



MAISONS-ALFORT LABORATORY FOR
ANIMAL HEALTH

AND

DOZULÉ LABORATORY FOR EQUINE
DISEASES



***EU REFERENCE LABORATORY
FOR
EQUINE DISEASES***

2015 Scientific program of the European Union Reference Laboratory for Equine Diseases

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This document describes the 2015 scientific program 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

Objectives: Equine Infectious Anemia (EIA)

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 antibodies anti-EIAV 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 will take approximately 2 years since EIA viruses will have to be sequenced at first.

- ✓ **Expected outputs :**
 - Determine conserved regions in EIAV genome between strains isolated in Europe, in Asia, in North and South America
- ✓ **Performance indicators :**
 - At least one primer/probe set developed and validated.

Sub-activity 2: Training and support to NRLs

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 EIA 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 Viral Arteritis (EVA)

Epidemiological studies:

A collaboration with the Faculty of Veterinary Medicine Warsaw University of Life Sciences (Dr. Lucjan Witkowski) in Poland will start in 2015. The aim of this collaboration is to determine the seroprevalence, performing the virus neutralization test (VNT), of EVA in Polish horse population as well as to characterize virus that would be isolated from shedder stallions.

✓ **Expected outputs :**

- Sero and molecular epidemiology of EVA in Poland

✓ **Performance indicators :**

- Number of seropositive horses in Poland
- EVA characterization

Specific studies:

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. Moreover, EVA may persist in the reproductive tract of stallions following the primary infection. Those stallions can shed the virus for life and can transmit the virus during breeding or when their semen are used for artificial insemination. Shedders stallions act as a reservoir of EVA. Two vaccines are available, one attenuated and one inactivated which is authorized in Europe. The development of a drug able to prevent viral replication in infected horses, especially in stallions, would be of great interest.

A collaborative research program with Pasteur Institute, Paris (PO. Vidalain) aims at identifying small molecules with a broad antiviral spectrum, expected to be active against viruses belonging to different virus families and genus. The Normandy Centre for Drug Studies and Research (CERMN, Caen) has identified a promising molecule in its chemical library (sr1057). In 2015, we will test the antiviral activity of sr1057 against EVA *in-vitro* in different cell lines (RK-13, Vero, ED and BHK-21).

✓ **Expected outputs :**

- Determine sr1057 efficacy against EVA *in vitro* in different cell lines

✓ **Performance indicators :**

- Concentration at which sr1057 may be active against EVA.

Objectives: Equine Infectious Anemia (EIA)

Specific studies:

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 EIA 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. In 2015 we will start by using NGS to sequence the full length genome of 15 EIAV isolated in different countries (France, Germany, Romania...).

- ✓ **Expected outputs :**
 - Full genome sequence of different EIAV strains

- ✓ **Performance indicators :**
 - Full genome sequence of 15 EIA strains

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

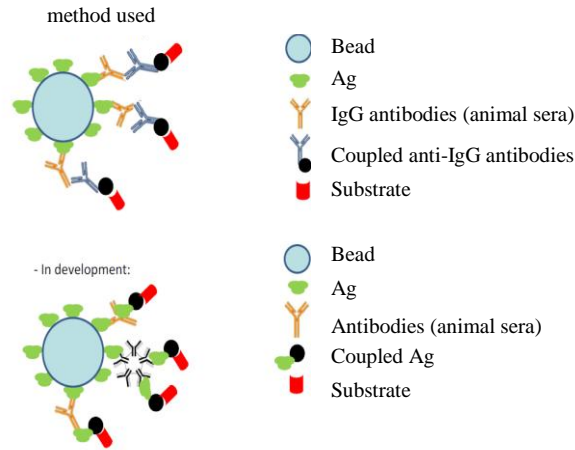
- ✓ *Novel diagnostic tools = Implementation of Luminex assays for the multiplex detection of flavivirus antibodies in varied animal species and development of "syndromic" Luminex assay to investigate viral encephalitis in horses*

The 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. The difficulty in establishing flavivirus infections through serology is due to antibody cross reactivity among members of a specific serocomplex, as well as to a lesser extent between flaviviruses belonging to different serocomplexes. The Luminex offers the possibility to multiplex flavivirus antigens and to compare the antibody response against different flaviviruses in a single run. Recombinant envelope Domains III (DIII) 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 (manuscript in preparation).

We propose in the coming years (2015-2017) to widen the range of analysis of the flavivirus Luminex serological tool, with regards to two aspects:

1. analysis of flavivirus infections in reservoirs (mainly in birds, also in pigs for JEV) and susceptible hosts (mainly mammals) = multispecies diagnostics
2. serological diagnostics of viral aetiologies responsible for equine encephalitis in a single assay = "syndromic" approach on horse sera

For the first approach, we will use either the competition concept, by using monoclonal antibodies coupled to the revealing fluorochrome that will bind the Luminex bead-antigen complex if the animal sera does not contain antibodies reacting with the antigen or an even more promising concept (developed for bluetongue Luminex serology for example) relying on the addition of antigens coupled to the revealing fluorochrome (see the following figure for more information). Flavivirus DIIs will be used as antigens (the same as for the protocol on horse sera) and well-characterized animal sera, sampled in varied bird species, in pigs or in horses will be used for the validation of this new Luminex assay.



For the syndromic approach on equine viral encephalitis cases, antigens corresponding to viruses most at risk to affect European equids (Borna, Louping ill (LIV) viruses, equine herpesvirus 1 (EHV1)...) and/or corresponding to major health threats for horses (exotic equine encephalitis, rabies viruses,...) will be added in the flavivirus Luminex serological tool. Antigens are already available (E2 proteins for alphaviruses responsible for exotic equine encephalitis synthesized in our laboratory, LIV antigens available in collaborating institutes (Pasteur,...), or will be synthesized. Well-characterized sera sampled on infected horses will be provided by reference centers and collaborators (ANSES Nancy, Veterinary Faculty of Vienna,...).

The corresponding tools will be developed by a master student (to be engaged in 2015 on the EU RL budget).

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

A few European NRLs implement WNV VNT, which is pivotal for the confirmation of horse cases found positive in WNV ELISA or IHA. 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 Vesicular Stomatitis Virus (VSV) harbouring the WNV Envelope protein will be constructed (2015-2016) and comparative tests between standard VNT and VSV-E WNV VNT will be conducted thereafter.

Objectives: equine exotic encephalitis viruses

✓ *Improvement of diagnostic tests for equine exotic encephalitis viruses*

Concerning exotic encephalitis viruses, JEV positive horse sera have been collected in 2014 thanks to the strong commitment of Takashi Yamanaka from Japan Racing Association. In 2015-2016, we will boost collaboration initiatives with American laboratories, in order to get and collect field sera positive for E/W or VEEV antibodies.

✓ **Expected outputs :**

- Implementation of multiplex luminex tools for the serological diagnosis of flavivirus infections in varied animal species and for the investigation of nervous syndromes in horses (2015-2016)
- Implementation of a BSL2 protocol for WNV VNT (by 2017)

✓ **Performance indicators :**

- A new operating procedure for the 2 Luminex tools will be disseminated to NRLs (2016-2017).
- Construction of a pseudotyped VSV-E WNV virus

- Number of E/W/VEEV horse positive sera collected in 2015

Objectives: Vesicular Stomatitis Virus (VSV)

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

A one-step duplex real time RT-PCR was developed and implemented for detection and typing of VSV. A SOP has been written and published on the EU-RL website. This method will be evaluated within the framework of the proficiency test that will be organized in October/November 2014. Since this method based on previously published primers and probes (Hole 2006) may not detect all circulating VSV strains, new primer and probe sets allowing detection of a larger VSV strains have been designed by bioinformatics analysis of available sequences. Assessment of these primers and probes in the one step duplex method is in progress. Selected primers and probe sets will be then used to evaluate and validate the method.

Recombinant glycoprotein from VS, serotype New Jersey (VSV-G-NJ) has been produced using baculovirus expression system. Recombinant glycoprotein from VS, serotype Indiana 1 (VSV-G-IND) will be produced using the same eukaryotic expression system. Both proteins will be used as antigen to develop serological luminex assay (simplex and duplex serological immunoassay) and Elisa tests.

For identification and quantification of neutralizing antibodies against VSV, the (Virus neutralization) test is a prescribed test for international trade. According to the OIE terrestrial manual of diagnostic tests, the VN test is carried out using inactivated serum as test sample, 1000 TCID₅₀ /25µL (50% tissue culture infective dose) of VS NJ or IND virus, and Vero M cells to test for the presence of unneutralised virus. An alternative protocol using virus at 100 TCID₅₀ /25µL is also applicable. We aim to standardize the VN methods used in the different NRLs, using namely reference horse sera recently obtained from USDA with the objective to organize a proficiency test on the standardized method in 2016.

✓ **Expected outputs :**

- Evaluation of the one-step rRT PCR method for VS detection and typing.
- Development of a one-step rRT PCR method for VS detection and typing of a larger panel of VSV strains
- Prototype of Luminex immunoassay for VSV antibodies detection (serotypes NJ and Indiana1)
- Standardization of the VN methods between laboratories

✓ **Performance indicators :**

- A new operating procedure for the one-step rRT PCR method for VS published on the EU RL website
- Number of NRLs testing the one-step rRT PCR method for VS detection and typing and moving to this method

Sub-activity 2: Training and support to NRLs

Objectives : West Nile Virus WNV and VSV

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

✓ **Expected outputs :**

- Positive and doubtful cases will be confirmed or invalidated;

✓ **Performance indicators :**

- Number of confirmed and invalidated cases
- Supply of biological samples to NRLs.

Sub-activity 3: Epidemiological and specific studies

Objectives: West Nile Virus (WNV)

Epidemiological studies:

A great diversity of flaviviruses is known to circulate in Europe among birds and/or mammals and is probably underestimated (new flaviviruses identified in insects, European regions or countries with surveillance systems to strengthen,...). We would like to estimate the feasibility of identifying flavivirus circulation patterns in European countries (France, Spain in the first place) through the screening of wild species (wild boars, cervids or birds) (collaborations with Elsa Jourdain, INRA, Philippe Gourlay, ONIRIS, Marta Cerdà Cuéllar, CRESA). Collections from wild birds or mammals from 2009-2014 will be analysed with flavivirus serological tools and RT-PCR.

A new flavivirus, close to a tick-borne flavivirus, Meaban, initially isolated from gulls in Brittany, France, has been isolated from soft ticks sampled recently in Medes Island, Spain (collaborative work with Thierry Boulinier, Centre d'Ecologie Fonctionnelle et Evolutive and Elsa Jourdain, INRA). Its genetic evolution along the years and among geographic areas (France, Spain) will be assessed in 2015 (no infectious virus could be obtained in 2014 from the 2013 samples, hampering the genetic characterization of flavivirus positive samples).

Japanese Encephalitis Virus is a major public health flavivirus in South-East Asia. In collaboration with epidemiologists from ANSES (B. Durand) and CIRAD (V. Chevalier), we will initiate collaborative programs for the genetic characterization of JEV isolates excreted by amplifying hosts (pigs, poultry) and for the identification of risk factors for JEV amplification (age, breeding conditions, excretion intensity and duration,...).

Specific studies:

1. WNV and JEV can cause severe meningo-encephalitis in infected horses. Effective vaccines, either inactivated or recombinant vaccines, have been developed against these 2 flaviviruses, however they are sparsely used (WNV vaccines) or not used (JEV vaccine, not authorized in Europe). Consequently the development of antiviral drugs efficient at reducing neurological symptoms and preventing virus replication in horses after WNV or JEV infection would be of great value.

A collaborative research program with Pasteur Institute, Paris (PO. Vidalain) aims at identifying small molecules with a broad antiviral spectrum, expected to be active against viruses belonging to different virus families and genus. The Normandy Centre for Drug Studies and Research (CERMN, Caen) has identified a promising molecule in its chemical library (sr1057).

In 2014, we tested the antiviral activity of sr1057 against WNV and JEV in the equine ED cells and set up a JEV murine model reproducing clinical signs and CNS lesions associated with JEV infections in mammals. Since the chemical compound appears to be effective at reducing WNV and JEV replication *in vitro*, we would like to investigate in 2015 the pharmacokinetics and antiviral activities of sr1057 *in vivo* in mice. Finally, the ultimate goal will be to evaluate this molecule in infected horses to prove its safety and efficacy (in coming years).

2. 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 has been obtained in 2011 in our laboratory (see Bahuon et al, 2012) and we would like 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. We will seek in 2015 to investigate the infection steps that are impacted by this mutation during the infection process *in vivo* and to characterize other modified viruses (growth kinetics will be evaluated in cell-culture systems, virulence will be evaluated in Balb/cJ mice, as well as in birds (corvids and hens, in collaboration with B. Lambrecht, CERVA CODA)).

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.

✓ **Expected outputs :**

- Horse positive sera well characterized for WNV and JEV neutralizing antibodies
- Better characterization of circulating flaviviruses in Europe and of JEV in Asia
- Demonstration of the safety and efficacy against WNV and JEV of the sr1057 molecule *in vitro* and *in vivo* in mice
- Molecular markers of WNV virulence for European WNV strains

✓ **Performance indicators :**

- Number of positive WNV and JEV sera received
- Number of positive samples for flavivirus antibodies or RNA

Objectives : Vesicular stomatitis virus (VSV)

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

Cloning of VSV-G protein coding genes from serotype New Jersey synthesized as codon-optimized gene, into a non-replicative canine Adenovirus vector previously developed in our laboratory, is currently in progress. A similar work will be performed with the gene encoding the VSV-G protein from serotype Indiana 1. Production of the recombinant VSV-G proteins (VSV-G-NJ and VSV-G-IND) will be assessed *in vitro* after transfection of the corresponding recombinant Adenovirus vector into canine kidney cells (DK-E1). Immunogenicity will be 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 and then horse could be planned (in 2015 or 2016)

✓ **Expected outputs :**

- Production of a recombinant non replicative VSV-Canine Adenovirus

✓ **Performance indicators :**

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

Activity 3 : Program in Parasitology (Dourine)

Sub-activity 1 : Towards a global diagnosis of equine trypanosomosis: can dourine be cured the same way as surra and nagana?

Working program on dourine focused up to now on discrimination of the agents of the *Trypanozoon* sub-genus, *Trypanosoma (T.) equiperdum*, *T. evansi* and *T. brucei*, respectively causing dourine, surra and nagana. The goal was to develop and validate more specific methods and tools for the serodiagnosis of dourine. But the three causative agents are very close and cross reactions are observed, demonstrating the absolute inadequacy of current diagnostic tools. Moreover dourine is officially not treatable. According to the OIE code, confirmed dourine cases have to be slaughtered or castrated. In this context, cross reactions leading to false-positive or false-negative serodiagnosis results of dourine may have a high impact. But base on current knowledge and sequencing results of whole genome of four *T. equiperdum* and one *T. evansi* strains, most of the experts from the NTTAT (Non Tse-tse Transmitted Animal Trypanosomoses) OIE ad hoc group support that differential diagnostic tests of these three parasites are probably not feasible.

The goal of the present study is to demonstrate using a consensus protocol that dourine cases can be treated. A similar studie* has been performed, but some aspects of the methods used remain discussed. The first point is the number of

animals implicated in the experimental protocol. We plan to use 12 Welsh ponies (8 infected and treated), which represent twice more. The second point is the time for the treatment depending on the criteria of the clinical stage of the disease. It's very important to prove that neurological lesions are induced by the parasite before the treatment. Many treatments are efficient against trypanosomes *in vivo* but many less are able to target the parasite penetrating the blood-brain barrier. Definition of the time for treatment in agreement with experts from the NTTAT group will be the strength of this protocol in order to answer to the question of the curability of dourine.

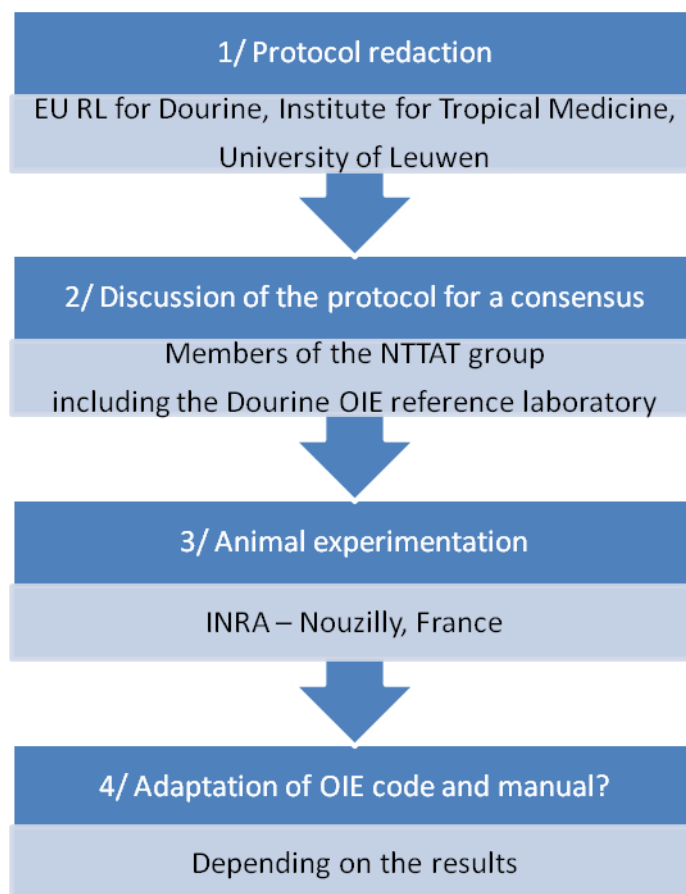
If confirmed, the main consequence would be that differentiation between these three parasites would not be necessary anymore. In this case, it should lead to modify the OIE code to speak of "equine trypanosomoses". Positive diagnosed horses for equine trypanosomoses could then move again after treatment and adapted observation period with controls after treatment.

If the possibility to treat dourine effectively is stated, working program for the next years should be adapted; development and validation of serodiagnostic methods and tools should be specific for the *Trypanozoon* sub-genus.

Depending on the results obtained for the treatment, ELISA development and validation will be adapted.

* Hagos A, Goddeeris BM, Yilkal K, Alemu T, Fikru R, Yacob HT, Feseha G, Claes F. Efficacy of Cymelarsan and Diminasan against *Trypanosoma equiperdum* infections in mice and horses. *Vet Parasitol.* 2010 Aug 4;171(3-4):200-6. doi: 10.1016/j.vetpar.2010.03.041

Protocol milestones



✓ Expected outputs :

- Consensus protocol validated by expert members from the NTTAT group.
- Production of positive sera
- Infection and treatment of 12 ponies with four different strains (2 *T. equiperdum*, 1 *T. evansi*, 1 *T. b. brucei*)

✓ **Performance indicators :**

- Regular serological follow-up and analyses of samples throughout the duration of the experimental protocol
- Regular hematology and biochemistry analyses of samples throughout the duration of the experimental protocol during six months
- Regular clinical follow up of the animals throughout the duration of the experimental protocol
- Number of samples included in the serum bank

Sub-activity 2 : Support to NRLs

- Confirming or invalidating positive and doubtful cases upon NRLs requests
- Supply of biological samples upon NRLs requests
- A third proficiency test will be organized in order to evaluate the performance and homogeneity of the NRLs. Positive serum produced by the EURL will be included in the blind samples that will be proposed to the laboratories.
- A third workshop on dourine will be organized. Results and analysis of the ring trials will be discussed as well as diagnostic improvement and epidemiological situations.

✓ **Expected outputs :**

- High participation of NRLs for the workshop.
- Good quality of proficiency test restitution and good quality of debates for the workshop

✓ **Performance indicators**

- Number of NRLs with satisfactory PT results
- Number of confirmed and invalidated cases
- Number of participating NRLs to the workshop
- Number of positive satisfaction surveys for the PT test and workshop.

Activity 4: Contagious equine metritis (CEM)

Sub-activity 1: Support to NRLs and epidemiological study

- A proficiency testing will be organized for culture and PCR methods;
- A second workshop on CEM will be organized;
- Biological elements (reference and field strains) will be provided according to NRLs requirements;

- CEM positive cases will be confirmed according to NRLs requirements;
- Started in 2014, the characterization of the molecular diversity of CEM isolates circulating in Europe will be continued in collaboration with the NRLs. The aim of the study is to better understand and describe the different *Taylorella equigenitalis* population circulating in Europe (and if it is possible *Taylorella asinigenitalis*). In addition to the EU-RL, seven NRLs have agreed to participate in this project, which takes into account 84% of the CEM cases reported since 2009. NRLs could perform themselves the MLST method previously developed by the EU-RL or send to the EU-RL their *Taylorella* strains. In addition to publication of the results planned for late 2015 / 2016, a report on this work will be presented at the second CEM workshop organized in 2015 by the EU-RL.
- ✓ **Expected outputs:**
 - One proficiency test (PT) organized for CEM for both culture and PCR methods;
 - One workshop organized for CEM;
 - Better knowledge of *Taylorella equigenitalis* (and *Taylorella asinigenitalis*) strains circulating in Europe.
- ✓ **Performance indicators:**
 - Number of NRLs with satisfactory PT results;
 - Number of NRLs attending the workshop;
 - Number of positive satisfaction surveys for the CEM PT test and workshop
 - Number of *Taylorella equigenitalis* (and *Taylorella asinigenitalis*) strains received and typed by MLST.

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

The complexity of the metabolism of the CEM agent and the difficulty of limiting the growth of the horse genital microflora without limiting those of the CEM agent prevented the entire development of a new culture medium in 2014. Accordingly the development of a new culture medium to improve the CEM bacteriological diagnosis will be continued in 2015 in collaboration with the AES chemunex company (manufacturer of culture medium).

- ✓ **Expected outputs:**
 - Development of a new culture medium to increase the sensitivity of the CEM diagnosis.
- ✓ **Performance indicators:**
 - Testing at least one new composition of culture medium.

Activity 5 : Glanders

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

Collect of sera from truly infected animals and Constitution of a reference serum

Thanks to the Collaboration initiated with LANAGRO of Brazil, Recife (Dr V. Lucia) and University of Agriculture of Pakistan, at Faisalabad (Dr M. Saqib), sera from naturally infected horse have been collected. These sera were mostly used for the ring trial organized in 2014 and for the new prototype ELISA's evaluation. More sera, in larger volumes should be collected from outbreaks. The objective is to collect at least 5L of sera (from different animals) 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.

In parallel, contacts have been established with the OIE Glanders reference laboratory of Dubai. It is envisaged to establish a common reference serum for EU-RL and OIE.

Development and validation of an indirect ELISA for Glanders.

A new crude antigenic fraction-based ELISA has been developed for the detection of *B. mallei*. Specificity and sensitivity determinations will be fully determined thanks to our collaborations established with Brazil and Pakistan, in the framework of outbreak investigations. The commercialization of this test is under progress and the kit should be available as complementary test to CFT.

A bilateral research project (between France and Brasil) has been submitted in 2014.

Implementation of a Luminex assay for the multiplex detection of *B.mallei* antibodies .

The antigen developed for the novel indirect ELISA (crude antigenic fraction) was tested by Luminex. This microsphere immunoassay technology would offer possibility of multiplexing different antigens, targeting different horse diseases.

Preliminary tests have been conducted and interesting results were obtained. Antigen concentration should be improved, and multiplexing with microspheres coated with other antigens targeting other equine diseases should be now tested.

Collect of tissues from naturally infected horses.

B. mallei and *B. pseudomallei* were recently added to the French list of "classified" pathogens. An authorization from the French administration is now required for the manipulation of these bacteria and any associated material (DNA, biological samples from infected animals). After ANSES has been authorized to hold and manipulate these two *Burkholderia* species, contacts have been established with our Brazilian and Pakistani colleagues to import tissues from infected animal (also submitted to export authorization). These samples are necessary for PCR methods' validation and also to initiate molecular typing studies.

Selection and validation of a PCR for the specific detection of *B. mallei*.

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. An 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 has 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.

Preliminary molecular typing of *B. mallei*.

Next-generation sequencing technologies are powerful tools for the unbiased typing of any pathogen. By interrogating nearly every base of a genome, individualizing genetic markers can now be reliably discovered at a reasonable cost. Sixteen genomes of *B. mallei* and more than thirty genomes of *B. pseudomallei* are currently available in public databases and will also be used for in silico comparisons. SNP markers that discriminate or specifically define a particular group of strains (geographical distinct) will be searched and SNP discriminative assays developed using technologies such as High Resolution Melting (HRM) or allele-specific PCR (AS-PCR).

✓ **Expected outputs:**

- Validated indirect ELISA for the serological diagnosis of glanders;
- Standard sera from CFT analysis;

✓ Specific *B. mallei* PCR methods validated on naturally infected tissues.

Performance indicators

- Implementation of an indirect ELISA test for the serological diagnosis of glanders;
- Lyophilized reference sera from *B. mallei* naturally infected horses, 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.

Activity 6 : Training

Training sessions for the NRLs will be planned. A priority will be accorded on the NRLs who have failed Glanders or EIA proficiency tests but other trainings could be proposed by the EU-RL in function of remaining budget and on demand of NRLs.

✓ **Expected outputs :**

- Improve the diagnostic of equine diseases in the NRLs

✓ **Performance indicators :**

- Number of training 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

- ✓ An 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, proficiencies 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 request and to have trained personnel available for emergency situations occurring within the Union
- ✓ **Performance indicators**
- Number of website visits
 - Positive feedback of the project officer concerning the equine diseases EU RL

Sub-activity 2 : annual NRLs workshops

On 2015, two workshops on one day will be organized in ANSES Maisons-Alfort and will be focused on Dourine and MCE diseases. Results and analysis of the ring trials for will be discussed as well as diagnosis improvement and epidemiological situations of the two diseases. Following the two workshops, final reports and all presentations will be published on the website.

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