA plates
10:30-10:45	**Break**
10:45-12:00	**Practical work 6c (in groups):** Incubation of the ELISA plates
12:00-13:00	**Lunch break**
13:00-15:00	**Practical work 6d (in groups):** Incubation of the ELISA plates, reading and interpretation
15:00-16:00	**Practical work 6**: Reading of the AGID test performed on Tuesday October 16th
16:00-17:00	**Discussion**

Thursday, 18 October 2012

Time	**Activity**
09:00-09:30	**Practical work 6a (in groups):** Principle of the IDEXX EIA
09:30-10:30	**Practical work 6b (in groups):** IDEXX EIA - Preparation of the buffers and adding samples into the ELISA plates
10:30-10:45	**Break**
10:45-12:00	**Practical work 6c (in groups):** Incubation of the ELISA plates
12:00-13:00	**Lunch break**
13:00-15:00	**Practical work 6d (in groups):** Incubation of the ELISA plates, reading and interpretation

15:00-16:00	Practical consideration in the use of different tests for EIA in the control strategies of the disease
16:00-17:00	General discussion

Friday, 19 October 2012

Time	**Activity**
09:00-10:00	**Practical work 7a:** Test the strong positive, weak positive and negative sera produced on Monday, 15 October 2012 in the West Nile ELISA test (each group 4 wells/serum)
10:00-11:00	**Practical work 8b:** Incubation of the ELISA plates
11:00-12:00	**Practical work 8c:** Washing ELISA plates and adding conjugate solution, incubation
11:30-12:30	**Lunch break**
12:30-13:00	**Practical work 8d:** Washing ELISA plates and adding chromogen/substrate solution
13:00-14:00	**Practical work 8e:** Reading and interpretation of the results
14:00-14:15	Break
14:15-15:15	**Practical work 8f:** Draw a separate chart for each group, in percentage of positivity or percentage of inhibition, as appropriate (PP or PI-values)
15:15-16:15	**Practical work 8g:** Drawing up a common chart of all the participants in Microsoft Excel, in percentage of positivity or percentage of inhibition, as appropriate (PP or PI-values)
16:15-16:30	**Break**
16:30-17-00	**Questionnaires**
17:00-17:30	**Certificates and closure**