MAISONS-ALFORT LABORATORY FOR ANIMAL HEALTH

AND

DOZULÉ LABORATORY FOR EQUINE DISEASES

EU REFERENCE LABORATORY FOR EQUINE DISEASES

2016-2017 Scientific program of the European Union Reference Laboratory for Equine Diseases

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This document describes the scientific program for the years 2016 and 2017 of the European Union reference Laboratory (EU-RL) for equine diseases. The described program is established in accordance with seven main activities which are listed as follows:

Activity 1: Equine Viral Arteritis (EVA), Equine Infectious Anemia (EIA) and Equine Herpes Viruses (EHV)
Activity 2: West Nile and other exotic encephalitis and Vesicular stomatitis
Activity 3: Dourine
Activity 4: Contagious equine metritis (CEM)
Activity 5: Glanders
Activity 6: Training
Activity 7: Coordination and management

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

Sub-activity 1: Development and validation of diagnostic methods

2016-2017:

Objectives: Equine Infectious Anemia (EIA)

- ✓ *Improvement of molecular diagnostic test*

Equine Infectious Anemia Virus (EIAV) is a *Lentivirus* exhibiting substantial genetic variation. Following primary infection, the virus will persist in the horse. Persistently infected horses, also called asymptomatic carriers, are the reservoir of the virus and may transmit EIAV to other horses even in absence of overt clinical signs. Positive horses are diagnosed when anti-EIAV antibodies are detected following an Agar Gel Immuno Diffusion (AGID) Test. This test recommended by OIE is known to be very specific but rather not sensitive. Moreover, this method is easy to perform for few hundreds of samples but becomes cumbersome when thousands of samples have to be tested. AGID reading is tedious and requires experienced people for good reading interpretation. In addition to those technical difficulties, antibodies in infected horses appear between 3 weeks to several months after infection. Thus an infected horse may be contagious long before to be tested seropositive for EIA. In this condition, a molecular tool able to detect infected horses before the appearance of anti-EIAV antibodies is crucial. Some molecular tools, such as PCR and qPCR, were produced to diagnose EIAV in asymptomatic horses. PCR methods were developed during the 90's to detect mainly strains isolated in North America and, in 2007, to detect the strain responsible of the outbreaks that occurred in Ireland and in Italy in 2006. Nevertheless, those PCR were designed to detect specific EIAV strains. We have decided to develop a new molecular diagnostic tool for the detection of the vast majority of strains circulating in Europe. To do so, we will take advantage of the full genome sequencing project described below, to determine conserved regions inside EIAV genome between several strains that have been isolated in Europe, in Asia, in North and South America. These data will be used to design a real time quantitative PCR that will be able to detect EIAV in infected horses even when viral load is very low, which is the case in asymptomatic horses. This project started in April 2015 and will continue in 2016 and should be done by the end of 2017. Indeed, first of all EIAV strains will need to be sequenced using Next Generation Sequencing method.

- ✓ **Expected outputs**:
  - Determine conserved regions in EIAV genome between strains isolated in Europe, in Asia, in North and South America

- ✓ **Performance indicators:**

- One primer/probe set developed and validated.

✓ *Improvement of EVA serological diagnostic test*

EVA is of major interest for equine industry. Indeed, clinical signs associated to the infection varied from nasal discharge to the death of young foals or abortion of pregnant mares. Virus Neutralization Test (VNT) is used to detect antibodies anti- Equine Arteritis Virus (EVA) in horse sera. This serological method is recommended by the World Organization for Animal Health (OIE) for international trade. Horse trade is increasing regularly over time leading to the potential spread of EAV around the world. Harmonization of official methods is a key component to ensure a better control of EAV and prevent costly outbreaks. VNT implementation in laboratory appears to be somewhat difficult and results interpretation is cumbersome and need experienced and very well trained people. Moreover, some horse sera are cytotoxic for RK-13 cells, which are used to perform VNT, and it is very difficult to determine horse status in such cases. Thus, we have decided to develop a new method for VNT results interpretation independent on microscope observation. To do so we will take advantage of EAV capacity to kill RK-13 cells within 72 hours upon infection. RK-13 cells viability will be assessed by measurement of ATP present in live cells by bioluminescence using an enzymatic reaction. Emission of bioluminescence is proportional to the amount of ATP present in each well and will be measured using a high throughput system allowing a quick analysis of several sera in few minutes. This project will start in 2016 and will take approximately 2 years to be fully developed and validated.

✓ **Expected outputs**:
- Development of a new method for VNT results interpretation

✓ **Performance indicators:**
- SOP for the NRLs available on the EURL website

2017:

Objectives: Equine Infectious Anemia (EIA)

✓ *Production of reference sera*

Production of reference samples through experimental infection of horses with Equine Infectious Anemia viruses, including Wyoming strain and one European strain. Two horses will be infected in BSL3 facilities (INRA PFIE platform) and monitored for 3 months maximum after infection. For transport and for longer lifespan the lyophilisation of those sera will be performed. At first, it will be checked whether lyophilisation steps modify antibody titer in sera. Then, lyophilized sera samples will be sent to NRLs upon request.

✓ **Expected outputs:**
- Two (2) reference sera for Equine Infectious Anemia

✓ **Performance indicators:**
- Numbers of lyophilized sera vials produced

Sub-activity 2: Training and support to NRLs

2016-2017:

Objectives: Equine Viral Arteritis (EVA), Equine Infectious Anemia (EIA) and Equine Herpes Viruses (EHVs)

- Positive cases will be confirmed according to NRLs requirements.

- Biological samples (viral strains, cell lines, sera, blood) will be provided upon NRLs requests.
- With reference to EVA PT results, training sessions will be organized for NRLs who faced sensitivity or specificity issues with their diagnostic tools.

✓ **Expected outputs :**

- Positive and doubtful cases will be confirmed or unvalidated
- Training of scientists and technicians from NRLs to varied diagnostic tools

✓ **Performance indicators :**

- Number of confirmed and unvalidated cases
- Supply of biological samples to NRLs.
- Number of scientist/technicians trained

Sub-activity 3: Epidemiological and specific studies

Objectives: Equine Infectious Anemia (EIA)

Specific studies:

✓ *Molecular characterisation*

Lentiviruses are capable of undergoing extensive genetic variation and a number of studies have documented complex evolutionary patterns in horses experimentally infected with EIAV. However very little is known about variation that occurs within field isolates of this virus. To date complete genomic sequence information has only been published for 4 field strains of EIAV from the US, Europe, Japan and China. These strains share less than 80% nucleotide sequence identity and phylogenetic analysis suggests each comprises a separate monophyletic group or clade. The fact that just four complete genomic sequences have provided evidence for the existence of four distinct clades suggests that variation among EIAV field isolates is both extensive and highly complex. Characterization of the extent of genetic variation occurring among EIAV field strains currently circulating in different EU countries is essential for the development of molecular diagnostic tools and for the eventual design of effective vaccines. Furthermore this information can be used to track the spread of EIAV between countries and assist in the identification of geographical risk factors for transmission. This project will take approximately 2 to 3 years to be completed. The first attempts to sequence the full EIAV genome failed. Yet samples were from a positive horse that exhibited clinical signs, when viremia is the highest. In 2016, we will develop first a protocol to relatively enriche “viral RNA target” from our samples compared to the contaminants (RNA from the host). Then in 2017, we will continue to sequence EIAV isolates by using NGS method.

✓ **Expected outputs**:

- Full genome sequence of different EIAV strains

✓ **Performance indicators:**

- Full genome sequence of 5 EIAV strains

Sub-activity 4: Proficiency test and Workshop

2016:

Objectives: Equine Viral Arteritis (EVA)

In 2016, two proficiency tests for EVA will be organized. One will be about serological diagnosis using Virus Neutralization test (VNT) and the second will be about virological diagnosis using virus isolation (VI) on cell culture and RT-PCR.
This PT will be followed in September or October 2016 by a workshop.

- ✓ **Expected outputs**:
  - Success of NRLs in the organized EVA ring trial

- ✓ **Performance indicators:**
  - Number of participants and average rate of success in PT test

Activity 2: West Nile, other exotic encephalitis and vesicular stomatitis

Sub-activity 2.1: Development and validation of diagnostic tools

Objectives: West Nile Virus (WNV) and other flaviviruses causing meningo-encephalitis in equids

- ✓ *Novel diagnostic tools = Implementation of Luminex assays for the multiplex detection of flavivirus antibodies in varied animal species and development of "syndromic" Luminex assay to investigate viral encephalitis in horses*

The serological diagnosis of infections by neurotropic *flaviviruses* is challenging due to their antigenic cross reactivity. The microsphere immunoassay multiplex technology (Luminex technology) has been implemented in 2013 at the ANSES Animal Health Laboratory in order to help in WNV (and more widely in *flavivirus*) serological diagnosis. Recombinant envelope Domains III (EDIII) of different *flaviviruses* (WNV, Japanese Encephalitis Virus (JEV), Tick-Borne Encephalitis Virus (TBEV) and Usutu Virus) have been produced (collaborative work with P. Desprès, Institut Pasteur) and used for the validation of a new Luminex protocol for the analysis of anti-flavivirus IgGs in horse sera. This Luminex protocol has proved useful for the differentiation of *flavivirus* infections, with sensitivity and specificity close to virus neutralization assays (C. Beck; BioMed Research International, 2015).

We propose now to follow two different strategies for the development of our Luminex diagnostic tool:

1. analysis of *flavivirus* infections through multispecies diagnostics (started in 2015; continue in 2016 and 2017)

Flavivirus epidemiological cycles are characterized by an important diversity of reservoir hosts belonging to different taxons (birds, mammals for Japanese Encephalitis Virus for example…). Many flaviviruses (WNV, Usutu, Bagaza, TBEV, Louping ill, Meaban viruses) have already been described in Europe and many others (Dengue, JEV,…) are at risk for introduction and emergence. In order to get insight into flavivirus emergence and spread in European countries, it would be of upmost importance to rapidly characterize flavivirus infections through

serology in diverse animal species. Currently, only virus neutralization tests can be used to determine the infecting flavivirus whatever the animal species considered (rapid tests, such as ELISAs, allow for the rapid identification of flavivirus infections in diverse animals without providing information on the flavivirus(es) involved), but these tests are long and heavy to carry out and require high amounts of serum (problematic for small bird species for example). Our first aim is to develop a multispecies flavivirus serological diagnostic test by using the Luminex technology. For this purpose, some new EDIII antigens from flaviviruses described in Europe like EDIIIs of Bagaza virus/Turkey Meningoencephalitis virus (BAGV/TMEV) and Meaban virus are being synthetized. Once produced, they will be tested with a panel of well characterized sera with the aim to validate their antigenic properties.
A first multispecies approach has been tested in 2015, by using biotinyled antigens for the detection of IgG binding to magnetic beads coupled to specific flavivirus antigens (WNV, JEV and TBEV) but the sensitivity of the method was found to be low and requires improvements. We will continue in 2016-2017 to improve the multispecies Luminex assay and to test other multispecies approaches like the use of biotinylated G or A proteins for the detection step

2. serological diagnostics of viral aetiologies responsible for equine encephalitis in a single assay = "neurological syndromic" approach on horse sera

A syndromic diagnostics approach for the rapid characterization of neurological cases in equines will be developed by broadening our Luminex tool. For this purpose, antigens corresponding to viruses most at risk to affect European equids (WNV, equine herpesvirus 1 (EHV1)...) and/or corresponding to major health threats for horses (exotic equine encephalitis, rabies viruses,...) will be added to the *flavivirus* Luminex serological tool.

One EHV1 antigen, designed in collaboration with Labeo Frank Duncombe (Caen, France), is being synthetized but some more antigens not yet available in our laboratory (a second EHV1 antigen and one rabies antigen in priority) will be synthesized in 2016. Well-characterized sera sampled on infected or vaccinated horses will be or have been (rabies) provided by reference centers and collaborators (ANSES Nancy, Veterinary schools…) to evaluate these new antigens. The luminex-based assay incorporating these new antigens will be validated afterwards.

- ✓ *Improve the diagnostic capabilities by developing molecular tools able to detect and identify many different flaviviruses (2016-2017)*

In order to develop molecular diagnostic tools for horse diseases, rtRT-PCR microarrays will be designed and developed in collaboration with the Identypath unit at ANSES on the Light Cycler (LC) 1536 platform, which allows to perform 1536 simultaneous reactions in a single plate. Primers and probes targeting horse pathogens of interest will be adapted to the LC1536 format and validated on positive reference samples. This Light Cycler 1536-based approach will be used in priority for the differential diagnostic of neurological syndroms in horses (EHV1, Rabies, WNV and exotic equine encephalitic viruses)

- ✓ *Simplification of Virus Neutralization Tests (VNT) for the confirmation of WNV infections (2016-2017)*

A few European NRLs implement WNV VNT, which is pivotal for the confirmation of horse cases found positive in WNV rapid serological tests. The requirement of BSL3 facilities for WNV VNTs precludes the generalization of this gold standard technique in European NRLs. NRLs will have in the future the possibility to further screen horse sera for WNV antibodies through Luminex or through WNV VNT achievable in BSL2 conditions. To reach this latter objective, a pseudotyped virus harbouring the WNV Envelope protein will be

constructed (collaboration with INRA Lyon) and comparative tests between standard VNT and WNV-E pseudotyped VNT will be conducted thereafter.

- ✓ **Expected outputs** :
  - Production of *flavivirus* antigens for Luminex technology (2016)
  - Protocol for the multispecies detection of *flavivirus* antibodies in varied animal species (2017)
  - Design of primers and probes on LC 1536 for encephalitic viruses (2016)
  - Protocol for the LC 1536-based molecular diagnostic of encephalitis-causing viruses (2017)
  - Implementation of a BSL2 protocol for WNV VNT (2017)

- ✓ **Performance indicators :**
  - Novel Luminex assay for multispecies *flavivirus* diagnostic (2017)
  - Novel Luminex assay for a syndromic approach in horses (2017)
  - Validation of the Light Cycler1536-based approach for the molecular diagnostic of encephalitis-causing viruses in horses (2017)
  - Validation of a BSL2 protocol for WNV VNT (2017)

Objectives: Vesicular Stomatitis Virus (VSV)

- ✓ *Improvement of diagnostic tests for Vesicular stomatitis virus (VSV)*

In order to provide accurate detection of vesicular stomatitis viruses improvement of the one-step real-time RT-PCR will be continued. For this purpose, new primers set and probes allowing detection of viruses from the four serotypes will be evaluated on VSV strains available in the laboratory, purchased or provided through collaborations.

A prototype Luminex test for serological diagnostic of VSV NJ has been developed. This prototype will be evaluated on a panel of sera from infected or naive animals (equine, bovine, porcine). In parallel production of recombinant glycoprotein using baculovirus expression system will be implemented in the laboratory, namely in order to produce recombinant glycoprotein serotype Indiana 1 (VSV-G-IND) whose customized production has failed. Once obtained, this recombinant antigen will be included in the development of Luminex test.

With the aim to develop improved diagnostic test, monoclonal antibodies raised against VSV-G protein NJ and VSV-G protein IND will be produced (sub-contracting) and then evaluated in the laboratory. These antibodies will be used to develop Elisa tests (2016-2017).

- ✓ **Expected outputs :**
  - Improvement of the one-step rtRT PCR method for VS detection of a larger panel of VSV strains (2016)

- Evaluation of Luminex immunoassay for VSV antibodies detection (serotype NJ) (2016)
- Production of recombinant VSV Ag (2016)
- Production of anti-VSV-G NJ and anti-VSV-G IND monoclonal antibodies (2016-2017)
- Development of Elisa Assays (by 2017)

✓ **Performance indicators :**

- An improved operating procedure for the one-step rtRT PCR method for VS detection (2016)
- Batch of recombinant Ag (2016-2017)
- Monoclonal antibodies available to develop serological tests (by 2017)
- A prototype of Elisa test (by 2017)

Sub-activity 2: Organization of Proficiency tests

Objectives: West Nile Virus (WNV) and VSV

- Organization in 2016 of a serological PT for VSV VNT (virus neutralization test) to evaluate and compare the two alternative protocols based on the use of 100 TCID50/25µl or 1000 TCID50/25µl of virus doses described in the OIE manual.
- Organization of a virological PT (VSV) for molecular detection of VSV genomes by rtRT-PCR

- Organization in 2017 of a proficiency test to evaluate the diagnostic performances of NRL methods for the detection of WNV RNA genome (matrix: infected and inactivated samples) and/or antibodies (matrix : horse sera)

✓ **Expected outputs :**

- PT report on serological diagnostics (VSV and WNV)
- PT report on rtRT-PCR (VSV and WNV)

✓ **Performance indicators :**

- Number of NRLs participating to the PT (VSV and WNV)
- Number of NRLs with satisfactory PT results (VSV and WNV)
- Number of positive satisfaction surveys for the PT tests and workshops (VSV and WNV).

Sub-activity 3: Training and support to NRLs

- Support to NRLs in confirming or invalidating positive and doubtful cases upon NRLs request (VSV and WNV)
- Biological samples for WNV and VSV (reference and field strains, cell lines, sera, blood) will be provided upon NRLs requests.

- Organization in 2016 of a workshop on VSV
- Organization in 2017 of a workshop on WNV

✓ **Expected outputs :**

- Positive and doubtful cases will be confirmed or invalidated;
- Improvement of diagnostic methods in NRLs for WNV and VSV and reduction in the number of NRLs failing the PT tests.
- High participation rate of NRLs to the workshop.
- Good quality of proficiency test restitution and good quality of debates for the workshop

✓ **Performance indicators :**

- Number of confirmed and invalidated cases
- Supply of biological samples to NRLs.
- Participation rate of NRLs to the workshop.

Sub-activity 4: Epidemiological and specific studies

Objectives: West Nile Virus (WNV)

Epidemiological studies:

Without enough positive field sera, the correct validation of new diagnostic tools for exotic encephalitic diseases is problematic. We'll continue our efforts to obtain more positive field sera from horses infected with Japanese, Eastern, Western and Venezuelan equine encephalitis viruses with collaborations in USA (Dr M. Long, University of Florida College of Veterinary Medicine and USDA; Ames in Iowa) and Asia (Pasteur Institute of Cambodge, Japan racing association in Japan…)

Specific studies:

We are also pursuing our efforts towards the characterization of WNV virulence determinants. A reverse genetics system for the production of de novo recombinant WNV particles, strain IS-98-ST1 (Is98), has been obtained in 2011 in our laboratory (see Bahuon et al, 2012) in order to address the impact of specific point mutations observed in emerging European WNV strains for WNV virulence in incidental (mammalian) and reservoir (avian) hosts. For this purpose, mutant viruses have already been generated. In particular, a mutant in the helicase part of NS3 is severely impaired in its replication capacities, as well as in its virulence for birds and mammals. In 2014-15 investigations of the infection steps that are impacted by this mutation during the infection process *in vivo* were conducted (growth kinetics in cell-culture systems, virulence in Balb/cJ mice) (publication in progress). Other mutant viruses, incorporating mutations that have recently appeared in European WNV strains and suspected to modulate WNV virulence, will be generated and characterized *in vitro* and *in vivo* in 2016-2018.

In 2008, a less pathogenic strain emerged in Italy, strain It08. Therefore, we wish to study the importance of different genomic regions by substituting Is98 genomic regions in the Is98 infectious clone with the homologous It08 genomic region. Constructions with regions including NS4A, NS4B and a part of NS5, and another one with most of NS5 and the 3'UTR were obtained in 2014, andviral particles were recently obtained. A particularity of our Is98 infectious clone is the presence of a stop codon in the NS1 region, which could hamper the generation of recombinant virus particles, in particular if coding sequences from other WNV strains are inserted. In order to speed up the production of chimeric viruses, in 2016, a new approach, the Circular

Polymerase Extension Cloning (Quan and Tian, 2014; Setoh et al, 2015) will be tried to obtain Is98/It08 chimeric constructs.

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.

Quan J, Tian J. 2014.Circular polymerase extension cloning. Methods Mol Biol;1116:103-17. doi: 10.1007/978-1-62703-764-8_8.

Setoh YX, Prow NA, Rawle DJ, Tan CS, Edmonds JH, Hall RA, Khromykh AA. 2015. Systematic analysis of viral genes responsible for differential virulence between American and Australian West Nile virus strains. J Gen Virol;96(Pt 6):1297-308.

- ✓ **Expected outputs :**
  - Horse positive sera well characterized for exotic encephalitis viruses
  - Production of chimeric Is98/It08 West Nile viruses (for structural and different non structural genes) and their characterization
  - Production of mutant Is98 West Nile viruses and their characterization

- ✓ **Performance indicators :**
  - Number of positive EEEV, WEEEV, VEEEV and JEV sera received
  - Number of positive samples for flavivirus antibodies or RNA
  - Publication of one article in an internationally recognized journal

Objectives : Vesicular stomatitis virus (VSV)

Specific study : development of a recombinant VSV- Canine Adenovirus based vaccine

The VSV-G protein coding gene from serotype New Jersey synthetized as codon-optimized gene, has been cloned into a non-replicative canine Adenovirus vector previously developed in our laboratory. *In vitro* production of the recombinant VSV-G protein (VSV-G-NJ) is currently tested after transfection of the corresponding recombinant Adenovirus vector into canine kidney cells (DK-E1). Immunogenicity will be then evaluated by Elisa and / or Luminex method. The ability of the recombinant virus to induce VSV neutralizing antibodies will be tested in a murine model. In positive case, the evaluation of this candidate vaccine in pig will be carried out in 2017. Challenging experiment in horses could be further planned if results obtained in pigs are satisfactory.

- ✓ **Expected outputs :**
  - Production of a recombinant non replicative VSV-Canine Adenovirus

- ✓ **Performance indicators :**

- Success in induction of VSV neutralizing antibodies in a murine model
- Success in induction of VSV neutralizing antibodies in a porcine model

Activity 3: Program in Parasitology (Dourine)

Sub-activity 1: Support to NRLs and network coordination

In 2016, a training session will be organized for two national reference laboratories. Based on the results obtained at the 2015 ring trial, it will be proposed to two laboratories considered as less efficient than the others to follow a training session adapted to their needs and to the qualification of the participants (veterinarian, technician or biologist).

Reference serum and antigens have been produced by the EU-RL. Up to now one high titer and one low titer serums are available for the European NRLs. In 2016, our own antigen production (strain OIE) will be finalized and validated. Antigen production is highly time consuming and quality vary among producers. In order to be more efficient, collaboration will be set up with the USDA (Ames – Iowa). The first step of this collaboration was the one week training in June 2015 in our laboratory of the veterinarian in charge of the dourine at the USDA (diagnosis, reagent production). Then, official setting up of this collaboration will be planned during a visit at the USDA in December 2015. In order to harmonize the serologic diagnosis of dourine, in 2016 and 2017, EU-RL will provide to USDA the reference strain OVI for the antigen production. EU-RL should also validate the batches of antigens produced by USDA.

In 2016 and 2017, EU-RL will participate as a core member to activity and coordination of the OIE Non Tsetse Transmitted Animal Trypanosomoses network (NTTAT) that has been created in May 2015. EU-RL will also contribute to the organisation of a joined NTTAT scientific meeting by providing a conference room at the Anses (Maisons-Alfort).

- Support to NRLs in confirming or invalidating positive and doubtful cases upon NRLs request.

✓ **Expected outputs**:
- Positive and doubtful cases will be confirmed or invalidated.
- Harmonization of diagnostic methods among EU NRLs and other laboratories.
- Optimization of antigen production.

✓ **Performance indicators:**
- Number of confirmed and invalidated cases;
- Supply of biological samples to NRLs;
- Harmonization of practices between EU-RL and USDA for antigen production (e.g. use of the *Trypanosoma equiperdum* OVI strain at the USDA for antigen production).

Sub-activity 2: Optimization of diagnosis

One of the main problems for dourine research activity is the lack of *Trypanosoma equiperdum* strains and sera from naturally and experimentally infected horses. This makes impossible the correct validation of new diagnostic tools.

Sample campaign in Tajikistan in 2016:

Following the development in 2014 of an efficient collaboration with the OIE reference laboratory in Moscow, the goal of this campaign is to sample horses naturally infected with trypanosomes in Tadjikistan in order to get well documented sera and strains.

It is expected that about 50 confirmed infected horses will be sampled in April or May 2016 during the mating period when the probability to isolate parasites in the blood is higher. In 2016 and 2017, the blood samples will be inoculated in rodents in order to propagate the parasites and isolate strains for research purposes.

Optimization of serological diagnosis:

Working program on dourine focused up to now on discrimination of the agents of the *Trypanozoon* sub-genus. The goal was to develop and validate more specific methods and tools for the serodiagnosis of dourine. But the three causative agents of dourine, surra and nagana are very close and cross reactions are observed, demonstrating the absolute inadequacy of current diagnostic tools. Thus, during the last meeting of the new OIE NTTAT (21-23 July 2015) it was decided to firstly focus the work on obtaining a reliable serological test for the *Trypanozoon* sub-genus as a basis for the detection of equine trypanosomes. Positive results with this test could then be confirmed with existing tools (CATT surra, dourine CFT, PCR etc.) and the epidemiological context. In a first time, current serological tools will be evaluated from sera collections of dourine and surra reference laboratories, and if the results are not conclusive, an ELISA will be developed.

- ✓ **Expected outputs:**
  - Provision of biological samples for research on dourine
  - Optimization of a serological method to support the official complement fixation test
- ✓ **Performance indicators:**
  - Number of sera sampled and strains isolated
  - Number of serological methods optimized

Activity 4: Contagious equine metritis (CEM)

Sub-activity 1: Support to NRLs and epidemiological study

- Biological elements will be provided according to NRLs requirements: reference and field Taylorella *equigenitalis* and *Taylorella asinigenitalis* strains; polyclonal anti-*Taylorella equigenitalis* serum for slide agglutination test.
- CEM positive cases will be confirmed according to NRLs requirements.
- According to results of the 2015 inter-laboratory proficiency test on CEM, training sessions will be organized in 2016 for NRLs who faced sensitivity or specificity issues with their diagnostic tools.
- After the development by the EU-RL of a specific MLST for the *Taylorella* genus (tool published in 2013 and transferred to interested LNRs in 2014), the characterization of the molecular diversity of *Taylorella*

equigenitalis isolates circulating in Europe started in 2014 in collaboration with seven NRLs which alone cover 85% of CEM cases reported between 2009 and 2013. The aim of the study was to characterize the different *Taylorella equigenitalis* population circulating in Europe. Currently, the EU-RL performed the MLST typing (after bacterial species verification and DNA extraction) on the Belgian, French, Polish and Swiss *Taylorella equigenitalis* and *Taylorella asinigenitalis* strains selected by NRLs for the study. For the remaining three countries, the NRLs should perform themselves the MLST typing; if finally they do not have enough time or means to do it, the EU-RL will propose to perform the work from strains or DNA that will be provided from these NRLs. A preliminary report will be presented to the European NRL network during the European workshop organized by the EU-RL on October 1st 2015. This presentation may motivate other NRLs to join the project in order to have a more comprehensive view of the different *Taylorella equigenitalis* population circulating in Europe.

Consequently, in 2016 the EU-RL may need to continue the MLST typing from strains or DNA which would be sent from new NRLs involved in the project, and will continue to provide technical support for the NRLs who want to carry out the MLST typing themselves. After that, the EU-RL proposes to valorize the results by writing and submitting a publication in an international peer review newspaper (planned in 2016 or 2017 depending on the number of strains to be analyzed in 2016). In the future, any strain sent to the EU-RL for confirmation will be typed by MLST.

✓ **Expected outputs:**

- Meet the scientific and technical needs of NRLs;
- In 2016, training of scientists and technicians from NRLs to CEM diagnostic tools (culture, PCR, immunofluorescence);
- Better knowledge of *Taylorella equigenitalis* (and *Taylorella asinigenitalis*) strains circulating in Europe.

✓ **Performance indicators:**

- Number of actions carried out to meet the NRLs needs compared to the number of NRL scientific and technical solicitations;
- Number of scientist/technicians trained in 2016;
- Number of *Taylorella equigenitalis* (and *Taylorella asinigenitalis*) strains received and typed by MLST.

Sub-activity 2: Production and validation of diagnostic methods and tools

- ***Molecular screening tools.*** Several 16S rRNA PCR were developed among others:
    - Bleumink-Pluym et al. (1994): conventional PCR for detecting the *Taylorella* genus. The PCR product must be sequenced to differentiate *Taylorella equigenitalis* from *Taylorella asinigenitalis*;
    - Duquesne et al. (2007) and Breuil et al. (2011): conventional PCR for detecting *Taylorella equigenitalis* and *Taylorella asinigenitalis*, respectively;
    - Arata et al. (2001): multiplex PCR; Premanandh (2003): real-time PCR without probe (the melting curve is necessary to discriminate *Taylorella equigenitalis* from *Taylorella asinigenitalis*) and Wakeley et al. (2006): real-time PCR with two TaqMan® probes (the commercial Qiagen cador

Taylorella equigenitalis PCR kit validated by the AHVLA corresponds to the reformulated *Taylorella equigenitalis* assay from the original Wakeley et al. real-time PCR) for detecting both *Taylorella*.

These PCR have already been evaluated and show a very good sensitivity. However, false positive results can be observed in the presence of samples highly contaminated with genital microflora, the explanation being that the DNA region targeted is common among all prokaryotes.

Several non-16S rRNA PCR now exist for detecting *Taylorella equigenitalis* among others:

- Anzai et al. (1999): semi-nested PCR in unknown DNA region;
- Commercial Genekam Biotechnology AG: nested PCR;
- Commercial genesig real-time PCR in DNA gyrase subunit B gene.

Moreover Kinoshita et al. (2015) recently published two loop-mediated isothermal amplification (LAMP) for detecting *Taylorella equigenitalis* and *Taylorella asinigenitalis*, respectively in the 23S rRNA.

In this context, the EU-RL intends to evaluate in 2016 the non-16S rRNA PCR and LAMP mentioned above, and compare the results with those obtained for 16S rRNA PCR. This will be done with the supervision of a student in master degree (to be engaged in 2016 on the EU RL budget).

- ***Culture method.*** The proposition of a more sensitive culture medium (improving the *Taylorella equigenitalis* growth rate and/or reducing the overgrowth of media by the horse genital microflora) is not still relevant despite much investment for this. Indeed, several research tracks have been followed since 2014 without being able to determine a new medium composition due to a complex metabolism of *Taylorella equigenitalis* and its sensibility to most antibiotics tested. Nevertheless, some of these research tracks should be continued, as the study of the *Taylorella equigenitalis* growth in suitable media for eukaryotic cells, or the addition of a chromogenic substrate to better differentiate *Taylorella equigenitalis* colonies from equine vaginal microflora.

  Since several years the EU-RL tries to develop a culture medium for *Taylorella equigenitalis* in close collaboration with an industrial partner, manufacturer of culture media: BioMérieux. The objective is to improve the sensitivity and specificity of the bacteriological diagnosis of CEM. Three lines of research have been investigated:

  Improve the basis of the culture medium. Following the analysis of the nutritional needs of *Taylorella*, panels of selected culture media were tested. A *Taylorella* growth was observed only with i) a bacteriological culture medium (*Haemophilus* Test Medium), ii) commercial milk and iii) two culture media for eukaryotic cells culture (Schneider environment and L15 Leibovitz). Nevertheless, the productivity of these media remains limited and variation between strain growth were observed.

  Improve the antibiotic composition of culture medium. A very large antibiotic panel was tested on a selection of a representative panel of *Taylorella equigenitalis* (n= 19) and *Taylorella asinigenitalis* (n= 8) selected on the basis of molecular typing data obtained using MLST. None of the tested antibiotics was proved to be suitable for its use in CEM diagnosis. These results highlight the low capacity of *Taylorella* to develop an antibiotic resistance and therefore this way of culture medium improvement was excluded.

  Improve the Taylorella visualization by addition of a chromogenic substrate. The *in silico* enzymatic capacity of *Taylorella* and expertise of our industrial partner were used to select a panel of chromogenic substrates to test. Two substrates showed the capacity to induce a coloration of the *Taylorella* colonies on agar plates but this coloration was not specific and many other tested bacterial species were also colored. Consequently, and given the limited metabolic capacity of *Taylorella*, it was decided to seek a

chromogenic substrate to color every member of bacterial flora except *Taylorella* (negative staining). In this purpose, a second panel of chromogenic substrates was selected and preliminary tests revealed that two substrates had the capacity to color a large panel of bacteria without inducing coloration of the Taylorella colonies. This research continued in 2015 with a study on a larger scale (in terms of quantity of Taylorella strains and species of bacterial flora) of these two chromogenic substrates. Depending on the results, the production of media containing these substrates should be performed and a validation step should be conducted.

Accordingly the development of a new culture medium to improve the CEM bacteriological diagnosis may be continued in 2017. The perspectives are to identify and study the factors allowing the growth of *Taylorella* in these very different media (HTM, milk and eukaryotic cell culture media).

- ✓ **Expected outputs:**
  - Conduct an evaluation of molecular screening tools recently developed for detecting *Taylorella equigenitalis*.
  - Improve the sensibility and selectivity of the culture media used for the bacteriological diagnosis of CEM.
- ✓ **Performance indicators:**
  - Number of molecular screening tools evaluated.
  - Number of media evaluated towards Taylorella growth

Activity 5 : Glanders

Sub-activity 1: Development and validation of diagnostic methods and tools

CFT harmonization (2016-2017)

The harmonization of the CFT protocols within EU will be continued. In 2015, laboratories which obtained weak results for some sera included in the 2014 IPTL panel and/or those which used antigens or methods known to have an impact on the sensitivity of the results were invited to compare their protocol with that of the EU-RL. Results obtained from this comparison will be individually analyzed in collaboration with each participant.

Querying EU laboratories showed that practices regarding the antigen titration procedure varied between laboratories, when done. A standard serum is absolutely necessary for this purpose. As for other diseases, harmonization of the antigen batches at the manufacturer level might be a step in the harmonization of methods, the laboratory only having to make a simple control on each new batch, with a reference serum. With this objective, the main producers (Bioveta, Ccpro, CVI, USDA) will be contacted to see if such an approach is feasible.

Standard sera

Given the difficulty to access large volumes of positive sera (related to outbreak accesses, fluctuating collaborations), an experimental infection of 4 horses with an inactivated bacterial suspension of *B. mallei* is programmed (2016).

The objective is to collect at least 5L of sera which will be pooled, filtered, re-tested by CFT and ELISA then lyophilized. This will constitute our reference serum for CFT and ELISA analysis. This reference serum is necessary for the CFT standardization among EU reference laboratories.

End 2015, visits will be organized in Dubai (OIE Glanders reference laboratory) and Brazil (Ministry of Agriculture of Brazil (MAPA)) to strengthen the established collaborations. The constitution of an international serum standard will be discussed.

Consortium for the validation of CFT alternative tests (2016-2017)

The EU-RL has integrated a research project to validate, according to OIE procedures, the currently available CFT alternative serological tests. This consortium of 2 years funded by the FEI (International Equestrian Federation) and IFHA (International Federation of Horseracing Authorities) incorporates Brazilian, Pakistani, and Indian teams, the OIE laboratory of Iena and our laboratory, led by the OIE laboratory. This project includes 2 indirect ELISA (EU-RL, Brazil), 1 recombinant ELISA (India) and the Immuno Blotting (Germany). These different candidates will be tested in parallel on a range of free and infected sera which will be specifically collected as part of this project. Ultimately, the project aims to establish the characteristics of each candidate tests (sensitivity, specificity, reproducibility). The most relevant tests may be proposed to the European laboratory network.

Selection and validation of a PCR for the specific detection of *B. mallei* to be continued (2016)

The major challenge of developing a reliable assay for the detection of B. mallei strains is its high similarity to the other strains in its genus. A extremely high degree of genomic homology exists between *B. mallei* and *B. pseudomallei*. To evaluate the wide range of PCR methods used in laboratories for *B. mallei* identification, a computer-based comparative analysis of PCR-target sequences reported in the literature has been conducted. Sequences have be compared against all publicly available *Burkholderia* genomes and sorted for specificity. PCR primer sets with highest in silico specificity have now to be tested on *Burkholderia* strains and directly in tissues from naturally infected horses.

Selection and Design of candidate genes (2017)

A comparative analysis of all available whole genome sequences in databases (16 genomes of *B. mallei* and >30 genomes of *B. pseudomallei*) will be performed to identify the most relevant potential targets to test (10-20). We will focus in particular on genes encoding products found on the cell surface, such as outer membrane proteins. Several softwares will be used to predict the subcellular localization of the encoded proteins or to predict the presence of signal peptides or transmembrane domains [SignalP v3.0 (Bendtsen et al, 2004), v.2.0 TMHMM (Krogh et al, 2001), PHYRE V.0.2 (Kelley & Sternberg, 2009) and PSORTb v.3.0.2 (Yu et al, 2010)]. Genes with high sequence homology with other bacterial species (except those related to *B. pseudomallei*) will be discarded. B. mallei proteins will be synthetized and tested with a collection of sera collected from naturally infected horses to reveal reactive antigens.

✓ Expected outputs:

- Standard serum from CFT analysis;
- Better harmonisation of the CFT within EU;
- Validated CFT alternative serological methods;
- Specific *B. mallei* PCR methods validated on naturally infected tissues.

✓ Performance indicators

- Lyophilized reference serum, sent on request to NRLs.

Sub-activity 2: Training and support to NRLs

- Training sessions will be organized according to NRLs requirements;
- Positive and doubtful cases will be confirmed or invalidated according to NRLs requirements;
- Reagents and/or support will be supplied to the NRLs upon request.

✓ **Expected outputs :**

- Positive and doubtful cases will be confirmed or invalidated;
- Training of scientists and technicians from NRLs to Glanders diagnosis tools

✓ **Performance indicators :**

- Number of trained NRLs;
- Number of confirmed and invalidated cases;
- Supply of biological reagents to the NRLs.

Sub-activity 3: Glanders Proficiency test and Workshop (started in 2017)

In 2017, two proficiency tests for Glanders will be organized. One will be about serological diagnosis using Complement Fixation test and the second will be about molecular detection of *B. mallei* using PCR. This PT will be followed in September or October 2017 by a workshop.

✓ **Expected outputs** :

- Success of NRLs in the organized Glanders ring trial

✓ **Performance indicators :**

- Number of participants and average rate of success in PT test

Activity 6: Training

Training sessions for the NRLs will be planed.

In 2016, a priority will be granted to NRLs who have failed dourine or CEM proficiency tests and training on Glanders will be also organized for 2 NRLs. Other trainings could be proposed by the EU-RL depending on the remaining budget and on demand of NRLs.

In 2017 training sessions will target in priority NRLs who have failed EVA or VSV proficiency tests. Other trainings could be proposed by the EU-RL depending on the remaining budget and on demand of NRLs

✓ **Expected outputs:**

- Improve the diagnostic of equine diseases in the NRLs.

✓ **Performance indicators:**

- Number of trainings organized.

Activity 7: Coordination and management

Sub-activity 1: Coordination, management and communication

- General coordination by the EU-RL of the NRL network (dispatch of documents, coordination of the scientific and technical support to NRLs…)

- Assist Commission with scientific and technical advice

- In house follow-up of EU-RL activities, expenses, support to laboratory units involved in EU-RL activities

- Preparation of the activity report, workshop reports, scientific program and performance indicator reports

- Improvement and up to date of the EU RL website

  ✓ A regular up to date of NRL contacts database

  ✓ A scientific monitoring of the equine diseases managed by EU-RL and by the updating of parts "news" or "documentation" as necessary

  ✓ The addition of all next workshops, proficiency tests, training sessions and documentation

  ✓ The addition of SOPs for each equine diseases diagnostic methods

✓ **Expected outputs:**

- Good communication between the EU-RL and the NRLs network

- To assist the Commission in case of specific requests and to have trained personnel available for emergency situations occurring within the European Union

✓ **Performance indicators**

- Number of website visits

- Positive feedback of the project officer concerning the EU-RL on equine diseases

Sub-activity 2: annual NRLs workshops

On 2016, two workshops on one day will be organized at ANSES Maisons-Alfort and will be focused on EVA and VSV diseases. 2 speakers from USDA (Dr Luis Rodriguez and Dr Sabrina Swenson) will be invited for the VSV workshop.

On 2017 two workshops on one day will be focused on Glanders and WNV disease. One speaker (Dr M Long from University of Florida) will be invited for WNV workshop and one speaker from a country infected with *B. mallei* will be invited for Glanders workshop.

Results and analysis of the ring trials for will be discussed as well as diagnosis improvement and epidemiological situations of the diseases. Following the two workshops, final reports and every presentation will be published on the website.

- ✓ **Expected outputs :**
  - High participation of NRLs for the two workshops/year .
  - Good quality of Proficiency tests restitution and good quality of debates for the two workshops/year
- ✓ **Performance indicators**
  - Number of participating NRLs in the two workshops/year
  - Number of positive satisfaction surveys for the two workshops/year.