

PROJECT FINAL REPORT

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4.1 Final publishable summary report

4.1.1 Executive summary

The aim of the NADIR project was to organise European animal infectiology centres to achieve economies of scale, modernise existing facilities and improve cooperation and complementarities. The project proposed to:

- Set up an Internet-based platform designed to organise the collaboration between partners in a secure and optimal way and provide internal as well as external organisations with access to the network's infrastructures
- Strengthen the exchange of best practices, training, and the validation of protocols
- Coordinate the management of material resources, including animal lines, and in-house developed tools in relationship with related European project and initiatives
- Jointly undertake research activities aimed at upgrading the services provided by NADIR infrastructures: characterise animal lines, customise imaging technologies, develop monitoring molecular tools and create new animal models.
- Conceive, structure and manage transnational access to the NADIR infrastructures.

The initial objectives were to prioritize Nadir research activities, to implement exchanges of resources between partners and to allow access to the 14 NADIR animal facilities. Eighteen meetings have been organised to complete these tasks from May 2009 to the end of the project in December 2013.

Exchange of best practices has been initiated through the Biosafety/biosecurity/best practices meetings attended by Nadir partners and an invited institute (IVI, Mittelhäusern, Berne). Very fruitful, these exchanges of experience between leading European institutes in the field allowed reaching a consensual report advising animal facilities for the containment of farm animal species infected with class 3 pathogens as well as for ethical aspects of experimental infections. During the second period a range of training course for improvement of use of animals has been settled. Next to coordination of studies performed within the NADIR research activities and the transnational access, a listing of animal models available within the consortium was compiled

The joint research activities of the project had been designed to sustain the improvement of the services provided by the infrastructures through (i) better characterisation of animal lines, (ii) progress in detection and diagnostic of diseases, (iii) development of infection process monitoring tools. It has produced more than 60 research publications and contributed to more than 80 communications.

According to the first objective, chicken, sheep, pig and fish lines have been genotyped and the susceptibility of different animal lines have been assessed by infections with various pathogens. Several immunological tools specific for farm animals have been produced and their use optimized.

In the frame of the second objective, experience in telemetric transmission of physiological parameters that can be used to monitor the animal response to infectious diseases was exchanged between partners. Devices dedicated to these tasks have been compared and several have been successfully tested to be used in pigs and ruminant species. Imaging in vivo the infectious process in small animal through fluorescent or bioluminescent markers has been achieved in pilot experiments conducted in a BSL3 animal facility.

A number of new diagnostic procedures using immunological tools and molecular technologies (microarrays for pathogens and their host) were developed as well as multi-diagnostic assays for monitoring infection processes in animals.



Recent evolution of the European sanitary status and of institutes' objective needed to prioritise a limited number of infectious diseases to work on. A common consensus was reached to prioritize emerging viral zoonoses. The documentation of cell culture systems and the challenge experiments of vector borne disease agents in different host species were performed, in order to generate defined positive tissue samples, and to study the pathogenicity of these agents, which included the most relevant fish pathogens towards a better understanding of their species spectrum and transmissibility. The “Call for Transnational Access” allowing external user to perform experiments in the BLS3 facilities proposed by NADIR has been disseminated by partners and through the communication channels developed within the project. A blog-like website has been created and has been promoted through LinkedIn Groups in order to increase the people in contact with NADIR. A total of 60 transnational access proposals from 16 European countries have been evaluated by the scientific panel and 43 have been realized and reported. Among these, four accesses were headed by private companies and 39 by public laboratories. The most frequent demands concerned studies on the pathogenesis of infectious diseases or vaccine development. Improvement of diagnostic and characterization of pathogen isolates were a second objective of many studies with the aim of developing an improved control of farm animal infectious diseases and zoonosis.

4.1.2 Project context and objectives

WP2: NA1- Fostering joint management of resources and interchange of services

Each NADIR partner has optimized the functionalities of their proposed services according to the main research topics in which they are involved. This has resulted in research groups developing original animal lines, animal cell lines, laboratory reagents and investigation techniques, for every farm and laboratory animal species which are investigated. These resources, had to be made available to all Network participants and potential external users of the NADIR facilities. The major objectives of NA1 were thus to make an inventory of available resources to study host-pathogens interactions using animal models of infectious diseases (D2.1). These lists were made available to all NADIR participants and after updating thanks to NADIR research activities made available to the scientific community (D2.4). In coherence with these inventory and linked research activities, the NA1 WP aimed to promote exchanges of resources among NADIR participants, i.e. animal lines, animal cell lines and reagents specific for farm animal pathogens and host proteins, cells and tissues. Exchanges of resources' objective were improving the NADIR partners' capacities as well as to make available the improved tools to the users of their facilities, e.g. through offered transnational accesses. Information about tools and materials have been presented to the scientific community through workshops dedicated to the description and discussions of each tool category. This has been achieved through specific seminars, that have been organized as often as possible as joint meetings and /or during the NADIR annual meetings. The workpackage has been carried out in consultation with other related European or world-wide projects in close relationship with the NA3 task dealing with communication with other programmes involved with research in animal infectiology and the RA1 task, which also involved the Immuno-tools project and the International Veterinary Symposium headed by NADIR partner MRI.

WP3: NA2-Virtual college for improvement of animal infectious disease studies

The quality and societal acceptance of animal infectious disease studies is on one side determined by the quality of the science, but to a large extend also by the quality, professionalism and



experience of the infrastructure and the awareness of ethical considerations and challenges towards the use of animal for research purposes. The major objectives of NA3 during the project period were to come to a better understanding on best practice, biosafety and biosecurity measures, to intensify the discussion on ethical issues and animal welfare and to set up training courses for improved use of experimental animals. Based on experience exchange and discussions an on-line available collection of documents, which touch on practical issues of best practise and design of BSL-3 facilities, dedicated to work with farm animals has been established. Principles for the design of BSL3 buildings were addressed by the use of the Hazard and Operability studies (HAZOPs) and risk analyses were initiated. In order to map and explore the wider ethical and social issues raised by infectious disease research using large animals, a series of research activities, peer-review literature reviews, workshops and network interactions have been exerted and resulted in a report mapping and analyzing ethical issues of (12) important key aspects like for example animal welfare, 3Rs principles or implementation of Animal Ethics committees, especially in regard to EU Directive 2010/63. Based on literature review and discussions during workshops guidelines for humane endpoints and pharmacological refinement in animal infectious disease studies have been suggested. Six training courses and workshops in regards to infectious disease studies were successfully performed, which constitutes an important tool for future networking for the improvement of methods and also facilitates consultancy for specific problems in the field of animal infectious diseases. The coordination of animal studies was committed in first instance to the organization and management of the transnational access requests, additionally the availability and experience on current animal model was investigated and the development of animal models for new emerging disease like SBV supported.

WP4: NA3 - Internal and External Communication

WP4-NA3 dealt with communication, with specific actions related to the following objectives:

- internal communication, the main objective of which was to provide suitable instruments to enhance communication between partners in order to harmonize the different project activities, including networking, research and management;
- external communication, the main objectives were to promote collaborations with external partners and to facilitate and promote the exchange of information with similar activities.

These objectives were achieved through the project dissemination tools that were developed and resources, which included the web-based electronic portal, the project newsletter, the blog-like website and specific activities carried out by the project participants.

In order to improve the efficiency of external communication, especially within the Animal Health Community, links with many related initiatives have been established and the blog-like website has been constantly updated; LinkedIn Groups (related to Animal Health Science) have been regularly used as a mean of dissemination and promotion of the project activities.

WP5: RA1 - Characterisation of animal lines

We urgently need animal models, particularly those based on farm species, to study relevant livestock infectious diseases. If such animals are to be used as reliable tools for experimental infection, it is required a previous accurate characterisation of them to obtain valid results. In this regard, definition of health status of animal lines used as model is a significant parameter to avoid interference with the studied pathogens. In addition, the availability of inbred strains allow the study of genetically identical cohorts and facilitate genetic approaches to understanding molecular mechanisms of disease. Therefore, the genetic characterisation of the animal lines, along with its immunological characterization is crucial for the use of these animals as feasible models in animal and even human health research. Genotypic and/or immunological differences



of animal lines in their response to pathogen infection can be exploited to analyse the genetic/immunological basis of infections.

Accordingly, the objectives of WP5 in this project have been focused on the characterisation of animal lines including chicken, pig, sheep, mice and fish, and on the development of immunological tools, valuable for animal characterisation. Therefore, the specific objectives of this WP have been: i) Definition of sanitary status; ii) Genetic characterisation and comparisons between lines; iii) Disease susceptibility/resistance of lines and iv) Development and validation of immunological reagents useful for immunological characterisation of animal lines and to support the development of vaccines, diagnosis test and pathogenesis studies. The three first objectives have been shared among five different tasks, each one corresponding to an animal species (chicken, pig, sheep, mice and fish).

WP6: RA2 - Development of infection monitoring tools

The general objective of WP6 is to jointly make the latest technologies suitable to be used in the field of animal infectiology with its specific demands (large or small animals, species specific material etc.). To achieve this, imaging and telemetric technologies were upgraded and customized for use in experimental infections in large animals. In addition, new molecular tools were developed as well as multidagnostic assays for monitoring infection processes in animals.

In tasks WP6.1 and WP6.2 activities were undertaken to upgrade imaging and telemetric technologies. To achieve this, INRA bought a fluorescent and bioluminescent imager dedicated to the imaging of small animals in a high contained facility (BSL3). INRA also developed and applied successfully imaging technologies to monitor the dissemination of pathogens during experiments on small animals, either model species as mouse or farm animals as young chicken. In NADIR also several telemetry approaches were tested. This concerns especially equipment for measuring body temperatures in farm animals. Experiments were successfully performed in sheep, cows, ponies and chickens. All these tests have shown the possibility of using a wide range of thermometers as “chips” or bolus adapted to various species and for various purposes.

In tasks WP6.3 and WP6.4 novel molecular tools for monitoring infection processes were developed and implemented; also single assays were upgraded into cost-effective, easy multiplex assays.

In animal infectiology there is constantly a need to improve and extend diagnostic, clinical and pathobiological tools using the relevant recent developments. Availability of these tools optimises and improves the efficiency of disease studies in animals, and thus serves the community at large. For these reasons several new tools were developed and applied by the NADIR partners. The newly developed tools include the set up and evaluation of real-time PCR either in single- or in multiplex, micro-arrays, suspension array (Luminex) for detection of highly virulent viruses as for example RVFV, CSFV and WNV. Furthermore immunohistochemical techniques have been established to detect a variety of immune cell types, cytokines and chemical mediators in tissue sections of different animals. Immunohistochemistry and In Situ Hybridisation techniques have been developed to detect different viral and mycoplasma strain in porcine tissues. Tests and techniques have been used to set up diagnostic tests for detection of infectious agents in animals and to study the pathogenesis and the host-pathogen interaction in animals.

WP7: RA3 - Development of infection models

The objectives of this WP were i) to provide the project partners with well characterized cell culture systems (primary, permanent and established cell lines) as well as stem cells for their specific applications; ii) to intensify the work with vector borne diseases and therefore to



optimize the availability of sample material and diagnostic protocols for these diseases, and iii) to reinforce the cooperation in fish disease research by generating standardized samples of fish infected with exotic and non-exotic diseases.

This was addressed by the preparation and characterization of cell lines for the specific needs of virological work, as well as the development of protocols for the preparation of stem cells. Moreover, different partners have performed challenge experiments with vector borne disease agents, in order to prepare defined positive and negative tissues for further analysis and for the development of optimized diagnostic methods. Due to the broad spectrum of diseases that are relevant in this topic, the research activities performed under this WP are rather various. These studies also revealed important information about the transmissibility of these agents, and on the pathogenesis. The major emphasis was put on Rift Valley Fever Virus (RVFV), West Nile Fever Virus (WNV), and Bluetongue Disease Virus (BTV). The common efforts in the RVFV challenge experiments performed by CReSA and INIA are a good example of the close cooperation of the NADIR partners in the field of vector borne diseases. Finally, the work on aquatic disease agents that pose a threat to the European aquaculture was intensified and cell culture as well as animal challenge experiments were performed with the most relevant agents, namely VHSV and IHN.

4.1.3. Main S&T results/foregrounds

WP2: NA1-Fostering joint management of resources and interchange of services

This WP is closely linked to research activities allowing updating NADIR shared resources. The completion of animal line characterization through genotyping and experimental infection were performed in WP5-RA1, and improvement of reagents specific for farm animal infectious diseases studies were performed through WP5-RA2. Results from the research activity have allowed a better characterization of animal lines, which was one of the main objective of NADIR as it is one way to improve sharing of resources between partners. In the case of Chicken, pigs and fishes line comparison with genetic tools have allowed to identify similarities and differences among lines. According to results from these investigations, which have shown e.g. that chicken lines with the same origin have conserved their characteristics after years of breeding in different colonies, and the one on the resistance/ susceptibility to diseases (deliverable 5. 3) the lines' characteristics have been completed (D2.4). These investigations have also allowed characterizing polymorphisms and/or Major histocompatibility complexes from lines of fish, sheep, pigs, chicken and even cattle.

Results have been made available online through the NADIR web sites. Several TA proposals have used several among these better characterized lines as a resource to investigate host-pathogen interactions.

The listing of available resources has been established through two meetings at the beginning of the project, organized in Lelystad (NL) in November 2009 and in Lodi (I) in February 2010. Constant updating have been done through annual meetings sessions and a dedicated meeting organized in Paris (July 2013).

Task NA1.1: Exchange of animal lines

The exchange of DNA from animal lines has been completed in the framework of WP5-RA1 activities. The planned characterization of animal lines has been performed, allowing the updating of animal lines characteristics. Lines of pig, chicken and cattle have been exchanged and/or



provided for transnational access. Sheep MHC has been better identified and new polymorphisms submitted for publication. Two additional AU chicken lines selected for High or Low level of Mannose binding lectins have also been characterized. The characterization by INIA of transgenic lines of mice has also been achieved and reported in deliverable D2.4. The characterization of trout lines used genomic molecular tools through exchange of DNA between DTU and INRA, as well as challenge experiments, which necessitate transportation of lines achieved through the exchanges of eggs. Expected work from this task as thus been fully completed.

Task Na1.2: In-house material exchange

Expected reagents have been developed by every group involved in WP6-RA2 and thus represent new reagent available for the research community. These have been exchanged has required on a collaborative basis or through MTA. Such documents have been used to suggest a NADIR MTA developed through deliverable D2.3. A number of chicken reagents have been developed by UEDIN and are commercialized through abd Serotec. IAH and MRI have also commercialized kit for studying cattle dendritic cells. VLPs, recombinant chemokines, anti-chemokines antibodies have also been obtained by INIA and made available. INRA has developed antibodies recognizing the cattle Il-17. Aside the development of reagents, techniques for immunohistochemical staining have been developed by AH-VLA, IAH, AU, and INIA and are also available on request. Exchange of reagents have also allowed research experiments to be performed by NADIR partners (e.g. on vector borne diseases) as well as through Transnational accesses.

Task NA1.3: High-throughput molecular tools acquisition and validation.

PTP and MRI have developed a number of tools for investigating the host-pathogen interactions. PTP has created whole genome micro-arrays for transcriptome analysis in cattle and chickens. The chicken array has been tested and validated by examination of gene expression in mesenchymal stem cells created in RA3. The use of the bovine array has also been validated in other ruminant species. In particular to examine expression patterns in milk somatic cells and blood of goats. This work was developed in the second period through a number of microarrays, but it has been extended to the use of New Generation sequencing during the third period of reporting. FLI has created microarrays as an aid to the diagnostic for the identification of emerging zoonotic pathogens including zoonotic arboviruses (flaviviridae, togaviridae, bunyaviridae and paramyxoviridae). CVI developed a Luminex multiplex assay with high diagnostic potential. Its flexible and open format makes it possible to increase the sensitivity of the assay by incorporation of additional microbead sets with immunodiagnostic antigens to *Streptococcus suis* and *Mycobacterium avium paratuberculosis*.

Task NA1.4: Sample Bank

NADIR partners have stored a number of tissue samples from various species infected naturally or experimentally with a large variety of pathogens. These represent a large sample bank, which completes the AH-VLA archive, mainly dedicated to prion infected tissues. Infections performed in the framework of NADIR research activities and several related to transnational accesses have also provided a number of samples made available to the scientific community. The following collections have been completed:

- KVI H9N2 Influenza Collection: A collection of about 1000 Isolates of AIV, obtained from wild and domestic birds. Collection of other AIV subgroups: A collection of about 140 isolates, collected between the years 1971- to 2013, includes almost all HA and NA AIV



types, included reference viruses and their respective antibodies, obtained from Dr. Robert Webster, Memphis, TN, U.S.A.

Newcastle Disease Virus Collection: A collection of about 3000 isolates was obtained between the years 1991-2013 from domestic and from wild birds, as well as about 10 reference viruses, obtained from Dr. D. Alexander, VLA, UK.

- INIA: Improvement of the number and variety of samples from animal infected with different viruses stored Blood, serum, brain and other tissues have been collected from mice infected with different prion strains and scrapie isolates. Furthermore sera and tissues from experimental infections with BTV, PPRV and Rift Valley have been stored.
- FLI: samples archived at FLI (partner 5) that may be relevant for other NADIR partners have been reviewed and updated. Samples from prion disease affected or from vector borne disease infected animals can be made available to NADIR partners upon request. Samples collected from animals challenged with vector-borne diseases have been reviewed for their suitability to be exchanged with other partners.
- INRA has updated the list of TSE samples from sheep BSE, goat BSE and tissues (mainly Spleen and brain) from mice infected with a collection of TSE isolates.
- CRISA has serum samples from animals experimentally infected with RVFV, CSFV, ASFV, BTV, PRRSV and Swine and Avian influenza. These samples are available for all participants. A collection of different isolates (prion samples, BTV, AIV, IBV, IBDV, and Pigeon Paramyxovirus) is also available for all participants. Histological sections of representative microscopic lesions of all these diseases may be also shared.

During the NADIR period experiments with RVFV, BTV, WNV and HPAI H7N1 and H5N1 have been performed. Sera samples from infected animals but also infected chicken homogenates and several tissues have been obtained. A bank of frozen PBMC and lymph-node cells preserved in liquid N2 from RVFV infected animals, but also BTV and ASFV experiments, has been set up. DTU has, following infection trials, produced positive control material for use in validating diagnostic tests for the following pathogens: VHSV, IHNV and *Aphanomyces invadans*, which is available upon request.

VLA continues to offer samples through the Biological Archive, the largest and most comprehensive store of TSE material worldwide and operates to a quality management system compliant with ISO9001: 2000. The Archive receives and stores a range of animal tissues and fluids collected ante- and post-mortem from UK field cases and some experimental programmes. The VLA also has a wide range of bacterial isolates through AHVLA scientific projects. This extensive collection has over 29,000 recent and historic bacterial isolates of large animal pathogens including both common and less common anaerobes from all livestock species.

The collection represents typical and atypical isolates from respiratory, enteric, mastitis and other animal diseases.

WP3: NA2-Virtual college for improvement of animal infectious disease studies

The quality and societal acceptance of animal infectious disease studies is on one side determined by the quality of the science, but to a large extent also by the quality, professionalism and experience of the infrastructure and the awareness of ethical considerations and challenges towards the use of animal for research purposes. Although most NADIR partners exert similar activities in the field of animal infectious disease studies, it was remarkable, that requirements for example for facilities or biocontainment or the approval criteria and approval processes can vary considerably between NADIR partners. The major objectives during the project period were to



come to a better understanding on best practice, biosafety and biosecurity measures, to intensify the coordination on performance of animal studies between the different institutes, and also of the transnational access requests, to intensify the discussion on ethical issues and animal welfare and to set up training courses for improved use of experimental animals. These objectives were approached in five different tasks, in which through discussions between NADIR partners, specific workshops, desk top studies and training courses issues on best practice (task NA2.1), biosafety (task NA2.2) animal welfare, legal and social aspects (task NA2.4) and professional development (task 2.5) were addressed. In a separate tasks (NA2.3) the management and administration of requests for transnational access (TA) for animal and other research studies was organized, animal studies within the research programme of NADIR coordinated and the availability of animal models at the partner institutes investigated.

Task NA2.1: Best practice and procedure exchange

Major activities were focused around the improvement and harmonization of procedures in regard to best practice in animal facilities and studies, biocontainment requirements and validation by exchange of information between partners. It was aimed to discuss and agree on specific aspects of the design and operation of BSL 3 facilities. Based on the results of a workshop on best practices the exchange of procedures was initiated and standard operating procedures for infection trials have been established and for accessibility and comparability translated into English language. A survey on currently used quality accreditation systems (i.e ISO 9001, GLP etc.) in BSL3 accommodation of NADIR partners was done. Mandatory procedures in regard to quality policy, quality objectives and quality manuals are often followed, but one key issue appeared to be the availability of an independent audit process. Nadir partners often rely on internal quality management groups. To this end, for example principles for design of buildings, use of the Hazard and Operability study (HAZOPs) in design were discussed and risk analysis by mathematical modelling for quantifying risks were initiated. An on-line available collection of documents, which touch on practical issues of best practise and design of BSL-3 facilities, dedicated to work with farm animals has been established in close cooperation with several partners. The on-line character is chosen to create an easy access for members of NADIR institutes and to establish an evolving collection of supportive documents, which go beyond the guidance of work in BSL-3 facilities for laboratory animals and address specific issues of facilities working with BSL3 microorganisms in farm animals. Although, due to the individual specificities of the different institute, the guidance documents do not necessarily reflect a general agreement, they are meant as support for the discussion and decision on best practise and operation in each facility. Next to best practice approaches in farm animals, also, biosafety measures and disinfection procedures have been established and validated for fish facilities and virus decontamination procedures.

Task NA 2.2: Biosafety and Biosecurity

Working with exotic infectious diseases or emerging zoonotic diseases regularly require a BSL3/3+ level of containment and especially working with farm animals in these containment put high demands on the knowledge about bio-safety and biosecurity. In this task specific national and international requirements for biosafety and biosecurity in the different countries of the NADIR partners were compared and discussed in a workshop (in 2010 at IAH) and in following meetings. In this discussions a range of topics concerning biocontainment, environment protection and personal protection were addressed and special attention was paid to the national regulations for biocontainment requirements for different animal pathogens, the



strategies and materials used in the various institutes in regard to personal protective equipment , decontamination of rooms and waste and carcass disposal.

On basis of the discussions of the workshop a report (DNA2.2) on the workshop on biosecurity and biosafety (DNA2.2) was compiled. It constituted the basis for a follow-up discussion on harmonization of bio-containment requirements for animal pathogens, personal protection measures, decontamination measures, containment validation and animal welfare issues in animal studies with exotic pathogens. There was a strong consensus, that biosecurity research to underpin relevant biocontainment controls with evidence would benefit NADIR partners and other organisations in the field. The discussions during the project period revealed that more standardization in the biosafety and biocontainment systems would enhance collective learning from existing systems and enable partners to defend their systems of work on the basis of evidence and meeting good or best practice in the veterinary containment. To accelerate this process the above-mentioned online database (see task NA2.1) with guidance documents was developed, in which specific biosafety relevant documents were in a common effort compiled and made available for NADIR partners.

Task NA 2.3: Coordination of Animal studies

The coordination of animal studies comprised the actual animal studies within the project and information on on-going and planned animal studies within RA1, RA2 and RA3 was periodically compiled to coordinate an effective use of animals, tissues and samples. An analysis of available animal models at the partner institutes was initiated and the validation status of infection models for research on OIE listed diseases at the NADIR partner institutes was assessed in a survey. A distinction was drawn between infection models used infrequently for special research questions, those considered as established and the ones considered as validated (based on a regular use). A list of available animal models within the partner institutes is being drawn up and part of D3.6. Such information should enable discussions, exchanges and improvement of procedures and also highlight the limited number of available animal models. From this task, NADIR actively participated in the discussion around newly developed animal models for the in 2011 new emerging virus disease in sheep and cattle, the Schmallenberg Virus Infection (SBV) and in cooperation with the EU project Epizone several NADIR partners were part of an international EU consortium on SBV. During annual meetings and specific workshops several aspects of experimental design and validity of animal studies were discussed and the outcomes of the discussions and desktop studies resulted in the report for deliverable D3.6.

In the second half of the project period the organisation of the requests for animal studies in the transnational access part was allocated to this task. A data base with all relevant communications concerning the proposals and a continuously updated master schedule was established on the NADIR management site. SOPs for the user selection panel and the executive committee for evaluation procedure of TA proposals were developed. In the reporting period 60 study requests were evaluated and discussed in 13 telephone conferences and two face to face meetings with the User Selection Panel. Results of these discussions were communicated with the requesting party and the access supplying NADIR partners before an approval procedure by the executive committee was initiated.

In October 2013 a mini-symposium was organized at 7th EPIZONE Annual Meeting in Brussels. Title of workshop was: ‘NADIR, joined forces to optimise animal and public health innovation’ and researchers from academia, research institutes and industry actively participated. Objective was to present the opportunities of infrastructure projects and to discuss the benefits and needs for the availability of a NADIR like infrastructure project for the European Research Area. Users



and stakeholders of NADIR underlined the need for an European network of high containment animal facilities as an infrastructure of major interest for animal and public health research.

Task NA2.4: Animal welfare and ethical, legal and social aspects (ELSA)

In this task ethical, legal and social aspects of work in BSL3 facilities, including experimental design of studies and specifically the application of human endpoints under scientific and animal welfare aspects as well the pharmacological refinement of animal studies were addressed. In order to map and explore the wider ethical and social issues raised by infectious disease research using large animals, a series of research activities, peer-review literature reviews, workshops and network interactions have been exerted to support both the deliverables DNA2.4 and DNA2.5. An Ethics workshop on “Research Ethics and Animal Use” was organized by UNOTT and held in Weybridge, UK on 14-16 September 2011. This event supported the mapping and analysis of ethical issues and a number (12) of important key aspects have been identified, such as for example: - Significance of developing an active sustained network that can be responsive; - Societal responsibilities and benefits of animal infectious disease research; - Managing the dual use dilemma in zoonotic disease research; - Animal Research Ethical Committees Processes and Composition; – Supporting the application of refinement: Developing skills, sharing methods and innovation techniques relating to end points; – Systems approach to animal wellbeing: examine the ‘life’ experience of the animal to improve wellbeing; – Waste management in BSL 3/ 4.

This resulted in a report that has been produced from this perspective with support from the literature. This report (DNA2.4) has supported the internal and external communication tasks within the Consortium and lead to specific outputs and contributions, for example in the NADIR Newsletter.

As a result of the very prominent controversy around the H5N1 research (late 2011 / 2012) with the WHO intervention and the discussion about whether full results will be published, a short study on the Dual-Use Dilemma was conducted with NADIR partners to gauge their views on what has occurred and what should occur in the future. The importance of this discussion / work was flagged in the NA2.4 report.

In cooperation between several NADIR partners and supported by results of discussions on a workshop on humane endpoints in Nottingham 2012 ‘Guidelines for Humane End Points (HEP) in Infectious Disease Experiments on Farm Animals’ (D3.5) have been evolved. Emerging from this HEP work within the NADIR Consortium, it was recognized that due to the importance of ensuring good practice approaches in the application of humane endpoints, the more general part of the report was made publically available. The UNOTT team also extended this work to produce a tool for animal users, animal care teams, animal ethics committee reviewers, etc, referred to as ‘the endpoint matrix’. An article describing this tool has been published in one of the prominent Laboratory Animals 3Rs Journal; ALTEX (available on-line in February 2014).

In the above mentioned workshop also half-a-day was committed to pain and pain reduction in study animals and the provided information and the outcome of the discussion was used to suggest guidelines on pharmacological refinement of infectious disease studies in farm animals (deliverable D3.7). To disseminate the ELSA expertise partner UNOTT arranged and ran together with FRAME, a UK based 3Rs NGO a 3Rs training event (Nottingham, January 2013: Training School in the Animal Experimental Design & Statistical Analysis). The feedback by participants and the networking aspects of the event were deemed to be very useful.

Task NA2.5: Training and professional development

After selection of a number of training courses, which are aimed to improve the use of animals in infectious disease studies or to enhance the use of available samples and materials from these



studies 6 training courses and workshops have been initiated. By this, the use of animals in studies can generate better scientific data and moreover, contribute to the ethical justification of animal experiments. The following courses were performed in the run-time of the project:

1. Hands-on cell culture work and classical virological methods
2. Intracellular detection of cytokines by flow cytometry
3. Assessment of chicken T cell mediated immunity by flow cytometry
4. Mice management
5. Protein identification by peptide mass fingerprinting
6. 3Rs training workshop

The five hands-on training courses (1-5) were successfully offered to 26 trainees from 9 different institutes from within and outside NADIR. For two other, offered courses on specific diagnosis methods (Cryo-IHC rapid diagnosis of “Peste des petits ruminants”) and on challenge studies in certain types of fish (“How to perform challenge tests with Atlantic salmon, seabass and cod”) were cancelled due to an insufficient number of registrations.

The other courses were held with success and the practical training increased the contact within the animal disease field. Furthermore relevant contacts of the different institutes were made known by the training courses and this constitutes an important tool for future networking for the improvement of methods and also facilitates consultancy for specific problems in the field of animal infectious diseases.

WP4: NA3 - Internal and External Communication

Task NA3.1: The network electronic portal

The objectives of this task were related to the development of the project electronic portal and the system to disseminate information on the project resources (e.g. materials, reagents, tools, databases) that were available among the project partners.

The NADIR Network Electronic Portal (www.nadir-project.eu) was created by INRA, which is based on the project website, hosted by INRA Network infrastructure in Jouy-en-Josas (France), which was developed using EzPublish, an open-source Content Management System.

The different sections of the project website, Home Page (news and editorial), About NADIR (objectives, organization and coordinator), The Project (concept and project structure), Call for Access (call for proposal for Trans-National Access projects), The Partners (list of partners), Links (websites of the partners), Newsletter (NADIR newsletter), have constantly been revised and updated.

Task NA3.2: Outreach to new users

The objectives of this task were to establish a Panel of Experts to review TA proposals, and organise the NADIR Call for Access, in order to stimulate researchers to submit proposals for projects that would be carried out within the participating NADIR facilities.

The dissemination of the NADIR Call for Transnational Access was achieved using all the means of communication developed within the NADIR Consortium (e.g. project website, blog-like website, project newsletter, contacts with related initiatives) and by each of the partners, by publishing the call on their websites and orally at national and international meetings and conferences. A specific website for online submission of the proposals was set up. The evaluation of the proposals was performed by an independent Panel of Experts, whose members are: Dr. Jan Langermans (Head of the Panel), Chairman of the Animal Science Department at Biomedical



Primate Research Center (BPRC), Rijswijk (Netherlands); Dr. Maria del Mar Blanco, Dpto. de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid (Spain); Dr Bertrand Collet, Immunology & Infection Marine Scotland - Science, Aberdeen (Scotland); Dr Arthur Summerfield, Head of Immunology, Research Department, Institute of Virology and Immunoprophylaxis (IVI), Mittelhausern, (Switzerland); Dr. Juan Badiola, National Reference Centre for Transmissible Spongiform Encephalopathies, University of Zaragoza, Zaragoza, (Spain) and Dr. Michel Pépin, Ecole Nationale Vétérinaire de Lyon, (France).

During the whole of the NADIR project, 60 TA pre-proposals were submitted and evaluated by the Panel of Experts. 45 proposals were accepted and the related project were hosted by the NADIR consortium facilities.

Task NA3.3: The network collaborative platform

The main objective of the task was the development of the network collaborative platform, an internet-based space to enable optimised and secure exchange of information between partners.

The NADIR Collaborative Platform was developed and implemented: the tool, launched by INRA, is structured into two separate parts, a Scientific Platform (www.nadir-network.eu) and a Management Platform (workspaces.inra-transfert.fr/nadir). The network collaborative platform is restricted to NADIR members: requiring a login and password in order to access to the online materials.

The NADIR Resources Dissemination System, a web tool was created to share and publicise data, services, products and protocols available in each research group of NADIR, consists of two complementary parts (a Keyword database and a Web Content Management System). Both these components are accessible through a web interface that has links from the project portal.

The Keyword database contains schematic descriptions of the resources within the project and a database of information related to resources available from each partner, which is organized according to the following fields: species, cell line, description, source/availability, email contacts and a free text notes field. A query facility is available through a user-friendly web interface which allows boolean operators.

The Content Management System (CMS) is structured as follows: every partner is the owner of a website area, where text, images and tables can be published. The area can be updated directly by each partner, through a user-friendly web interface tool. Guest users accessing this CMS area can surf the data published by the partners using the search facility of the CMS web interface.

The following technologies and languages were used in the development of the resources: Linux, MySQL, Php, Phpmyadmin and Joomla.

Task NA3.4: Exchange with related initiatives

The main aim of this task was to identify, select and establish contact with the most relevant related initiatives, mainly among EU-funded projects, in order to establish fruitful interaction, e.g. by exchange of experiences, best practices etc.

Interactions with related initiatives were carried out during the entire project period: NADIR partners have directly contacted EADGENE, EPIZONE, SABRE, MedVetNet, ERIN – European Ruminant Infrastructure Network, TRANSVAC, PROVAX and PIROVAC.

Negotiations with EADGENE and EPIZONE were carried out in order to define the most effective way to share access to the database resources.

In order to strengthen the exchange with related initiatives, the NADIR project was presented during the following events: Africa-EU Research Infrastructure Conference, held in Accra (Ghana) in December 2012; ANIHWA/SCAR Joint Meeting at OIE, held in Paris (France) in February 2013; EADGENE_S Stakeholder Meeting, in Brussels (Belgium) in May 2013;



TRANSVAC Meeting, in Brussels (Belgium) in June 2013; One Health Summit, in Davos (Switzerland) in October 2013; NADIR Session at EPIZONE, in Brussels (Belgium) in October 2013.

The communication materials developed within NADIR have been disseminated to the following EU-funded projects and related initiatives: ETPGAH, STAR-IDAZ, NeuroPrion, SEAFOODPLUS, BTVAC Project, TB-STEP, EUPRIM-NET, EMIDA, FERFLU EXPRESS, DISCONTTOOLS, VIRULENCE EVOLUTION, PNEUMOPATH, FMD-DISCONVAC, COMBINE, ANIMBIOGEN IN EU, POSTICK, INTERPOD-CRG, BOLD, EIGIS, GIPIO, SAPRO, PIGSNP, HYPOTHALAMIC T3, ROBUSTMILK, LUPA, MISS-SA, ESGI, DOGPSYCH, FLUARRAY, E PIAF, INTERPLAY, EVONET, APO-HSV-2/HIV, INCOME, CLARA, NEW-FLUBIRD, FLUTEST, FLUPATH, TRIOH, MVECTOR, INTRANASAL H5VACCINE, FLU-LAB-NET).

Task NA3.5: Workshops

The objectives of this task were to organize workshops on topics related to NADIR activities (e.g. diagnosis methods, biosafety recommendations, ethical issues, etc.) to provide the partners with an instrument to foster internal interactions.

Eighteen NADIR project meetings and workshops related to project WPs were held during the project period. Some of these workshops created working groups focussed on specific NADIR objectives.

In May 2009, the NADIR Kick-Off Meeting took place in Tours (France).

A Website tools Meeting was organized in June 2009 in Toulouse (France).

A NADIR RA1-RA2-RA3 Workshop was held in November 2009 in Lelystad (Netherlands).

In February 2010, a NADIR Meeting was held in Lodi, to discuss animal cell lines and molecular tools for genotyping.

In May 2010 a NADIR Biosafety and Biosecurity Workshop was held in Weybridge (U.K.).

A NADIR RVFV, ASFV, AHSV Meeting was organized in June 2010 in Madrid (Spain), to discuss animal the experiments to be performed for establishment of animal infection models for Rift Valley Fever Virus, African Horse Sickness, African swine fever.

In August 2010, a NADIR Influenza Meeting was held in Paris.

In October 2010, the NADIR Annual Meeting was organized in Paris (France).

A NADIR Workshop on Telemetry took place in Tel Aviv (Isr) in March 2011.

In September 2011, a NADIR WP2 Meeting and a NADIR Ethics Meeting were held in Pirbright (U.K.).

In November 2011, the NADIR Annual Meeting was organized in Barcelona (Spain), hosted by CReSA.

In August 2012, the NADIR Annual Meeting was organized in Insel Vilm (Germany), hosted by FLI.

A meeting to discuss the contents of a letter of intention to be prepared for Horizon 2020 was held in Paris, in September 2012: Letter of Intention for Horizon 2020 Meeting.

In November 2012, two events took place at UNOTT in Nottingham, U.K.: a Workshop on Pain Management and the Human End Point Workshop Meeting.

A workshop to discuss the activities carried out and results obtained within the project WP6 was organized in Paris, France, in July 2013: WP6 Meeting.

The NADIR Final Meeting took place in November 2012 in Tours, France, hosted by INRA.

In addition to these meetings regular telephone conference meetings and User Selection Panel meetings for the evaluation of the proposals for the TA activities took place during the entire period of the project.



Task NA3.6: Newsletter

This task was related to the periodic publication of a project newsletter, in order to inform partners of the project activities and to spread information regarding NADIR, by disseminating the project results to the scientific community active in the field of animal infectology research.

The first issue of the newsletter (November 2009) included a message from the coordinator of the project, Frederic Lantier; a description of the Moredun Research Institute (MRI), United Kingdom; a brief list of upcoming events of interest; a report on the Final Meeting of the project EADGENE, held in Paris, France (October 13th and 14th 2009) and The Call for Proposals for Transnational Access Projects.

The second edition of the newsletter (May 2010) included a report on the NADIR meeting held in Lodi on February 15th and 16th; a description of ANSES (formerly AFSSA), France and INRA, France; a list of the upcoming international scientific events and NADIR meetings and a reminder of the Call for Proposals for Transnational Access Projects.

PTP published the third issue of the NADIR project newsletter, featuring the creation of ANSES, the French agency for food, environmental and occupational health safety; Aarhus University – Department of Animal Health and Bioscience as the highlighted institution; a summary of the achievements over the last 5 years of the EADGENE project; a report on the Plant and Animal Genome (PAG) Meeting; a reminder of the Call for Proposals for Transnational Access Projects and a list of the upcoming scientific events and NADIR meetings.

The fourth issue of the Newsletter featured articles on the NADIR Annual Meeting, held in Barcelona on November 2011; the Training Courses organized within NADIR project and planned for the whole 2012; PTP and CReSA as highlighted institutions; an entire page on the upcoming animal experimentations conferences planned for 2012 and a reminder of the Call for Proposals for Transnational Access Projects. This fourth issue was published on NADIR website and disseminated through the NADIR mailing list, to related initiatives at a European Level and through the blog-like website.

PTP published the fifth issue of the NADIR project newsletter, featuring the following articles: NADIR Annual Meeting 2012, the NADIR annual meeting 2012 was held on the small Island of Vilm in the Baltic Sea, in proximity to the larger Island of Rügen, Germany; Animal Use in Research and the new EU Directive, the Animal Use in Research and the new EU Directive Conference, addressed the ethical, legal and scientific issues raised by the new EU Directive (2010/63/EU) on the protection of animals used for scientific purposes; Highlighting the Value of the ARRIVE Guidelines, developed by the UK NC3Rs, the ARRIVE guidelines aim to improve the reporting of animal experimentations to ensure the data can be fully evaluated and used by other animal researchers; a reminder on the Call for Proposals for Transnational Access Projects and a list of the upcoming conferences on animal experimentations.

The sixth issue of the NADIR project newsletter was produced by PTP with materials provided by UNOTT, featuring a guide on National and International 3Rs Centres, as a resource for researchers working in large animal experimentation: accessing international sources of good alternatives tools and data can be an important part of applying the 3Rs.

All the newsletter issues have been published on NADIR website and disseminated through the NADIR mailing list and to related initiatives at a European Level.

The fifth and sixth newsletter issues have also been disseminated through the blog-like website and LinkedIn Groups. The blog-like website (<http://nadirproject.wordpress.com>) was specifically developed during the second reporting period to increase the dissemination of the information related to the project, providing a way to promote individually each web entry.



The following LinkedIn Groups were chosen for the dissemination: Animal Health and Nutrition Network; Animal Health Forum; Animal Health in the UK; Animal Health Nutrition and Production Professionals; Animal Science Monitor; Animal Science Professionals; Dairy Health, Nutrition and Production Professionals; European Animal Professionals; Global Dairy Innovation Network; Hi-tech Dairy Farming; National Mastitis Council; Poultry Science Network. Dissemination through these Groups reached approximately 64,000 professionals active in the field of animal health science.

WP5: RA1 - Characterisation of animal lines

The WP5 has achieved all the Scientific & Technological target objectives established at begin of the activities.

As main result, based on the activities carried out at this WP, we can state that now better characterised animal lines are available for all the scientific community. These well characterised animals are robust tools that will allow scientists to continue to discover innovative ways to control and prevent livestock diseases. Their use as animal models, together with the developed and validated immunological reagents obtained in this WP5, will allow a significant reduction on the number of experiments required to obtain solid results (in agreement with the 3Rs rule) and on the time needed to achieve them.

The main results achieved in this WP5 are indicated in the following.

1.- Characterization of inbred and/or histocompatible lines of chicken, pigs, sheep, mice and fish (Genotyping, immunological characterisation, sanitary status analyses).

NADIR project has created a very effective platform for animal lines characterisation, which has enabled to share animal lines, their nucleic acid material and also the procedures and reagents for animal characterisation.

Chicken lines from UEDIN/IAH, INRA and AU have been genotyped by an improved SNP panel developed at INRA, to characterise the large variability of MHC region in this animal species. Genotyping results has shown that the 66,6% of analysed samples were homozygous, corresponding to at least 84 unambiguous haplotypes. The new SNP methodology was able to detect new alleles, which is highly relevant since it allows inferring evolutive relationships between MHC alleles. Another important conclusion of this study was that there isn't underlying variability in MHC between animals with the same serologically defined allele, inside or between populations. That's mean that all animals from all population homozygous for one MHC allele are sharing the same SNP haplotype.

In addition to MHC characterisation, at AU chicken lines has been characterised at the molecular level the chicken gene encoding mannose-binding lectin (MBL). This gene is a potential candidate for innate disease resistance, and therefore it characterisation in chicken lines can be very important for correlation between this gene and disease susceptibility. To improve the methodology available for such characterisation, a new test named KASP-based genotyping assay, was developed. This new assay is able to distinguish between different alleles identified in the promoter region of MBL gene.

In addition to genotyping, chicken lines were further characterise immunologically (AU, UEDIN). The absolute numbers of relevant immune cells (T-, B, $\gamma\delta$ -T, etc) as well as the concentration of specific IgG antibodies have been determined.

Pigs, one of the most relevant farm species in terms of economic production, are valuable animal models not only for the study of veterinary diseases, also as models for human biomedical



research, due to anatomical and physiological similarities between pigs and humans. Furthermore, the immune system of pigs is also similar to that of humans.

During NADIR project, pig herds available at IAH (inbred lines) and at DTU-Vet (outbred line) were tested to confirm their unique high sanitary status. Such analyses confirmed these pig lines are free from more than 20 pathogens, including some highly prevalent viruses in domestic pigs, as Hepatitis E (HEV) or PRRSV.

IAH inbred pigs, Babraham, cc and dd lines, were analysed on their SNP genotypes. The degree of homozygosity across the genome of the three lines is high: 85% (Babraham), 70% (CC pig line) and 79% (DD lines).

Additionally, SLA-I specific alleles represented in such inbred pig population, and the more frequent alleles represented in the DTU-Vet outbred pigs, were characterized. These analyses are highly relevant for immunological research analyse. Incorporation of SLA-I sequence alleles to bioinformatics databases will made possible the prediction of CTL epitopes, as well as the synthesis of SLA- tetramers, which can be used for detection and quantification of T-cells specific for a given antigen. Therefore, these contributions enhance the value of these pigs models, allowing its use for example, in the development of new vaccines and deep analyses of immunological mechanisms involved in pathogenesis.

Similar characterisation analyses to those performed in chicken lines were performed in sheep lines at INRA and MRI. The genetic characterisation of the principal class I and class II MHC genes and haplotypes associated with the four MHC haplotypes was completed within MRI and INRA sheep lines. Full-length class II DQA and DQB transcripts from sheep lines at both locations were amplified and sequenced. This study is highly relevant since allowed the identification of locus specific characteristics and phylogenetic analyses. Data obtaining provide evidence about the likely origin of DQA3 genes, from a recombination between DQA1 and DQA2. Furthermore, such analyses have lead the development of a new locus specific allelic nomenclature system for DQA and DQB alleles, available to the international community through the IPD-MHC database (<http://www.ebi.ac.uk/ipd/mhc/index.html>).

Significant progress has been achieved in sheep lines genotyping methodology. In particular, a next generation sequence based genotyping methods for typing functional diversity within the multiple class I MHC genes of sheep have been developed. This method can now rapidly identify diversity in the class I region of sheep. In this context, Illumina MiSeq and Roche 454 platforms were evaluated and comparison with conventional Sanger based genotyping methods was carried out, performing well both sequencing platforms. The functional range of class I genes within MRI and INRA lines has been determined using this method and novel SNP's and alleles identified (Methodology and data will be submitted as paper in 2014). An additional achievement in this WP5 was the construction of a BAC library from a MRI homozygous animal. This library is a quite interesting tool that will allow determine the complete haplotype from a homozygous animal.

In parallel to sheep lines genotyping, defined flock and the four haplotypes in MHC heterozygous animals, obtained through selective breeding of MHC-typed sheep, have continued maintaining. Interestingly, an archive stock of primary fibroblast cell lines from MRI flock is maintained in liquid nitrogen. Now, the scientific community has available high value sheep lines as animal models, fully characterised.

Mice are the most commonly used vertebrate species as animal model, popular because of their availability, size, low cost, ease of handling, and fast reproduction rate. With the advent of genetic engineering technology, genetically modified mice can be generated. One of the main objectives of NADIR project was the development and characterisation of transgenic mice



expressing PrP from different species as models to study prion diseases and their transmission. INIA, INRA, FLI and CRESA have been involved in this task.

Each of transgenic mouse line was obtained in a background null for murine PrP. Transgenic mice were generated by DNA-microinjection following the procedures previously described. For each construction, at least one mouse line was selected based on their PrP expression levels, and bred to homozygosity in a murine *prnp* null background. To achieve this, the selected lines were crossed with *prnp* null mice (*Prnp* $\mu^{-/-}$) to achieve transgene-hemizygous lines (*Prnp* $\mu^{-/-}$ 113Lbo $+/^{-}$). The absence of the murine *prnp* gene was determined by PCR using specific primers. Transgene expression levels were then determined in brain homogenates by serial dilution and compared to the PrPC levels found in brain of the corresponding natural specie. These expression levels were biochemically assayed by Western blot after limit dilution of the sample from each transgenic mouse line. The work performed in regard mice lines have contribute significant to increase the mice model lines available for the study of different PrP strains. The use of such mice lines will allow to enhance the analyses of prions transmission, contributing to the improvement of disease control.

Fish lines characterisation was focused on rainbow trout, a relevant academic model for immunological studies in teleost fish as well as an important economical species in Europe. Two outbred populations of rainbow trout (French and Danish), used as references in a number of studies in DTU-Vet, VESO and INRA, were phenotypically and genetic compared. Genetic variability in these populations was carried out using a set of 88 SNP markers developed at INRA. In addition to both outbred populations, the comparison included further 12 domestic populations. The results of this study concluded that no critical differences were found among the both outbred lines; both have a high level of polymorphism and are genetically close to each other and to other domestic populations. Both lines constitute interesting models for pathogenicity and disease susceptibility studies, since differ in resistance when are infected with several viruses (see next section).

In addition to outbred fish lines, a set of isogenic homozygous lines, previously established from INRA outbred population, were characterized for their status (susceptible vs. resistant) at a major QTL for survival after VHSV infection. In this study interesting results were obtained, which provide key information on the possible mechanisms of resistance (role of innate/intrinsic factors). QTL governs both survivals after waterborne infection and virus multiplication on fin explants, which strongly suggested a major contribution of innate/intrinsic factors in resistance. Furthermore, established cell lines derived from the isogenic fish lines constitute a relevant tool to further decipher the antiviral defence mechanisms. Intercrossing information from fish and cell lines will greatly increase the power of future genetic and functional studies of immune and disease resistant traits while contributing to comply with the “3R” rule.

All these animal lines genotyping and immunological achievements have been described in detail in Deliverable 5.1 (DRA 1.1).

2.- Characterization of animal lines resistance/susceptibility to pathogens.

The animal lines characterised during NADIR project, now constitutes excellent animal models for the study of susceptibility/resistance to pathogens. In this regard, all the partners involved in this WP5 have collaborated efficiently and coordinated to carry out in vivo experimental infections with diverse pathogens, included virus, bacteria and parasites, avoiding duplication analyses.

Among others, the resistance to the following pathogens have been assessed in chicken lines: Coccidiosis (seven different strains), Salmonellosis (five strains), Infectious bronchitis virus (IBV), Infectious bursal diseases IBDV, Marek disease (MDV), *Ascaridia galli*, Newcastle



disease virus, etc. These results contribute significant to the study of such pathogens, providing useful information for development of new approaches for better control of the diseases.

In agreement to the objectives established in the NADIR DOW, IAH, DTU-VET and ANSES pigs herds were inoculated with an African swine fever virus strain non-pathogenic and boosted with a pathogenic one. In these experiments collaborated also other partners as INRA, INIA, FLI and CRESA. The experimental infections shown that pathogenesis of ASFV strains used, both isolated from ticks, was different depending on the pig background. These results highlight the relevance of use well characterised animal models and support the genetic role on ASFV replication *in vivo*.

In regard of disease resistance studies in sheep, Spanish autochthonous breed (Ripollesa) was inoculated with the Rift valley virus. Clinical signs, mortality and histopathological analyses were carried out at CRESA in collaboration with INIA. This study has demonstrated that sheep age is a clear factor for clinical and pathological display. Moreover, INRA sheep inbred lines were tested for their susceptibility to *Salmonella Abortusovis* and the results were correlated to the alleles ARQ (susceptible to Scrapie), AHQ (partially resistant) and ARR (resistant). Salmonellosis study showed that there is roughly a 10 fold difference in bacterial load of target tissues between two extreme lines.

Susceptibility of mice lines (transgenic, outbred and inbred lines) to different pathogens as prions, West Nile, Rift Valley virus and Bluetongue was tested. These studies provide interesting data about the suitability of such animal lines as models to carry out pathogenesis, transmission and other studies on the target pathogens.

A collection of Tg-mice expressing PrP from different species (bovine, porcine, goat and human) were inoculated with different prion strains from different species at INIA, INRA, FLI and CRESA. These mice lines are highly sensitive models for analysis of prion strains transmissions among different species. As expected, all the lines available at the start of the Project have been characterised for the relevant prion strains. Among others, a relevant result achieved in these studies should be highlighted. It have been demonstrated that a variety of infectious prion strains might spontaneously emerge in hosts displaying random genetic PrPC mutations. According to the relevance of this finding, the results have been published recently in the *Emerging Infectious Disease* journal (Torres et al., 2013).

Mice from different backgrounds were also characterised at INIA in terms of West Nile virus susceptibility. Pathogenicity of different WNV strains and other Flavivirus were studied in mice model. Interestingly, characterised mice model for WNV infection allowed to study vertical transmission of such viruses and the assessment of new recombinant vaccines.

Additionally, 129IFNAR KO mice were studied as models of Rift Valley and Bluetongue virus, demonstrating for both viruses the utility of this model to test the potency of vaccines.

Susceptibility of two rainbow trout strains (outbred populations) and two Atlantic salmon strains to infection with the viruses IPNV, IHNV, VHSV, SAV have been tested. A French and a Danish rainbow trout strain was compared, and two Norwegian strains of Atlantic salmon; one sensitive and another resistant to disease with IPNV respectively. Differences in susceptibility to VHSV and IHNV were seen for the two rainbow trout strains. The Danish trout strain showed higher susceptibility to VHSV than the French reversely the French trout strain showed higher susceptibility to IHNV than the Danish strain. None of the salmon strains showed to be susceptible to the rainbow trout pathogenic VHSV and IHNV strains used. The difference in susceptibility between the two fish strains is interesting and could be used to look further into host pathogen interaction and further host response and defence mechanisms to disease. Challenge of wild Danish Atlantic salmon with VHSV and IHNV has not been performed due to



unforeseen problems with the fry during the NADIR project period. Another attempt to obtain wild Danish Atlantic salmon fry will be done in the remaining project period.

These animal lines resistance/susceptibility studies have been described in detail in Deliverable 5.3 (DRA 1.3).

2.- Development and validation of immunological tools useful for monitoring immune response, pathogen detection and/or for improvement of vaccines.

A number of new reagents have been developed to monitor the immune response to infectious diseases in ruminants, pigs and poultry. Reagents and methods have been developed and/or adapted to measure T cell responses in cattle and pigs. Additionally, the same progress has been achieved in regard of chicken studies. New reagents have been developed to analyse specific dendritic cell, CD4 Th2 and B cell interactions in the chicken. Likewise, new procedures for evaluation of cytotoxic responses in these animal species have been set up. IAH, UEDIN, MRI and AU have highly contributed to such developments [Deliverable 5.2 (DRA1.2)].

Additionally, significant progress has been achieved in the NADIR framework in regard to obtention of plasmids that coded cytokines (particularly for ruminant and fish species) and antibodies against such cytokines. Rainbow trout recombinant chemokines (CK5B, 6, 7A, 9, 11 and 12) and polyclonal antibody (anti-CK12) have been obtained by first time. These reagents are now available to fish research community and will contribute highly to increase the basic knowledge on immune response of teleost animal species.

An additional relevant achievement reached in this WP was the obtention of IL-4 and GM-CSF ruminant cytokines, useful for in vitro obtention of dendritic cells from peripheral blood monocytes. These cytokines have recently been commercialised and are available to the research community through Bio-Rad AbD Serotec.

These developments were described in the Deliverable 5.6 (DRA.1.6).

An important development for improvement of new vaccines include the obtention of immunomodulatory molecules. In this regard, recombinant adenoviruses expressing functional MIP3 α and FLt3L were produced. These constructions were evaluated in vitro, showing promising results about their potential as immunoadjuvants in vaccine formulations [Deliverable 5.5 (DRA.1.5)].

The development of these new reagents is especially relevant to characterise the animal lines of different species used as experimental models of animal infectious disease. Therefore, some of described reagents were used as tools on analyses of healthy and infected tissues, using different methodologies. Immunohistochemical procedures were established for detection of West Nile virus in different bird tissues (FLI). This technique was also used to measure sheep NK cells in addition to Flow cytometry assay.

This last method was also used to determine fish chemokines release in different tissues, in response to VHSV infections.

All together these results highlight the relevance of obtention of immunological reagents, for better analyses of host pathogen interactions. [Deliverable 5.4 (DRA1.4)].

WP6: RA2 - Development of infection monitoring tools

The general objective of WP6 is to jointly make the latest technologies suitable to be used in the field of animal infectiology with its specific demands (large or small animals, species specific material etc).

This can be broken down into the more specific objectives:



- To customize imaging and telemetric technologies in order to make them suitable for the animals of interest or under certain circumstances.
- To implement novel molecular tools for monitoring infection processes
- To upgrade single assays into cost-effective, easy multiplex assays

RA2.1: Upgrading imaging technologies

The main objective of WP6.1 was the development of imaging technologies in order to make them suitable for the animals of interest or under certain circumstances, i.e. to provide imaging tools to study infectious processes during animal experiments.

At INRA, initially it was planned to use a gamma camera coupled with a X-Scanner that was adapted to the imaging of small and medium sized farm animals (from chicken to sheep and pig). Although the financial support was and is still available through the French Research Ministry, purchasing of the equipment was post-poned by INRA because of cumulating difficulties in refurbishing the building adapted to the confinement of both microbes and radioactivity. This delay was partly circumvented through the purchase by INRA of a fluorescent and bioluminescent imager dedicated to the imaging of small animals in high contained facility (BSL3). The IVIS® Spectrum CT from PerkinElmer was acquired and installed. It provides INRA with possibilities of imaging the infectious process in model species (e.g. rodents) or young poultry.

Using the IVIS Spectrum CT, at INRA experiments were performed with labelled *Salmonella enterica* serotype Enteritidis, *Escherichia coli*, *Mycobacterium bovis* strain BCG and *Eimeria tenella* in mice and/or chickens. These pathogens were chosen according to their availability and to their diversity representative of infection models used throughout the NADIR partners laboratories.

The IVIS Spectrum CT appeared to be able to distinguish the injected pathogens in all tested models when they were labelled with bioluminescence or fluorescent markers emitting in the “red –infrared” wavelengths, “green” markers such as GFP could not be discriminated from the tissue or intestine food content natural fluorescence.

The IVIS Spectrum CT is installed in a contained animal facility at INRA which allow investigation on small animals infected with pathogens up to the BSL3 level. Entry of investigators according to safety regulation is controlled. The facility as well as the apparatus can be disinfected with hydrogen peroxide and waste is decontaminated through autoclaving. Data and images can be exported through optical fibre network in any authorized laboratory.

It can be concluded that this platform offers good possibilities for studying infections in animals. CreSA reported the application of Multi-Detector Computed Tomography (MDCT) to evaluate extension of pulmonary lesions in a tuberculosis model in goats. The extent of pathology was expressed quantitatively as a percentage of the total pulmonary volume affected by lesions. The model has been used for assessment of vaccine efficacy of BCG and recombinant Adenovirus type 5 in a prime boost strategy.

RA2.2: Upgrading telemetry technologies

The main objective of WP6.2 was the development of infection monitoring tools. It included the development and customization of telemetry technologies in order to make them suitable for the animals of interest or under certain circumstances, i.e. provide telemetric tools to study infectious processes during experiments on farm animals.

Various telemetry devices have been used for years to monitor and record physiological parameters such as temperature, heart rate, blood pressure, respiratory volume and rate, activity, as well as biopotentials (ECG, EEG, EMG) in laboratory animals such as rodents, rabbits, dogs



or monkeys. But only few tools were available so far to monitor the same parameters in farm animals, especially in large species like cattle, small ruminants or pigs.

The use of existing devices or the development of new tools to monitor temperature in these species were the main objectives of this task. More precisely, our needs were the following:

- Perform automatic measurement, transmission and recording of body temperature
- Collect data continuously at a frequency that could be set up
- Send data at long distance (at least 20-30 m and beyond)
- Export data in an Excel-type format
- Possibly send information on mobile phone by GSM transfer of data (option)
- Use the telemetric thermometers in various farm species (cattle, small ruminants, pig, horse, poultry), but also in some wild species of interest (wild boars, badgers) in adult and young animals.

A workshop dedicated for telemetry and imaging technologies was organized in Israel at KVI, 13-15 March 2011. At the workshop different technologies were demonstrated and experiences shared.

Several NADIR partners reported successful use of telemetry equipment:

- KVI tested novel telemetry equipment for the acquisition of physiological parameters during experimental infections in animals. KVI acquired also a thermographic camera and is in the process for acquiring electronic detection system for following up of infection experiments in large animals.
- DTU tested microchips in pigs, but found that it is not a suitable to measure body temperatures of larger animals, however it could be useful for group evaluation.
- INRA reported the successful use of
 - rumen located thermometers in sheep
 - vaginal thermometers in pregnant cows for monitoring of calving
 - surgically implantable thermometers in pigs inside the peritoneum or under the muscles of the flank.
- a Microbolus® or “small Thermobolus” in vaginas of ponies, during an experimental infection in five mares with Flaviviruses in BSL3 containment.
- implantable thermometer in chickens. Either the thermometers were introduced under the skin, below the wing or swallowed by the chicken.
- All these tests have shown the possibility of using a wide range of thermometers adapted to various species and for various purposes.

RA2.3: New molecular tools for monitoring infection processes

The main objective of task WP6.3 was to implement novel molecular tools for monitoring infection processes.

Various activities were performed to develop new molecular tools of diverse types.

AU established methods for high throughput screening for Newcastle disease virus and Infectious Bronchitis Virus in infected chickens.

The aim of ANSES was to characterise the porcine endogenous retrovirus (PERV) integration in the pig genomes by using high throughput sequencing after specific amplification of the junctions between PERV and pig sequence (PERV junctions). A technique for fishing of the sequences was set-up during the first 2 periods of Nadir. This technique was validated with a hybrid systems constituted of human cell line infected with PERV. Experiments with the pig genome however did not allow the characterisation of the full population of potentially active PERV in the pig genome.



CreSA developed molecular tools for detection of Rift Valley Fever Virus and West Nile Virus to monitor animals undergoing experimental infection. A SYBR Green real-time RT-PCR assay for the detection and quantitation of Classical Swine Fever Virus has been developed and validated. The successful validation of the test on two real-time PCR instruments with the most divergent ramping conditions has demonstrated that the assay is not affected by the type of platform and that it can be used as a reliable tool for large-scale CSFV detection.

INIA developed strategies for generation of fluorescent proteins which are potential useful tools for virus-host and viral infection analyses in live cells. For this purpose a system has been developed for the production of recombinant CSFV-GFP viruses and fluorescent calicivirus VLPs. INIA showed also the potential application of fluorescent VLPs as tools to study virus-host interactions. The obtained fluorescence VLPs have been used to elucidate which antigen presenting cells are responsible for uptaking and processing. The information obtained in this study can be relevant for the design of new vaccine strategies, based on recombinant VLPs.

INIA also developed a competition ELISA for detection of anti Rift Valley Fever Virus (RVFV) antibodies in camel serum samples. Since antibodies against RVFV can be detected in many wildlife species, tests based on competition ELISA (c-ELISA) may be useful to cover a broader spectrum of RVF susceptible species. This is based on the fact that in a competition assay a species-specific secondary antibody is substituted by a competitor antibody.

Routine H&E histopathology defines the changes in cellular organisation within an organ that characterise specific lesions, but additional histopathological techniques permit in situ localisation of pathogens, host cell phenotypes and host molecules that extend interpretation to the subcellular level. In situ detection of pathogen proteins or nucleic acids is critical to differentiate the presence of infection in tissue cells rather than blood cells. The main results obtained by AHVLA within this work package are:

- Immunohistochemical techniques have been established to detect a variety of immune cell types, cytokines and chemical mediators in cattle, pig, fallow deer, badger and wild boar tissue sections.
- Immunohistochemistry and In Situ Hybridisation techniques have been developed to detect different strains of PRRSV and CSFV and *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* in porcine tissues.
- qPCR techniques have been developed to quantify mRNA levels of cytokines and chemokines using laser-capture microdissection technology in cattle and pig tissues.

These techniques have been used to study the pathogenesis and the host-pathogen interaction in tuberculosis in cattle, fallow deer, wild boar and badger and pigs with CSFV, PRRSV or *Mycoplasma* spp.

RA2.4: Multi-diagnostic tools

The main objective of task WP6.4 is to upgrade singleplex diagnostic assays into cost-effective multiplex assay.

Diagnostic tools remain important in the field as well as in animal experiments. Constantly there is a demand for testing a higher number of samples on more targets and in less time. To meet these demands several NADIR partners started to develop new multiplex tests.

FLI was working towards the optimization of real-time PCR protocols for the detection and quantification of alphaviruses (WEEV, EEEV, VEEV) and bunyaviruses (RVFV). A quantitative real-time RT-PCR for the detection of Venezuelan equine encephalitis virus (VEEV) was developed and a universal equine encephalitis virus control RNA was designed. In addition, a SYBRgreen based pan-flavi assay was developed for the detection of flaviviruses, genus flavivirus. Identification of viruses is carried out by melting curve analysis and sequencing. The



moment the assay is being validated by a set of flavivirus isolates including WNV, USUV, JEV, TBEV and others.

CVI developed a six-plex bead-based suspension assay that enables detection and limited serotyping of *Streptococcus suis*, using the Luminex platform. The assay targets a generic household gene (*gdh*), four serotype-specific genes (*cps1I*, *cps2J*, *cps7H*, and *cps9H*), and a virulence factor (*epf*). The six-plex assay consist of a multiplex PCR, which was adapted to increase specificity, and a target specific primer extension (TSPE), which is now fully functional, followed by hybridization to beads and subsequent analysis using the Luminex technology. This procedure is up and running. The assay was successfully evaluated with field samples and a comparison with classical PCRs was made. In this way knowledge was gained of designing and performing molecular assays in multiplex format.

KVI developed several PCRs:

- a real-time multiplex PCR for the detection of Chicken Anemia (CAV) and Marek's Disease (MDV) viruses. The multiplex real-time shows a similar sensitivity as compared to the mono-real-time PCR for each virus.
- a conventional RT-PCR and real-time multiplex assays for the detection of Israel Turkey Meningo Encephalitis Virus (TMEV) in turkey brain tissues was developed.
- two multiplex PCR assays which possesses a similar sensitivity as the most sensitive system, the NS5 gene, the conventional and the real-time RT-PCR for the simultaneous detection of both genes.
- a real-time quantitative real-time RT-PCR for the detection of the avian influenza (AIV), subgroup H9N2 M gene. The detection limit of the assay was shown to perform at a sensitivity of 1-10 molecules.
- a real-time multiplex assay for the detection of fowlpox (FPV) and infectious laryngotracheitis (ILT) viruses. As the clinical signs of chickens infected by both viruses, FPV and ILTV FPV overlap to a great extent, differential diagnosis is needed. For this purpose these PCRs are suitable.

WP7: RA3 - Development of infection models

Task RA 3.1: (Cell culture)

At the beginning of this reporting period, we continued our effort to prepare a list of cell culture systems at the partner institutes, so that all NADIR partners can benefit from the huge collection of cell cultures that is available. With the knowledge about the already available and well defined cell lines, we could then intensify our work on the optimization and further characterization of cell lines that will be useful for our future work.

First of all, a list of animal cell lines of numerous different species (mammals, birds, fish etc) that are well characterized and are therefore valuable for any kind of research activities has been prepared. This includes cell lines generated at FLI as well as other NADIR institutes (mainly partners 10 and 14). This list has been published through the NADIR website.

In the next step, several approaches were made to generate additional cell lines that can become valuable tools for future virological work. Attempts were made to generate permanent cell lines derived from pluripotent myeloid cells originated from porcine bone marrow. The aim of this activity was to obtain cell lines suitable to replicate relevant swine viral pathogens. Porcine bone marrow cells were differentiated to a macrophage-like cell subsets. Thus, an immortalized porcine macrophage-like cell line with capability to sustain replication of several viruses was obtained. Virus infection experiments confirmed replication of Suid herpesvirus 1 (SHV),



African Swine Fever Virus (ASFV), and Classical Swine Fever Virus (CSFV). The yields obtained from SHV and ASFV infections were similar than those obtained with their gold standard cell lines. The yield obtained from CSFV was lower than the one obtained with PK15. These cells also present Class II MHC in an interferon-inducible manner, and show some phagocytosis capacity.

We have also established procedures for the preparation of murine bone marrow dendritic cells. Ovine skin fibroblasts were obtained from animals used for a RVFV experimental infection (see RA3.2). They could stand multiple passages and are used now as autologous antigen presenting cells for immunological studies and growth of the virus.

It was found that BTV-8 infects mice bone marrow progenitors and interferes with their development to mature cells. BTV-8 interferes with dendritic cells' (DC) antigen presenting capacity and moreover, DCs undergo apoptosis during in vitro infection. All these new findings suggest that BTV infection of bone marrow progenitors and DCs can cause immune response impairment, leading to animal death or persistence.

We have established frozen stocks of pig kidney cells and alveolar lung macrophages from Lindholm pigs, as well as 1st passage bovine thyroid gland cells (BTY), and calf kidney cells. These cells are available to member state institutions free of charge. Vet Lindholm has intensified activities on the establishment of porcine aorta endothelial cell cultures. To overcome a problem of infiltrating fibroblasts, magnetic separation has been initiated. To test the susceptibility of frozen BTY cells; virus titration assays, including FMDV strain O and A, were carried out on different passages of BTY cells. However, the endothelial cells were found not to be susceptible to ASFV after 6/7 cell culture passage in our set-up. The results varied and single set-ups provided promising results, however, it was not possible to repeat and standardize these results. Thus, the use of porcine endothelial cell cultures does not provide an immediate option for a robust isolation and detection protocol for ASFV.

The characterization of the replication capacity of viral haemorrhagic septicaemia virus (VHSV) in the established RTS11 rainbow trout monocyte-macrophage cell line was addressed, in comparison to that of primary rainbow trout macrophages. We have found that when we extend the incubation period for up to 14 days, some RTS11 cells become susceptible to the virus and release viral proteins and infectious virus into the culture supernatant. This model in which a small percentage of macrophages become fully susceptible to VHSV closely resembles the in vivo model.

In another effort, we attempted to analyse the role of autophagy, a cellular pathway that can play important roles on different aspects of viral infections and pathogenesis, in USUV (Flavivirus) infection of cell cultures. The results indicate that USUV virus infection upregulates the cellular autophagic pathway and those drugs targeting this pathway can modulate the infection of this virus. We therefore aim to develop an in vitro assay using Vero cells for the assessment of antivirals against USUV strains, by analyses of the autophagic pathway.

Task RA 3.2: (vector borne diseases):

The aim of this task was to improve our understanding of vector borne diseases, in terms of species specificity, pathogenicity, pathogenesis, as well as diagnostic and preventive possibilities. To be able to develop diagnostic assays and preventive formulations, it was also necessary to collect samples from naturally and experimentally infected animals. This knowledge is crucial for a fast response after a possible introduction or emergence of such a disease in a region or country that has so far been free of the disease.



Rift Valley Fever Virus (RVFV):

We have worked on the determination of the pathogenicity and pathogenesis of RVFV in European sheep, in combination with a vaccine study. In collaboration with CRESA, we inoculated four different strains of RVFV in 8-9 weeks old lambs. viraemia was induced, but apart from fever, no other clinical signs could be observed in infected animals. With the aim of establishing an experimental model showing RVFV clinico-pathological changes, a second experiment has been carried in younger lambs (4 to 6 weeks old). The experiment was also used at the same time to test a possible vaccine candidate for RVF, a recombinant MVA encoding the RVF glycoproteins Gn and Gc (rMVA-GnGc). This vaccine had conferred full protection in the mouse model. We then performed an experimental RVFV infection of European with this new vaccine candidate. The experimental RVFV infection model and vaccine efficacy were assessed in terms of clinical signs, body temperature (pyrexia), viremia levels and viral shedding. In summary, the vaccine candidate resulted in a limited protection of lambs against a RVFV infection. Further immunological and histopathological studies are underway to better characterize immune responses and explore the pathogenesis. In another experiment, a DNA vaccine expressing RFV nucleoprotein joined to a ubiquitin (signal targeting the antigens to CTL pathway, to promote cytotoxic responses) conferred 70% protection in a mouse model against a RFV challenge.

Characterization of infection with RFV strains MP12 and 56/74, in knock-out mice IFNAR (-/-) and Balb/c has been performed. Mice were inoculated intraperitoneally (i.p.) the virulent South African RVFV strain 56/74. Mortality rates reached 100%.

Bluetongue Disease Virus (BTV):

IFNAR (-/-) adult mice were identified to be susceptible to BTV infection with two different serotypes (BTV-4 and BTV-8), causing similar symptoms but different virulence. Strain BTV-4 produced similar tissue lesions to those produced in the natural host, suggesting its relevance as model for pathogenicity studies. We have identified that T-cell responses to BTV are directed against multiple and identical T-cell epitopes (CD4+ and CD8+) from the VP7 core protein, in mouse and sheep. The characterisation of these novel T-cell epitopes may also provide an opportunity to develop DIVA-compliant vaccination approach to BTV encompassing a broad-spectrum of serotypes.

Several hundred individuals of *C. nubeculosus* were intrathoracically injected with a BTV8 strain. About 80% of midges survived the injection and 50% of them were infected by BTV8. These midges were used (25/animal) as challenging material in ovine for a TA experiment. Remaining infected midges were used to determine viral load.

We also worked on the development of a multiserotype BTV vaccine, and performed a challenge experiment with the BTV vector species, in order to follow up the actual transmission dynamics of the agent in vivo.

West Nile Virus (WNV):

Models have been developed for West Nile virus (NY99) in falcon (*Falco rusticolus*), by the oral and oculonasal routes in two independent experiments. Clinical signs and viraemia have been assessed. Two different commercially available vaccines were tested for their efficiency in protecting falcons against an infection with WNFV of the lineages I and II. The study revealed differences in the level of immune responses that were induced by the inactivated WNV lineage I virus vaccine and by a live canaripox-based vaccine expressing the prM/E-polyprotein of WNV lineage I. In neither vaccine a single vaccination was sufficient to stimulate a convincing



antibody level. Antibody titres were detected by neutralization test. Both experiments are complementary to each other.

African Horse Sickness (AHS):

AHS is considered to be a relevant threat to the European horse population and vaccine initiatives are undertaken to develop new and better vaccines. However, efficacy of vaccines can up to now only be tested in horses. The limited available animal experimental space and the socio-emotional constraints make the use of horses in trials difficult. Therefore, in a discussion between partners (Madrid, June 2010) it was concluded that a validation of an alternative animal model, making use of the IFNAR mouse is necessary. Two major questions were concerned, 1) the reproducibility of the model between partner institutes and between different serotypes and 2) the extrapolation of vaccine induced immunity in mouse to that in the target species. CVI took the initiative to invest the reproducibility of the model between different institutes (IAH, FLI, INIA) and between serotypes (serotype 1 – 9). It appeared that the dissemination of reference strains of the serotypes was very difficult for various reasons, therefore it was decided not further to proceed with this initiative. CVI performed an animal study in IFNAR mouse to establish the model and to compare effects between known virulent and avirulent strains of serotype 4. In this context, INRA tested the possibility of housing horses (ponies) in a BSL3 facility. Three ponies were kept during a period of 2 months in a BSL3 room (“INPREST” facility), especially prepared and adapted to this species. No obvious modification of behaviour or discomfort was observed in any of the ponies. We have shown that it should be possible to perform experimental infections in BSL3 rooms using ponies, in the PFIE facility “INPREST”.

Following the procedures to characterize a mouse model for BT we have started to develop a mouse model for AHS infection. The results indicate that $\alpha\beta$ receptor deficient mice have viraemia and reduce percentage of survival after inoculation with AHSV strain 4.

In addition, it could be shown that IFNAR $-/-$ mice are susceptible to an AHSV-4 challenge by a subcutaneous route, inducing clinical signs, lethality, viraemia and pathology that are appropriate for immunology and pathogenesis studies of AHSV. Moreover, we have conducted a vaccination and challenge experiment in this model, demonstrating the protective efficacy of the recombinant MVA-VP2 prototype vaccine. The development of such a mouse model for AHSV studies can contribute to speed up the development of alternative vaccines, so far restrained due to ethical and financial considerations relating to the infection of horses.

Collection of samples from infected animals:

In the frame of the above mentioned challenge experiments, samples from experimentally challenged animals were collected under defined conditions. These samples are now available for NADIR partners for future work.

In addition to this, some partners were also able to collect samples from naturally infected animals. KVI was able to prepare a collection of samples from large ruminants infected with arboviruses, including: African Horse Sickness, African swine fever, Akabane, West Nile virus, Bluetongue disease virus, bovine ephemeral fever, EHD, lumpy skin disease virus, peste des petits ruminants.

Task RA 3.3 (Emerging aquatic animal diseases):

There are some viral (VHSV, KHV, IPNV) and fungal (EUS) agents that pose a serious health risk for the European aquaculture. Therefore it was agreed to intensify the research on the pathogenic mechanisms in involved these diseases in order to allow the preparation of vaccines and therapeutics in the future.



We therefore concentrated on the preparation of infection trials for Viral Hemorrhagic Septicaemia Virus (VHSV) in wild European freshwater and marine finfish species (herring and turbot) as well as the infection of the rainbow-trout monocyte-macrophage cell line RTS11 with VHSV (see RA 3.1). First of all, supply and maintaining of herring in the infection facility at DTU for use in infection studies has been optimized. Infection studies with the pathogens *Aphanomyces invadans*, the infectious agent causing epizootic ulcerative syndrome (EUS), and Koi herpes virus (KHV) have been performed and an infection models has been established. Protocols for infection with EUS, the pathogen, training and material for growth of the pathogen have been obtained in collaboration with the EU Reference Laboratory for Fish Diseases.

For establishing a VHS disease model in herring, two batches of wild caught herring were received from an area where the disease is known to be absent or at least present with a very low prevalence. Similarly, a disease model was established in turbot, of which several genotypes were tested both by IP injection and bath infection. This demonstrated which genotypes were to be used as controls in the final infection model, and it also showed a peculiar infection pattern in which bath infected fish had a very long incubation period of 3-4 weeks before starting showing signs of disease.

The infection model for EUS were developed in collaboration with the European Reference Laboratory for Fish Diseases, and included extensive work on in vitro methods on cultivation and storage of the pathogens as well as induction of sporulation for obtaining infective material. Further and in parallel, diagnostic tools were developed and validated, among others with tissue material obtained from the infection trials. The model is among others to be used to evaluate the risk of infection for native European fish species, with unknown susceptibility, therefore infection trials were done in three species of gourami, a type of aquarium fish from Southeast Asia, suggested to be naturally susceptible to disease. From this the species of choice, the three spot gourami, was decided to be used as positive infection control, and further validating trials were done to verify the model. Further it was noted that dwarf gourami was highly susceptible, a characteristic that was not characterized further, but could be of importance for refining the model, or for infection trials with low pathogenic strains of *A. invadans*. The model was used for testing susceptibility to disease of rainbow trout, a highly valuable aquaculture species in continental Europe. And from the infection trials several types of material were collected and stored for use as reference material.

4.1.4 The potential impact

WP2: NA1- Fostering joint management of resources and interchange of services

NADIR contributes to structuring the European Research Area and the way research infrastructures operate, evolve and interact with similar infrastructures and their users. The consortium brings together the 14 most significant research infrastructures in the field of animal infectiology and includes in addition PTP as a leader in farm animal specific molecular tools and UEDIN, which considerably developed in recent years. NA1 activities were of importance in this contribution as they were complementary of the NADIR management activities through facilitation of exchanges of resources, animal lines as well as reagents. NA1 is also highly dependent of research activities in the domain. Thanks to the NADIR partners research activities, the quantity and quality of resources made available to NADIR partners has been largely increased during the NADIR project period. Furthermore, most of these improvements are available for the whole scientific community as it has been demonstrated in realised transnational accesses and through the mobilization of resources .



Each NADIR partner has now an excellent knowledge of resources available from other European institute in the field. It thus allow the NADIR consortium partners to purchase the resources adapted to each scientific question at the right place through known experts in a short delay. As a consequence, this allows the mobilisation of reinforced competencies for any research topic in the domain of transmissible infectious diseases of farm animals and zoonosis, but it also allows a rapid mobilisation of expertise, resources and infrastructures in case of emergency. The links established through NA1 with projects of world-wide audience as the immune-tool project headed by MRI (UK) and USDA (USA) allows an extended panel of resources being made available to the European researchers. The commercialization of a number of reagents by IAH and MRI partners is also helpful in making reagents available to the enlarged scientific community from the public or the private sectors. The improved knowledge of each other, the enlargement of resources, and their availability from every partners contribute to the specialisation of facilities and avoid duplication of efforts.

High throughput technologies have been applied by several partners to the analysis of host immune responses, thanks to the microarray and sequencing tools developed through NADIR research activities by PTP (chicken, cattle), INRA (Fishes, chicken), IAH (pig) and MRI (sheep), as well as to the pathogen diagnostic, thanks to PTP, INIA, CVI and FLI activities. These new tools are complementary of improved classical diagnostic tools developed by other partners and now represent a considerably enlarged panel of technologies that can be applied for infectiology research in farm animals.

Sample banks have also been enlarged by NADIR partners and include a number of tissue samples infected by most farm animal pathogens. All farm animal species, including fish, are concerned. The collections also include wild species involved as reservoir of pathogens as badgers and falcons for mycobacteria, or other species of birds for influenza.

It now represent a considerable resource, that both include collection of pathogen isolates, positive control for tissue investigations as well as for diagnostic purposes as the samples collected include blood (sera and at a lesser degree blood cells) or milk and in some cases feces, urine or saliva.

Sustainability and even further improvement of the NADIR site is assured by INRA and PTP.

WP3: NA2 - Virtual college for improvement of animal infectious disease studies

Compared to the number of research institutes, which work with BSL3 organism in laboratory animals, only a relative small number of institute throughout the EU are working with highly virulent BSL3 pathogens in farm animals and fish. For these institutes, the NA2 network activity enabled expertise and knowledge exchange and facilitated the discussion on best practise, biosafety and biosecurity in the NADIR partner facilities. Important in these discussion was, that they were not only focussed on the technical view of a biosafety department, but involved the daily user, the animal scientist and also the ethic scientist. This broadening of the view resulted in an increased awareness of the problems, which arise during the work in such facilities. One example of this was the conflicting discussion between the preferable offer of straw bedding to animals versus the technical difficulties which arise from the use of straw for waste disposal. The harmonization of some procedures and the description of online available guiding documents on best practise procedures and biosafety procedures in farm animal facilities are based on the collaborative expertise and will contribute to improved environmental protection and occupational safety.

Inherent to the type of studies with highly pathogenic organism, it is important to critically assess them in regard to ethical and legal issues and also in the light of the modern requirements EU Directive 86/2010) on the use of animals. In the NADIR project specific ethical problems associated with these studies were discussed, awareness raised and in several deliverables solutions and guidance offered. The reports on pharmacological refinement and on humane endpoints in animal studies in general, but specifically in studies with high pathogenic organism will contribute to an



underpinned decision on the termination of a study and substantially contribute to one of the 3r criteria, i.e. the refinement of an animal study. By the dissemination of knowledge on the 3Rs by training and workshops also an increased awareness and critical dispute on the use of animals in BSL3 studies was stimulated between young, but also experienced researchers.

The discussions within the virtual college for the improvement of animal infectious disease studies has led to a better understanding between partner institutes on what is available, for example on animal models and how are certain problems handled. It is expected, that as an effect of this, the network activity contributes to an improved safety and security in European infrastructures, an optimized use of animals in studies and with an even higher degree of exchange to an improvement of scientific results and stronger emphasis on the 3Rs principles.

WP4: NA3 - Internal and External Communication

Several dissemination tools were developed within the present WP, whose main objectives were related to internal and external communication.

In order to enhance communication between partners and to promote collaborations with external partners and similar activities, a web-based electronic portal, the project newsletter, the blog-like website and specific activities carried out by the project participants were completed within the present WP.

In detail, in order to improve the efficiency of external communication, especially within the Animal Health Community, links with many related initiatives have been established and the blog-like website has been constantly updated; LinkedIn Groups (related to Animal Health Science) have been regularly used as a mean of dissemination and promotion of the project activities.

WP5 : RA1- Characterisation of animal lines

Successful achievement of the WP5 goals have required a significant synergy among research partners involved in NADIR project. To this end, this WP5 presents an excellent contribution with added value at the European level, in particular on the characterisation of animal lines as models of infection.

Specifically, one of the developments foreseen in NADIR WP5, included the validation of better methodologies for animal genotyping. In this way, the work done provides significant progress in genotype methodologies used for farm animals. In addition to the intrinsic value of the new procedures/test developed, their use have allowed a better discrimination among different genotype backgrounds and interestingly the identification of new alleles. These findings shall contribute to establish and strengthen the use of genotyping technologies for animal characterisation, reinforcing the expertise of European laboratories and establish leaderships in this key research area. Moreover, these findings contribute to increase the knowledge on alleles sequences, disseminated through public databases, ensuring the correct transmission of the progress made to the entire scientific community, not just European.

Likewise, the obtention of immune reagents for immunological characterisation of such animals, together with the establishment of validated procedures, contributes highly to the characterisation of such animal lines. Some of the immunological tools developed in this project have been transferred to European industry, contributing to the innovation of such companies and reinforcing their roles improving market opportunities.

The deep knowledge on genotyping and immunology of different animal lines shall contribute to a more efficient selection of the adequate animal model for the study of a particular pathogen. This finding contributes to the implementation of animal experimentation studies in agreement with a better animal welfare, reducing the number of animals per experiment , diminishing the cost of such experiences and avoiding the duplication of animal experiments.



Dissemination work of WP5 achievements have been done as early stage through NADIR website. Deliverables including the more relevant results obtained, with detailed description of the procedures followed and lists of animal lines characterised are available at such website. Two type of lists have now available for scientific community: i) List of animal lines characterised (genotyped, immunologically and sanitary) and ii) List of resistance/susceptibility of animal lines to different viruses, bacteria and parasites.

Likewise, peer-reviewed publications also played an important role in the dissemination of NADIR WP5 results. A total of 37 peer-reviewed papers were already published or accepted for publication and three more are under review.

WP6: RA2 - Development of infection monitoring tools

Imaging and telemetry

To date, frequently invasive techniques are used to study animal diseases. For example, the dissemination of pathogens through the body of an animal can only be established when an animal is killed and organs are examined for lesions and or presence of the infectious agent. Although well established, there are several disadvantages to these techniques. These include: large numbers of animals are required and all of them have to be sacrificed; follow-up studies are generally not possible in the same animal nor can multiple samples be obtained from the same animal; and drugs administered as prodrugs, requiring metabolism for efficacy, are difficult to study with destructive methods. Moreover, stringent ethical regulations and economic demands (research time and cost of genetically manipulated animals) urge researchers to use other methods that restrict the numbers of animals involved.

As an alternative, a number of non-invasive imaging and telemetry technologies have been developed in the last years. In NADIR some of these technologies have been further developed, customized for use in animal infectiology and tested. INRA bought a fluorescent and bioluminescent imager and showed that it was possible to follow the spread of bacteria and parasites through the body of small animals.

There are numerous other applications for optical imaging. There are a high number of possibilities to apply this technique: distribution and clearance of molecules in therapeutics (drugs, vaccines, vectors) or regulatory molecules (cytokines, small RNAs); distribution and quantification of receptors/ targets; follow up of a cell population; location and evolution of lesions (inflammatory or chronic) and gene expression.

Application of telemetry technologies have also advantages and positive impacts on various domains:

- ethics: no needs to restrain animals, reduced number of interventions,
- biosafety: reduced contacts with infected animals,
- scientific results: accurate measured values (no stress induced artefacts), no interference with experimental protocol, continuous recording 24/7,
- improvement of breeders' work: monitoring of calving, animal health surveillance.

In NADIR also several telemetry approaches were tested. This concerns especially equipment for measuring body temperatures in farm animals. Experiments were successfully performed in sheep, cows, pony's and chickens. All these tests have shown the possibility of using a wide range of thermometers adapted to various species and for various purposes.

Diagnostic tools

In NADIR novel molecular tools for monitoring infection processes were implemented.

There is a need to constantly improve and extend diagnostic, clinical and pathobiological tools using the relevant recent developments. Availability of these tools will optimise and improve the efficiency



of disease studies in animals, and thus serve the community at large. The development of extended routines and procedures for monitoring disease progression and host response in studies with micro-organisms will contribute significantly to the refinement and ultimately reduction of animals used in infectious disease experiments.

In NADIR novel diagnostic assays were developed either in singleplex or in multiplex format. Sensitive and specific diagnostic tests to detect livestock pathogens are essential for disease surveillance and to support cost effective and targeted deployment of control measures. Such diagnostic tools enable collection of data for input to impact assessment and modelling of health constraints to livestock production and both regional and international trade. Furthermore, it helps in monitoring and controlling disease outbreaks of infectious agents and to learn about the epidemiology of the disease.

Main dissemination activities and exploitation of results

Results have been widely presented at International Conferences and several articles have been published.

WP 7: RA3 - Development of infection models

The work performed during this project and in this workpackage has resulted in a collection of cell lines and tissues that can now be made available to researchers working in the field, predominantly NADIR partners. Any material exchange needs to be agreed on the basis of the owning institution's material transfer agreement (MTA). The list of available cell lines has been published during the first year on the NADIR website. The tissue samples collected during this project have also been summarized.

The work performed on vector borne diseases represents important contributions to the field of disease pathogenesis, transmissibility and vaccine development. The work has been summarized in numerous publications and thus made available to the scientific community.

Finally, the work in the field of aquatic diseases is highly valuable, since only a few groups are working with these diseases, while the aquaculture is rapidly gaining importance and relevance. Therefore the challenge studies performed with the agents of the most important endemic and exotic fish diseases that have been performed during the NADIR project represent important basics for future work on fish diseases.

CONCLUSION

Every objectives of the NADIR project have been fulfilled:

- Set up an Internet-based platform designed to organise collaboration between partners in a secure and optimal way and provide internal as well as external organisations with access to the network's infrastructures
- Strengthen the exchange of best practices, training, and the harmonisation of protocols
- Coordinate the management of material resources, including animal lines, tools developed in house to facilitate relationships with European project studying specific diseases
- Jointly undertake research activities aimed at upgrading the services provided by NADIR infrastructures: characterise animal lines, customise imaging technologies, develop monitoring molecular tools and develop new animal models.
- Conceive, structure and manage transnational access to the NADIR infrastructures.

Eighteen meetings have been organised to perform these tasks from May 2009 to December 2013.

Exchange of best practices has been the aim of three Biosafety/biosecurity meetings and three ethical standards meetings and have produced useful guidelines that have been or will be soon published. A range of training courses to improve the design of animal studies have been achieved and a database



compiled of the animal models available within the consortium. From these animal models, a number of samples were continuously cumulated in NADIR partners' sample banks, which now includes thousands of pathogen isolates, infected tissues and fluids that can be provided as controls for the development of research topics or new diagnostic tests.

The joint research activities included (i) Better characterisation of animal lines, (ii) Progress in detection and diagnostic of diseases, (iii) Development of improved or new models of infectious diseases. Chicken, sheep pig and fish lines have been genotyped and the susceptibility of different animal lines have been assessed after infections with different pathogens, mostly viruses but also prions, bacteria and parasites. Several new immunological reagents have been produced (recombinant cytokines, Mabs).

A telemetry monitoring meeting was organised (Tel Aviv, 2011) which allowed exchange of experience and allowed a number of devices to be successfully tested in pigs and ruminants to monitor the progression of infectious disease, thanks to collaboration with a private company. The NADIR consortium has now the possibility to follow the infectious process in small animals (mice, chicken) through imaging technologies that are available at the BSL3 level of confinement.

New diagnostic procedures using immunological tools and molecular technologies (microarrays for pathogens and their host for example) were developed as well as multi-diagnostic assays for monitoring infection processes in animals.

The NADIR scientists have responded to the newly emerging viral diseases such as Blue Tongue, Rift valley fever, Schmallenberg virus, African swine fever and African horse sickness, chicken salmonellosis and the three main salmonids' viral diseases. Accordingly, the collection and documentation of cell culture systems that are a prerequisite for most virological work procedures was developed. Challenge experiments with vector borne disease agents in different host species were performed in order to study their pathogenicity and to generate defined positive tissue samples. Up to August 2013, date of closing accesses, 60 proposals have been evaluated for TA accesses and 43 were realized among which two requested the collaboration of two infrastructures.

Finally NADIR has:

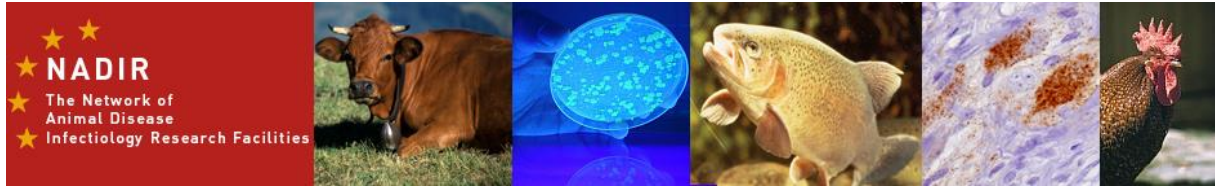
- Enhanced the collaboration between the most important European facilities dedicated to the investigation of farm animal diseases.
- Contributed to the global improvement of procedures taking into account biosecurity, biosafety and ethical issues
- Increased the use of new tools and new technologies for an improved follow up of experimental infections, optimizing the quality of results and consequently decreasing the number of animals used for improving our knowledge of infectious diseases.
- Reduced the "reaction time" of research departments and animal facilities in response to emergence of a new disease, as potential investigators know each other and are aware of available tools and resources throughout Europe.
- Offered improved facilities and improved resources, including the genetic and sanitary status of animal lines, to researchers from the private and public institutes. Their efficiency has been proved through the number and quality of transnational accesses from a wide range of European partners and approved by users.

4.1.5 Public website address

www.nadir-project.eu



4.1.6 Logo



4.1.7 Relevant contact details

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