TB-STEP FINAL PUBLISHABLE SUMMARY REPORT

A. EXECUTIVE SUMMARY

Tuberculosis in livestock is an infectious disease caused by microorganisms of the *Mycobacterium tuberculosis* complex. This mycobacterial infection represents a major concern worldwide because of its high economic impact due to mortalities, condemnations, decreases in productions, and its zoonotic potential. Eradication programmes based on a test-and-slaughter policy have been implemented for many years in the European Union. This scheme proved to be successful in some countries, however, has been unable to eradicate the infection in others despite the use of vast economical and human resources. In some countries, infection is endemic while in others sporadic outbreaks are still detected involving several properties and posing significant challenges to its elimination. To approach the eradication of this infection, the TB-STEP Project designed a multifaceted battlefront. The consortium was made up of 12 partners from eight countries which research on eight work-packages included in 5 thematic areas (Control of mycobacterial diseases through vaccination, improved diagnostic tools, wildlife management, epidemiology and risk assessment) devoted to improved tools and to develop strategies for the eradication of bovine tuberculosis in areas where the disease is present in both domestnic and wildlife populations.

Regarding **vaccination**, significant progress in developing TB vaccines for domestic animals (cattle and goats) has been made as well as developing DIVA reagents for bovine tuberculosis. In the case of UK, Ireland and Spain there is a growing body of evidence to implicate infected wildlife (badgers and wild boar) in the maintenance of tuberculosis in associated cattle. In this project progress in vaccination of wildlife has been made (delivery systems, vaccine/challenge protocols, monitoring disease, experimental model in ferrets, infection dose, type of vaccine, biosafety issues, biomarkers, role of environmental atypical mycobacteria, etc.).

The eradication campaigns are based in detection of the higher number of infected animals and therefore the **improvement of the diagnostic tools** was a key objective for the TB-STEP project. Progress has been made in this area developing new glycolipids antigens of *M. bovis* to validate an ELISA and defining their potential role as candidates for a vaccine. Moreover, a multi-species serological test for TB-antibodies detection (MPB83), including wild animals, has been developed as well as IFN-γ assay for other species (ie. pigs). An improved version of BOVIGAM®, BOVIGAM® 2G was developed with better reproducibility, repeatability and robustness. Studies to determine the sensitivity and specificity of the current oficial diagnostic tools (skin test and IFN-γ assay) have been performed in cattle and goats. And the impact of non-tuberculous mycobacteria in the outcome of these diagnostic tests was also studied.

The TB-STEP project has significantly contributed to confirm the role of **wildlife hosts** in bTB maintenance and that disease control strategies need to be integrated and consider both compartments. This project has contributed to (1) improved wildlife population monitoring and disease monitoring; (2) better understanding of wildlife TB epidemiology and identification of wildlife-related risk factors for bTB maintenance; and (3) development of wildlife disease control tools (ELISA TB test for wild boar, oral bait, selective delivery cage, and MdR vaccine).

Molecular typing of *M. bovis and M. caprae* isolates is a helpful tool to improved knowledge of epidemiology of animal tuberculosis that can be applied to the design and monitoring of the eradication campaigns; this would result in better use of resources and would reduce public health risks. Improvement of predictive power of **epidemiological and risk models of bTB** was one of the objective of this project mainly to study the (1) risk based bTB surveillance to protect and reduce the incursion risk in bTB-free areas; and (2) increased efficiency of bTB control through improved predictive power of epidemiological models used to inform the development of bTB control strategies.

B. A SUMMARY DESCRIPTION OF PROJECT CONTECT AND OBJECTIVES

Tuberculosis in livestock is an infectious disease caused by microorganisms of the *Mycobacterium tuberculosis* complex. This mycobacterial infection represents a major concern worldwide because of its high economic impact due to mortalities, condemnations, decreases in productions, and its zoonotic potential. Eradication programmes based on a test-and-slaughter policy have been implemented for many years in the European Union. This scheme proved to be successful in some countries, however, has been unable to eradicate the infection in others despite the use of vast economical and human resources. In some countries, infection is endemic while in others sporadic outbreaks are still detected involving several properties and posing significant challenges to its elimination.

The most relevant problems have been assumed to be caused by the existence of infected wildlife. Thus, it is considered that the role of wild animals in the maintenance and spread of *M. bovis* infection in domestic livestock is of particular importance in countries where eradication programmes have substantially reduced the incidence of bovine tuberculosis but where outbreaks still occur. In this sense, the best known examples are the European badger (*Meles meles*) in United Kingdom and the Republic of Ireland, and the wild boar (*Sus scrofa)* in Spain. Therefore, strategies to control wildlife infection in these areas are needed.

Besides this fact, there is only a limited knowledge about other potential underlying causes, such as (1) the real contribution of cattle-to-cattle transmission at the same area (neighbouring farms and communal pastures) or after movement of animals; (2) the role played in the epidemiology by other domestic animals, or (3) the effect of interferences in the diagnosis tests. The weight of these causes may also differ depending on the farming system and ecological factors.

The project aims at the design of rational strategies to achieve eradication of tuberculosis from livestock and wildlife. The research is focused on topics that will have a deep impact on the understanding and control of the diseases in a short future. The five thematic areas have been designed to share technology and expertise in order to both avoid research fragmentation.

The project is divided into five thematic areas which contain eight work-packages according to the different topics covered.

Thematic area 1. Management.

- **Work Package 0**. Management, dissemination and web-page.
    - Objectives**:**
        - Overall management of the Consortium.
        - Dissemination of the TB-STEP activities to the Consortium, Scientific Community and to general audience.

Thematic area 2. Control of mycobacterial diseases through vaccination.

- **Work Package 1**. Vaccination of domestic animals (cattle and goats): Assessment of protective efficacy in cattle of a heterologous prime-boost strategy based on BCG and a recombinant adenovirus expressing a fusion protein of protective antigens, and to assess the suitability of novel diagnostic reagents to distinguish vaccinated from infected animals (DIVA principle).
    - Objectives:
        - To generate a novel recombinant adenovirus vector expressing three protective *M. bovis* antigens

- To test its immunogenicity and ability to improve the efficacy of BCG in cattle using an heterologous prime-boost protocol
- To demonstrate that DIVA reagents developed in VLA to distinguish BCG vaccination from *M. bovis* infection can also be applied to this prime-boost scenario
- To determine safety and efficacy of *M. bovis* vaccine in natural and experimental infected goats by laboratory and field studies
- To assess the safety and efficacy of live and inactivated *M. bovis* (BCG) vaccines in experimentally infected goats
- To assess the safety and efficacy of live and inactivated *M. bovis* (BCG) vaccines in goat farms in a field study (depending on results obtained in former objective).

- **Work Package 2**. Evaluation of vaccines for use in wildlife populations in the European Union.
    - Objectives:
        - to evaluate the different strategies for vaccination of wildlife against *M. bovis*
        - to compile the results obtained with different vaccines and animal species to make recommendations to the Scientific Community
        - to determine the efficacy of oral vaccination in captive badgers
        - to develop a reliable bait delivery system for oral vaccination of badgers against tuberculosis
        - to recommend adaptations of oral vaccine for other wildlife species
        - to develop an experimental infection model in ferrets to evaluate protein sub-unit vaccines
        - to investigate the delivery of efficacious vaccines, in a manner suitable for use in a wild population
        - to evaluate the immune response in wild boar after oral vaccination
        - to study the safety of vaccination of wild boar for environment and the consumers
        - to disclose the effect of experimental atypical exposure on the level of protection of BCG vaccination.

Thematic area 3. Improved diagnostic tools.

- **Work Package 3**. Development of new glycolipid antigens of *M. bovis*.
    - Objective:
        - Isolation and characterization of antigenic *M. bovis* glycolipids as antigens for immunological diagnostic tests and/or vaccine candidates for bovine tuberculosis.
- **Work Package 4**. Improvement of immunology-based diagnosis in wildlife and livestock.
    - WP4a. Development of a tuberculosis antibody detection assay.

- Objectives:
  - to develop an affordable serological test for use in wild animals
  - to evaluation of its sensitivity and specificity
  - to clone and express specific and sensitive tuberculosis antigen
  - to develop, identify and characterise monoclonal antibodies (MAbs) specific of tuberculosis selected antigen
  - to develop immunoassays for interferon-g detection of different species.

- WP4b. Sensitivity and specificity of the antigens for the IFN-γ test.
  - Objectives:
    - to evaluate the performance of the new antigens regarding specificity and sensitivity in livestock
    - to increase the test specificity and study the effect of other mycobacteria in the performance of the immunological tests
    - to assess the effect of the paratuberculosis infection in the diagnosis of tuberculosis
    - to improve the practical use of the interferon-gamma test.

Thematic area 4. Wildlife management.

- **Work Package 5**. Control of populations.
  - Objectives:
    - to develop new management tools to identify wildlife overabundance and to monitor the effect of management changes on both population and disease indicators
    - to design adequate risk-control measures for each particular situation
    - to perform risk factor analysis of current wildlife management practices in Mediterranean habitats, including specifically the risks associated to carrion consumption
    - to analysis the ecology of wildlife feeding and watering in Mediterranean ecosystems, and the possibilities of reducing the associated disease transmission risks
    - to develop and characterize oral bait delivery methods to European wild boar and Iberian red deer under Mediterranean field conditions.

Thematic area 5. Epidemiology and risk assessment.

- **Work Package 6**. Molecular typing of *Mycobacterium bovis and M. caprae* isolates: focused epidemiological investigation.
  - Objectives:
    - to determine the relative contribution of each factor in the transmission of infection

        - to determine the effect of agricultural system and farm management practices on the infection in domestic livestock
        - to evaluate the real role of wildlife as reservoirs of the infection
        - to study the role of other domestic animals in the maintenance of the infection
        - to evaluate the recommended methods for molecular typing
        - to compare performance of Variable Number Tandem Repeat (VNTR) - mycobacterial interspersed repetitive units (MIRUs) with the new spacers for DVR-spoligotyping for *M. bovis* typing
        - to study the epidemiology of *M. caprae.*
- **Work Package 7**. TB risk and control in EU systems.
    - Objectives:
        - to quantify the role of badger vaccination in integrated tuberculosis eradication strategies
        - to provide an EU perspective on the role of wildlife in the persistence of tuberculosis in problem areas and develop best practice tuberculosis control strategies for problem areas
        - to quantify the effects of farming system on livestock exposure to tuberculosis from wildlife in the EU to inform strategies to limit wildlife-livestock contact.

C. A DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS

The description of the main S&T results/foregrounds will be divided in the different workpackages.

WP1. Vaccination of domestic animals (cattle and goats).

Over the last two decades the tuberculin test and slaughter strategy failed to prevent a dramatic rise in the incidence of TB in cattle in England and Wales. The urgency for new and improved cattle vaccines and diagnostic reagents has been acknowledged by the United Kingdom government. The only potentially immediately available TB vaccine is Bacillus Calmette-Guerin (BCG), the vaccine used to vaccine humans against tuberculosis. Whilst there can be no doubt that BCG can protect animals including cattle against bovine TB - indeed this has been known since 1912 - BCG has shown some of degree of efficacy variability to protecting cattle to a similar degree than in humans (reviewed by Waters *et al.*, 2012). One largely unknown quality of BCG in cattle is how long the duration of immunity will be following BCG vaccination. Recent studies using a stringent experimental challenge model have indicated that protection may last only 1 year (Thom *et al.*, 2012), whereas a study undertaken under field conditions indicated that protection may last at least 22 months (Ameni *et al.*, 2010). Thus, this identifies challenges that one is faced to improve on BCG vaccination: to increase its efficacy per se and to extend the duration of the immunity it induces. An additional challenge associated with the use of BCG in cattle is that its use in cattle and other domestic animals will require the development of a diagnostic test that can be used alongside vaccination to differentiate vaccinated and infected cattle (DIVA test). This work package is aimed to address these challenges. Conceptually there are two ways of improving BCG efficacy and/or extend it duration of immunity. Firstly, one could increase immunity (and by inference also it duration) at the time of primary vaccination by amplifying or boosting BCG induced immunity, or secondly, one could boost immunity at the time point of waning below non-protective levels.

Significant progress in developing TB vaccines for **cattle** has been made and it has been shown that the most effective vaccination strategy against bovine TB is based on priming the immune system with BCG followed by boosting with subunit vaccines containing protective antigens that are present in BCG (heterologous prime-boost strategy). The most consistently successful approach has been the use of virally vectored booster vaccines (Vordermeier *et al.*, 2009). The data generated over the last 5 years at AHVLA (DEFRA, UK) demonstrated that a replication-deficient recombinant human type 5 adenoviral vaccine expressing the mycobacterial antigen Ag85A (Ad-85) consistently improved BCG efficacy when applied in a BCG-prime/adenoviral booster vaccine scenario (Vordermeier *et al.*, 2009 and unpublished data). This strategy is in line with current approaches conducted in the human TB vaccine programmes with several of such vaccines in human clinical trials. Nevertheless, all these strategies resulted in at least a proportion of animals becoming tuberculin-test positive (both in tuberculin skin and IFN-γ tests). The aims of this work package in relation to cattle were therefore aimed improving BCG protection by producing and testing a multivalent recombinant adenoviral booster vaccine.

Strategies to developing **DIVA reagents for bovine tuberculosis** that would be applicable alongside BCG vaccination are based on the search for antigens whose genes are deleted from the BCG genome such as the RD1-encoded antigens ESAT-6 and CFP-10. Additional antigens are based on their secretion by *M. bovis* but not by BCG, such as Rv3615c (Sidders *et al.*, 2008). Thus, the development of complementary diagnostic reagents allowing the discrimination of infected from vaccinated animals remains an essential requirement so that a test and slaughter approach to TB control can be continued in the face of vaccination.

Domestic goats are another domestic livestock species highly susceptible to *M. bovis* and *M. caprae* infection and can therefore pose a risk to human health and constitute a reservoir of infection for domestic cattle and wildlife. Very few efforts to validate the

control measures used in bovines have been done in goats. Therefore a major goal of this workpackage was to apply the same vaccination and DIVA diagnostic strategies developed for cattle also to goats. Undertaking this task involved the development of a goat *M. caprae* vaccination and challenge model including the establishment of a novel way to determine protection in the lung (computer tomography), and harmonisation of methodologies between the cattle and goat models. This was achieved by the close collaboration and interactions between the laboratories in GB (partner 4) and Spain (partner 1).

This work package was divided into the following objectives: a) to generate a novel recombinant adenovirus vector expressing three protective *M. bovis* antigens; b) to test its immunogenicity and ability to improve the efficacy of BCG in cattle using an heterologous prime-boost protocol; c) to demonstrate that DIVA reagents developed in VLA (partner 4) to distinguish BCG vaccination from *M. bovis* infection can also be applied to this prime-boost scenario; d) to determine safety and efficacy of *M. bovis* vaccine in natural and experimental infected goats by laboratory and field studies; e) to assess the safety and efficacy of live and inactivated *M. bovis* (BCG) vaccines in experimentally infected goats; and f) to assess the safety and efficacy of live and inactivated *M. bovis* (BCG) vaccines in goat farms in a field study (depending on results obtained in former objective).

These objectives were achieved in full and thus the TB-STEP project has made significant progress in the **development of TB vaccine for livestock species such as cattle and goats**. Specifically, a recombinant attenuated adenoviral vaccine was produced that expressed the 4 protective mycobacterial antigens Ag85A, TB10.4, TB9.8, and Acr2 (Ad-TBF). This construct was based on the human adenovirus type 5. In a pilot experiment, the immunogenicity of this vaccine was determined in cattle. This experiment was also used to determine the optimal vaccination route and dose. Its outcome established that the intradermal route with a vaccination dose of 2 x 10^9 ifu induced the strongest IFN-γ responses specific for all 4 vaccine antigens.

Consequently, this dose and route were used to vaccinate calves in a second experiment designed to determine whether this vaccine, by boosting BCG primed responses, was able to induce protective immunity superior to that induced by BCG alone. A further group of calves were BCG primed followed by boosting with an adenoviral virally vectored vaccine expressing Ag85A alone (Vordermeier *et al.*, 2009) as additional control group (Ad-85A). The outcome of this experiment confirmed that both Ad-85A and Ad-TBF induced protection better than BCG vaccination alone although only the effects seen with Ad-85A were statistically significantly better than with BCG alone when protection was determined by the assessment of granuloma formation. However, the relatively small group sizes (which are mainly governed by the availability of BL3 challenge facilities for large animals such as cattle) meant that this study was of relatively small power. Therefore, to determine whether Ad-TBF is superior or equal in providing protection than Ad-85A will need to be determined in additional and potentially larger experiments.

These experiments also presented the opportunity to test the prototype DIVA reagents ESAT-6, CFP-10 and Rv3615c (Sidders *et al.*, 2008). The application of Rv3615c improved the sensitivity of ESAT-6 and CFP-10 by detecting animals that are ESAT-6/CFP-10 negative whilst not compromising specificity in either naïve or BCG vaccinated cattle. Thus, the same antigen preparations were also tested in the animals used in the vaccination and challenge experiment described in the previous paragraph. Blood samples were collected following vaccination and again after *M. bovis* challenge. Blood samples were stimulated with the DIVA antigens and IFN-γ responses compared to those after stimulation with avian and bovine PPD. The data demonstrated the high specificity of the DIVA antigens following BCG vaccination and adenoviral, which in contrast to tuberculin was not compromised by vaccination. Test sensitivity was also determined after *M. bovis* infection and the data generated confirmed not only the high sensitivity of these reagents in non-vaccinated/ *M. bovis* infected cattle but also that these test sensitivities were also

maintained in vaccinated/*M. bovis* infected animals and that these specificities are approaching those of tuberculin PPD.

These vaccination and DIVA approaches were also applied to a second domestic ruminant species, goats. To undertake this work, an experimental infection model was established and characterized based on infection of goats via the endobronchial route with *M. caprae*. This method allows the reproducible infection of goats with a therapeutic range large enough to determine vaccine-induced protection. The method, akin to that used in the cattle experiments described above was refined by introducing Multi-Detector Computed Tomography (MDCT) and 3D-imaging to assess the degree of lung pathology.

This model was then used in two independent experiments to determine the protective efficacies of BCG vaccination on its own compared to heterologous prime-boost vaccination using the same adenovirally-vectored vaccines described above used to vaccinate cattle (Ad-85A and Ad-TBF). Compared to unvaccinated controls, BCG was able to protect goats against infection by significantly reducing the observed pathology and bacterial loads. Moreover, boosting of BCG with Ad-85A or Ad-TBF improved protection considerably and significantly further than BCG vaccination alone, thus extending the results observed in cattle to another target species.

The DIVA reagents described in previous section for cattle were also tested in goats. This analysis was also extended to determine their performance in the face of vaccination against Johne's disease (caused by *M. avium paratuberculosis*) which is licensed and used in goats. The results demonstrated that in contrast to tuberculin-based reagents, these DIVA reagents are not compromised by BCG or BCG/adenoviral subunit or vaccination against Johne's disease. In addition, low responses to these DIVA antigens in BCG/adenovirally vaccinated goats are indicative of protection (correlate of protection).

In conclusion, this work package has addressed and delivered all objectives, milestones and deliverables. Specifically, the proof concept data generated in cattle demonstrating that heterologous prime-boost approaches using BCG and virally vectored subunit vaccines improved BCG protective efficacy were confirmed and extended. Further, the results described above demonstrated that this approach can also be applied to another domestic ruminant species, goats. Lastly, the application and superior specificities of novel DIVA tests compared to tuberculin-based reagents were confirmed and extended to cover both target species. Therefore, the outcomes of this work package have highlighted and underscored the potential of vaccination combined with suitable DIVA tests as effective component of innovative bovine TB control strategies aimed at domestic ruminant target species of bovine tuberculosis.

WP2. Evaluation of vaccines for use in wildlife populations in the European Union.

In many EU countries, the presence of tuberculosis in wildlife poses a threat to domestic livestock and contributes to economic hardship for farmers. Where wildlife has been shown to be a reservoir for transmission of infection, it is unlikely that current surveillance testing of livestock will be sufficient to eradicate the disease. This is particularly the case in the UK, Ireland and Spain where there is a growing body of evidence to implicate infected wildlife (badgers, wild boar) in the maintenance of tuberculosis in associated cattle. A strategic approach involving vaccination offers the opportunity of reducing the prevalence of tuberculosis in wildlife. Given the constraints involved with working with wildlife, it is likely that any mass vaccination campaign will utilize an oral uptake of vaccine. The development of oral delivery systems for vaccination is technically challenging but feasible means of targeting wildlife.

WP2 addressed tuberculosis in a number of wildlife species including badgers, wild boar and ferrets. The **badger** studies conducted by Partner 3 (NUiD) focused on refining the experimental vaccine/challenge protocol to evaluate the efficacy of oral vaccination against low-dose challenge with *M. bovis*. A group of badgers was vaccinated (BCG-lipid

mixture) and twelve weeks after all the badgers in both groups (vaccinated and non-vaccinated controls) were challenged with 300 CFU of *M. bovis*. At 52 weeks post-infection all the badgers were examined *post mortem* to assess the pathological responses to challenge. Regarding pathology, when compared with the control sham-vaccinated group, the vaccine group had significantly fewer (MW test, $p = 0.05$) lesions than the control group. In addition to the number of gross lesions observed, the severity of the lesions in the vaccine group was significantly lower than in the control group (t test, $p < 0.05$).

The cell mediated immunological responses in PBMC of all badgers were monitored *in vitro* by the lymphocyte transformation assay and IFN-γ ELISPOT assay using PPD-Bov and CFP10 as stimulating antigen. The response profiles of both vaccinated and control groups were similar using either assay and were consistent with the observed severity of pathology at the end of the study in both groups. Antigen specific responses were observed following vaccination with BCG. Following infection, there was a rapid increase in CMI responses to PPD-bov that peaked between five and eight weeks post-challenge in vaccine and control groups, respectively. A similar profile was observed in response to stimulation with CFP10, however the peak of activity was measured 14 weeks post infection. Thereafter, the CMI responses declined in both groups but remained higher than the pre-infection levels. When the data was evaluated in the context of the lesion score, it was observed that the highest responses were measured in those badgers, in both groups, with the highest lesion severity scores.

The serological responses of each badger were measured throughout the course of the study by the badger StatPak lateral flow immunoassay. Six of the nine control badgers were positive in StatPak during at least one time-point. In contrast, only three of nine vaccinated badgers showed evidence of seroconversion. Seroconverion appeared to be associated with severity of lesions, particularly in the vaccinated group; the three badgers that seroconverted in this group had the highest lesion score at post-mortem.

In summary, orally delivered lipid-formulated BCG vaccine induced a significant protective response in the vaccinated badgers when challenged with a low dose of *M. bovis*. The results are consistent with those obtained following subcutaneous and mucosal vaccination of badgers with BCG, and are also consistent with the development of a protective response obtained when lipid-formulated BCG vaccine was studied in mice possums guinea pigs and cattle. In developing and testing the low-dose challenge model, partner 3 (NUiD) was able to demonstrate that the disease produced was similar to that in naturally infected badgers. In the majority, the infection was mild and there was limited dissemination of infection to extra-thoracic sites in badgers of, particularly in the vaccinated group.

Badgers and ferrets are closely related members of the *Mustelidae*, with the advantage that ferrets are an available source of laboratory animals. A specific task involved the development of an experimental vaccine-challenge-model in **ferrets** (*Mustela furo*) to evaluate protein sub-unit vaccines (Partner 6, QUB). This novel model was based on the Madison aerosol chamber to deliver a precise dose of *M. bovis* (AF2122/96) to the lungs of ferrets. An infection dose was defined and used to test the vaccine H56 comprising Ag85B-ESAT-6-Rv2660c for use with CAF01 as the adjuvant, a protein sub-unit vaccine (SSI, Denmark) and compared with BCG vaccination.

Regarding infections dose, data suggested that at the lower infectious dose, at the longer time period, ferrets were controlling the infection compared to the higher dose. Although a number of tubercule-like lesions developed, histology was not conclusive, so lesion score and histology were not deemed reliable measures of infection. Following vaccination with BCG and challenge with *M. bovis*, 7/8 ferrets were infected with *M. bovis* but this finding was complicated by recovery of BCG from 7 ferret tissues. *M. bovis* was recovered from all 8 ferrets in the H56 vaccine group with 19 tissues culture positive. In the BCG-H56 co-vaccination group 6/8 ferrets were *M. bovis* positive, with a total of 11 culture positive

tissues. In contrast, all 8 ferrets in the non-vaccine group were culture positive with 26 tissues positive. The bacterial load was greatest in the non-vaccinate group (3.3 Log_{10}) compared to the other groups (1.5-2.5Log_{10}). The persistence and distribution of BCG in the vaccinated groups was somewhat surprising and VNTR analysis distinctly indicated the presence of BCG in certain tissues, in the absence of AF2122/96, but in addition, some tissues were co-infected with both the vaccine and challenge strains.

This study has developed a new understanding of *M. bovis* infection in ferrets. Delivery of aerosolised *M. bovis* initiates infection in the lungs and lung lymph nodes. However, there is significant spread of infection from the lungs to the liver, kidney and spleen. In addition, there is substantial progress of infection to the mesentery lymph node which, in a dose dependant manner, can induce faecal excretion. This infection model was further developed to evaluate a novel vaccine (H56) in comparison with BCG. Under challenge with *M. bovis*, vaccine efficacy was measured and was found to reduce the numbers of tissues infected and the total bacterial load.

In Mediterranean habitats of the Iberian Peninsula, the abundant and widespread native **Eurasian wild boar** (*Sus scrofa*) is the main reservoir host for *M. bovis*. It is likely that wild boar vaccination will be necessary for tuberculosis control in this region. In the studies carried out by Partner 2 (UCLM), oral vaccination with live Bacillus Calmette Guérin (BCG) in captive wild boar resulted in significant levels of protection against challenge with virulent *M. bovis*. The safety of delivering BCG-containing baits to wild boar, even at high doses, was also confirmed by Partner 2 (UCLM). A new heat-killed *M. bovis* vaccine showed that oral or parenteral vaccination with heat-inactivated *M. bovis* conferred a similar protection after challenge when compared to oral vaccination with BCG, and that the response of wild boar to both vaccines was similar. Although a high challenge dose was used (10^6 cfu), this vaccination protocol reduced the number and severity of lesions and the infection burden, particularly in the thoracic region. The dynamics of antibody production, IFN-gamma response and gene expression were similar in oral BCG- and inactivated *M. bovis*-vaccinated animals. Wild boar parenterally vaccinated with the inactivated vaccine responded to the MPB83 antigen but not to bPPD immediately after vaccination, suggesting potential use of these ELISAs to distinguish between parenterally vaccinated and exposed wild boar (Garrido et al., 2011). Moreover, Partner 2 studied the four main biosafety issues that must be considered before delivery of oral baits containing live vaccines such as BCG to wild boar: (1) potential effects of high vaccine doses (e.g. ten times the normal dose) on wild boar health; (2) potential survival of *M. bovis* BCG in vaccinated wild boar; (3) potential excretion of *M. bovis* BCG by vaccinated wild boar; (4) vaccine-containing bait uptake by non-target species, particularly by cattle.

The study of host-pathogen interactions by Partner 2 (UCLM) allowed identification of biomarkers of resistance/susceptibility to tuberculosis in wild boar. The expression of genes including complement component 3 (C3) and methylmalonyl coenzyme A mutase (MUT) was shown to correlate with resistance to natural *M. bovis* infection and protection against *M. bovis* challenge in vaccinated wild boar. Different baits were developed by Partner 2 (UCLM) and used for the oral delivery of vaccines and pharmaceuticals to wild boar or pigs. However, bait consumption rates and host specificity depend on the delivery method employed.

This WP also included a task to understand the role of **environmental atypical mycobacteria** in the reduction of the efficacy of vaccines in wildlife (Partner 12, UP) a problem under specific geographical areas that remains largely unstudied. This is a particular concern in southern Africa, where bovine tuberculosis in cattle and African buffalo affects communal and commercial livestock production and critical wildlife conservation programs. Partner 12 (UP) set out to evaluate the kinetics of the cytokines involved after presensitisation of *M. bovis* challenged mice with atypical mycobacteria including the slow growing opportunistic pathogen *M. kansasii* and the fast growing opportunistic pathogenic *M. fortuitum*. Their investigations showed that the balance in

expressions of classic Th1 cytokines (TNFa, IFNg) and Th17 cytokines may be pivotal in determining the immunological pathways required for immune protection.

WP3. Development of new glycolipid antigens of *M. bovis*.

Mycobacterial glycolipids (GL) play an important role in both the virulence of pathogenic mycobacteria as well as in the immune response of the infected host. This has been known more than 70 years but certainly in recent decades, the scientific exploration of glycolipid antigens has been neglected in comparison to the study of mycobacterial protein antigens. Using recombinant DNA techniques the analysis of proteins has become easily accessible, whereas the isolation and functional analysis of (glyco)lipids, posing greater technical difficulties thus requiring greater efforts, has been neglected, leading almost to the extinction of the available expertise in this area.

Nevertheless, there are a large number of indications that (glyco)lipids play an important role in pathogenesis of mycobacteria and are therefore potential candidates for both the development of diagnostic tools as well as the development of vaccines (for example, tuberculins, still a major diagnostic antigen, consist for about 20% of lipid material). Furthermore, the establishment of a LAM-based ELISA for *M. avium* subsp. *paratuberculosis* performing equally well as protein based conventional ELISAs gives another evidence for mycobacterial lipids as potential diagnostic antigens. In addition, there are numerous reports on the involvement of (glyco)lipids on the interaction of mycobacteria with a variety of T-cells of the immune system of the hosts.

Wildlife, such as wild boar and deer often exhibit a strong immune reaction against antigens of mycobacteria from the *M. avium* complex such as *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis* and *M. avium* subsp. *avium*. This is in addition to a non-specific response against a wide variety of atypical mycobacteria from the environment. Therefore diagnostic tests for *M. bovis* will suffer from **non-specific responses** if they are based on common mycobacterial antigens. Some mycobacterial lipid antigens however have been shown to be species-specific. It was found that a pentapeptidelipid (LPx) (Willemsen *et al.*, *in preparation*), highly similar to the species-specific L5P or para-LP-01 (Biet *et al.*, Eckstein *et al.*), is only present in *M. avium* subsp. *paratuberculosis* and could be used in a prototype ELISA. Another earlier findings showed that glycopeptidolipids (GPL) present in the cell envelope of *M. avium* subsp. *avium* define the different *M. avium* subsp. *avium* serotypes. In the course of this project it was shown that GPL4 has a potential use in diagnostics for *M. avium* subsp. *avium* infection.

Based on this foreground the objective of this work package was the isolation and characterization of antigenic *M. bovis* (glyco)lipids as antigens to be used in immunological diagnostic tests and/or as vaccine candidates for bovine tuberculosis.

The first objective (task 3.1. Isolation and characterization of **antigenic GL's for *M. bovis***) concerns the collection of (glyco)lipid antigens for *M. bovis*. This objective was met by the isolation of 87 lipids from 5 different *M. bovis* strains. Out of these lipids, 62 lipids were isolated from *M. bovis* strain AN5 (the strain worldwide used for the production of bovine tuberculins) and 25 lipids from different *M. bovis* field isolates. From this collection of *M. bovis* lipids a panel of potentially antigenic lipids was assembled, using a prototype ELISA in combination with pooled sera, obtained from infected animals. This panel was made available to other partners in the consortium.

The subsequent validation of **(glyco)lipid based ELISA and T-cell diagnostic tests** were investigated as task 3.2. As a first step, the best conditions were developed enabling lipids to be tested in an ELISA. When comparing a wide variety of polystyrene microwell plates, all exhibiting different binding characteristics, it was observed that when lipids were dissolved in methanol prior to coating, using Nunc "polysorb" microwell plates best results with regard to sensitivity and specificity were obtained. Additional steps in the ELISA protocol could be kept conveniently identical to the already available protein antigens

based ELISA protocols, except for the use Tween-80, which had to be omitted in washing steps.

In the course of this project an important laboratory tool for screening lipids for antigenicity was further developed. **Immuno-Thin Layer Chromatography (TLC)** can be used for the detection of specific antigenic lipids in crude lipid fractions, by showing specific binding of antibodies, present in sera from infected animals to those antigenic lipids after separation on TLC. Thus far however, all available protocols from literature for this method require large amounts of lipids and therefore either suffer from a low sensitivity or would require the expensive and time-consuming isolation of vast amounts of biomass of mostly slow growing mycobacteria for the isolation of the crude lipid fractions. Due to these complications, immuno-TLC therefore is only occasionally used for confirmation of antigenic properties of lipids.

The candidate lipid antigens were tested in a **prototype ELISA** using a large number of sera obtained from naturally *M. bovis* infected cattle and were compared with sera obtained from experimental infected cattle. Data shows that there were no significant differences in the magnitude of responses. Using 10 sera from natural infected cattle from AHVLA (partner 4) confirmed the antigenicity of the same set of lipids. However using sera from *M. avium* subsp. *paratuberculosis* infected cattle showed overlapping responses. Therefore it was concluded that the antigenic *M. bovis* lipids from the collected panel cross-reacted with sera from *M. avium* subsp. *paratuberculosis* infected cattle. In serology, none of the isolated *M. bovis* lipids could be shown to be unique for *M. bovis* with respect to their antigenic properties. Whether the lipids are not unique for *M. bovis* or whether different mycobacterial lipids harbour the same antigenic epitopes is unknown.

Given the fact that serology only has a very limited value with respect to *M. bovis* diagnostics caused by the general low observed humoral responses of infected animals (at least without prior skin testing, which certainly does not routinely take place in wildlife), more emphasis was put on the usefulness of lipids for diagnostics of cell mediated immune (CMI) responses during the course of the TB-STEP project. At the AHVLA (partner 4) a set of lipids was tested for CMI responses with ten reactors (naturally infected) by a proliferation assay, by means of incorporation of tritiated thymidine after stimulation by the lipid of PBMC's derived from reactor cattle. The lipids were first tested in suspension but in a second test (again with 10 reactors) lipids were tested after they were coated to microwell plates. Binding of lipids to microwell plates decreases the signal. Repeating the stimulation experiment using lipids in suspension with a smaller group of three reactors showed, despite a limited repeatability, several lipids such as lipid 10, 14 and PIM2 to stand out and being able to induce significant CMI responses. In a parallel study by partner 6 (QUB) another set of lipids was tested in two groups of experimentally infected cattle using the gamma interferon (IFN-γ) release assay (Bovigam assay). Again the results show that several of the lipid antigens are able to induce an IFN-γ response but none of them were specific for *M. bovis* infected cattle only.

In conclusion, a considerable number of the *M. bovis* derived lipids were shown to be antigenic both as B-cell and/or as T-cell antigens, but most lipids will show a high ***M. avium* subsp. *paratuberculosis* background** in both tests, limiting their use in diagnostic assays. Since serology is regarded to be of very limited use in the diagnosis of *M. bovis* infection, certainly without prior skin testing, measuring T-cell mediated immune (CMI) reponses remains the only option. However, CMI testing of lipids will need to be improved further, in particular with regard to repeatability. Improving the repeatability as well as a further analysis of the antigenic properties of the mycobacterial (glyco) lipids will be an important part of the new 2012 EMIDA project Mycobactdiagnosis.

In addition, even though the antigenic (glyco)lipids characterised thus far were shown to have only a limited specificity, thus hampering their use in a discriminatory diagnostic assay, a large number were shown to be able to provoke a significant CMI response. This makes these antigenic (glyco)lipids potential candidates for the development of a

vaccine against bovine tuberculosis, either alone or as part of such a vaccine. Their limited specificity would be less of a problem, given the fact that for the diagnosis of (bovine) tuberculosis sufficient potent protein antigens (e.g. ESAT-6, CFP-10) are available for use in a discriminatory diagnostic assay (DIVA-test), thus making (glyco)lipid antigens excellent **candidates for a vaccine**.

WP 4. Improvement of immunology-based diagnosis in wildlife and livestock.

WP4a. Development of a tuberculosis antibody detection assay.

The main goals of the WP4a have been, on one hand, the development and clinical evaluation of a **multi-species serological test** for TB-antibodies detection, including wild animals and, on the other, a **gamma-interferon detection test for different species**.

To achieve the eradication of tuberculosis is necessary not only to eradicate the disease in domestic animals, but also to control TB in wild animals, especially in those that are very important in the transmission of the disease. This is the case of European badger, one of the most important reservoirs of infection in UK and Republic of Ireland, and the wild boar in Spain. The use of the common diagnostic test for bovine tuberculosis such us intradermal tuberculin test or gamma interferon assay, requires handling the wild animals and an equipped laboratory and skilled personnel, respectively. Detection of specific antibodies could be an important alternative tool. To date, studies have shown that the antibody response to *M. bovis* is not uniform and different antigens are used simultaneously.

Different antigens, such as ESAT-6, CFP10, MPB64, MPB70 and MPB83, have been cloned and expressed using prokaryotic (*E. coli*) and/or eukaryotic (Baculovirus) systems. The complete ESAT-6 and CFP10 genes were amplified by PCR. In the case of the three MPB genes, a fragment without the signal peptide were amplified, cloned and expressed. In all cases, the genes were amplified from pQE30 plasmids kindly provided by Partner 8 (ISS). These plasmids were used to transform BL21 cells for expression of the proteins.

The five genes were also cloned in the baculovirus expression vector pAcHLT which add a His tag to the amino terminus to permit the tracking of the protein during the expression and purification steps. Moreover, genes **MPB70 and MPB83** were cloned without their signal peptide in the baculovirus expression vector described before and also in frame with the signal peptide of the baculovirus protein GP67 using pAcGP67 or pAcSecG2T vectors. The corresponding recombinant baculoviruses were obtained by cotransfection of Sf9 insect cells with the transfer vector and flashBACTM.

In order to obtain an immunologic assay able to detect specific antibodies to *Mycobacterium bovis* in different species, several assays have been tested: a) competition assays; b) double recognition enzyme-linked immunosorbent assay (DR-ELISA); and c) LFA (Lateral flow assay). A prototype was finalised for the DR-ELISA (multi-species). The MPB83 recombinant protein expressed in the baculovirus system, was selected and used as coated and enzyme-conjugated antigen in a double recognition enzyme-linked immunosorbent assay (DR-ELISA). The main aim of this study was to develop and optimize a rapid and sensitive assay to recognize not only IgGs but also other immunoglobulins, such as IgMs, allowing the early detection of *Mycobacterium bovis* infection in different species. Distinct sets of sera obtained from field samples and from experimentally infected animals were used to validate the new assay.

The study of the cellular response to *Mycobacterium bovis* is very important for the control of the TB disease and therefore, it is important to have assays, such as ELISAs, able to detect the gamma interferon secreted by T-lymphocytes from different animal species. In some cases (ie. determination of feline and canine G-IFN), this is not possible and for that reason different reagents useful for detection of G-INF have been developed. G-INF sequences from different species have been compared using the DS Gene 1.5 program. After analysing the sequences, it has been possible to define two conserved regions in the

amino-terminal half of the protein (corresponding with peptide 1 and peptide 2) and another conserved region in the carboxy-terminal region, corresponding with peptide 3 and peptide 4. Moreover, a specific region for canine and feline G-INF has been designed (peptide 5). All of them have been linked to ovoalbumin (OVA) for immunizations of mice and rabbits. The total immunoglobulins were purified by Protein G affinity column and female BALB/c strain mice (one for each peptide), were injected intraperitoneally with 30 μg of peptide conjugated to OVA in order to produce MAbs specific to this regions. Moreover, two DAS-ELISAs have been developed: 1) DAS-ELISA for interferon-γ of different species; and 2) DAS-ELISA for feline interferon-γ.

WP4b. Sensitivity and specificity of the antigens for the IFN-γ test.

BOVIGAM® is a blood-based *in vitro* laboratory test for bovine tuberculosis. It is based on the detection of cell mediated immune response to infection with *Mycobacterium bovis* in cattle. Lymphocytes in blood samples from *Mycobacterium bovis* infected cattle respond to specific tuberculin antigens with the production of interferon gamma (IFN-γ) which is detected in an enzyme-linked immunosorbent assay (ELISA).

An improved version of BOVIGAM®, **BOVIGAM® 2G** was developed with better reproducibility, repeatability and robustness. Lelystad tuberculin PPD antigens (avian and bovine) (Prionics, Switzerland) or a synthetic peptide cocktail, developed by Prionics AG during the funding period, were used for stimulation of whole blood cultures. Following stimulation, plasma was harvested and IFN-γ was measured with the BOVIGAM® 2G sandwich enzyme immunoassay (EIA). The peptide cocktail Prionics® PC-HP, formerly named PC-11, contained overlapping peptides derived from ESAT-6 and CFP-10 and in addition peptides derived from Rv3615c and 3 other mycobacterial proteins. Diagnostic sensitivity was assessed in naturally bovine TB-exposed animals in a high prevalence bovine TB herd in Ireland. Diagnostic specificity was evaluated in France from 12 herds free of bovine TB according to OIE criteria. Comparison data were achieved in reference herds from UK and Switzerland. Estimates for the test characteristics in the absence of a true gold standard were derived by Bayesian model as described by Branscum *et al.* 2005. Equivalency between BOVIGAM® and BOVIGAM® 2G was tested in all studies and in particular in a field trial in UK.

In France 390 animals were included into the **specificity** trial. Specificity estimates for the BOVIGAM® 2G with tuberculin PPD from Lelystad was calculated with 84% (95%CI: 81% - 87%) whereas using the peptide cocktail PC-HP for whole blood stimulation a specificity estimate of 94% (95%CI: 92% - 96%) was computed. The difference in the specificity estimates of PPD and PC-HP was highly significant ($p < 0.05$; two-tailed exact Fisher test). **Sensitivity** was evaluated from the sample of 201 animals from Ireland, with an apparent prevalence (depending on test) between 16% and 31%. Sensitivity in this trial was calculated for Lelystad PPD with 89% (95%CI: 75% - 97%) whereas with PC-HP a comparable sensitivity of 85% (95%CI: 63% - 96%) was obtained.

Partner 11 (Prionics) compared the diagnostic performance of a defined **antigen cocktail** with tuberculin PPD for the stimulation of blood cultures in the BOVIGAM® 2G IFN-γ derived from a Bayesian modelling approach. Based on the given results it is shown that diagnostic sensitivity of the peptide cocktail PC-HP is not significantly different from PPD whereas peptide cocktail PC-HP resulted in diagnostic specificity that was significantly higher than stimulation with PPD. These studies show that peptide cocktails have superior performance compared to tuberculin PPD in the BOVIGAM® 2G assay and open up new possibilities for highly specific diagnostic tools in the eradication of bovine TB.

BOVIGAM® 2G demonstrated an overall better sensitivity (89%) in comparison to BOVIGAM® (75%) by applied Lelystad PPD's. The difference in the sensitivity estimates of Lelystad PPD between the two IFN-γ tests was highly significant ($p < 0.05$; two-tailed exact Fisher test).

A total of 699 animals in four different countries could be tested in parallel with BOVIGAM® and BOVIGAM® 2G in order to prove equivalency between the two tests using Lelystad PPD's and two peptide cocktails (PC-HP and PC-EC). Based on this data set the **agreement** between the two tests in UK (N=319), France (N=109), as within the reference herds from UK (N=13) and Switzerland (N=48), are assessed to be very high (between 93.9% and 100%), depending of the used antigen and country. BOVIGAM® 2G recognizes up to 5% more positive reactors in comparison to BOVIGAM® using the same antigens. In Ireland (N=201), the agreement between the two tests was between 76% and 85%. The number of reactors which are recognized by BOVIGAM® 2G in high prevalence herds is increased in comparison to BOVIGAM® without affecting the specificity as shown in UK (equivalency up to 100% in exposed but skin test negative animals) and France (between 93.9 to 98,50 in a low prevalence herd). Conclusively, in low prevalence (exposed) or negative herd situations the percentage of agreement between BOVIGAM® and BOVIGAM® 2G is very high (between 93% and 100%). In a high prevalence situation BOVIGAM® 2G demonstrated a better sensitivity and thus more reactors could be detected in comparison to BOVIGAM®. Consequently, the percentage of agreement is here lower.

Regarding the **in-tube device** for BOVIGAM, a transportation device could be identified which ensures stable temperature of 37°C inside the box for 120 hours when the environmental temperature is between -10°C and 42°C. Temperature is over 24 hours constant within the TempShell, indicating that the identified system is suitable for a stable in tube stimulation for 24 hours over time. Data generated indicates that the stimulation between the two different approached achieved equivalent results. Thus, the transport device is suitable to be used as a transport device for diagnostic. The tested QuantiFERON-TB Gold whole blood stimulation tubes in combination with the Tempshell transportation device is a suitable tool to be used as a complete in tube stimulation solution for facilitating the collection of blood, transportation and stimulation in order to detect TB infected cattle.

Three commercial available IFN-γ assays were tested for their cross reactive antibody capabilities (BOVIGAM®, CERVIGAM® and PRIMAGAM®). A variety of species were tested. A variety of species could successfully be tested with each of the three IFN-γ assays. Thus, this IFN-γ assay could also be just to detect TB reactors in the above listed species. The development of a **pig IFN-γ test** for the detection of TB infected animals was done by Dr. Paolo Pasquali (ISS, partner 8). As a preliminary study to assess the significance of IFN-gamma test, ISS initially performed a study to assess the occurrence of bTB in pigs to study the potential role of such animals in the transmission of the disease. In this study 6.7% of pig carcasses at slaughterhouse were affected by gross tuberculous-like lesions (TBL) and 3.4% were *Mycobacterium bovis* culture positive. Most pigs with TBL showed generalized lesions in both gross and histological examinations (53% and 65.5%, respectively). Head lymph nodes were the most frequently affected in both localized and generalized TB cases observed macroscopically and microscopically. These data suggested a potential transmission of mycobacteria from domestic animals to black pigs and vice versa. These findings, along with ethological, ecological, and management considerations, suggest that the black pig might act as a bTB reservoir in the ecosystem under study.

Successively, partner 8 sensitized the blood of selected animals with bovine or avian PPDs and assessed the production of IFN-γ by using an ELISA Kit (R&D Systems, MN, USA). The results of the immune-diagnosis were correlated to the findings of the post mortem inspection and the bacterial culture of lymph nodes. In particular, blood samples of 146 pigs, belonging to a local breed of Sicily reared in free or semi-free roaming conditions, were collected to assess the specificity and the sensitivity of the IFN-γ assay. Thirty-one pigs, from *M. bovis* free herds, did not react to the IFN-γ assay, yielding a specificity of 100%. The IFN-γ assay identified 15 out of 19 animals positive to the bacterial culture and 22 out of 26 animals with tuberculous lesions, with a sensibility of 78.9% to 84.6%, respectively. Out of 26 reactors to the test, 15 pigs (57.7%) confirmed to be infected after the bacterial culture and 22 (84.6%) had tuberculous lesions. The IFN-γ assay was able to

reveal 4 animals with no visible lesions (NVL). These findings support the feasible use of the IFN-γ assay as an *intra vitam* tool for the surveillance and management of *M. bovis* infection in swine populations.

Partner 1 (UCM) performed three studies in deliverable 4b.1. Optimized antigens for stimulation in the Bovigam assay. A preliminary study was performed to evaluate the **Se of the IFN-γ assay** for tuberculosis diagnosis using PPDs and a peptide cocktail (ESAT-6 and CFP-10) in caprine flocks. In the assayed samples the sensitivity (Se) of the IFN-γ assay using the PPD tuberculin was 88.46%, whereas using the antigenic cocktail the Se decreased to 80%. ODs obtained using the cocktail were lower than those obtained using the PPDs in most of the animals (Bezos *et al.* 2009). To complete this preliminary study, a second experiment using 38 female adult goats selected randomly from two naturally *M. caprae*-infected Spanish dairy flocks with a high prevalence (more than 30% of positive reactors) of infection but with a different clinical history and different breeds. Flock 1 (n=27 goats, mixed breed) had a clinical history of tuberculosis for at least 3 years. Flock 2 (n=11 goats, Guadarrama breed) was recently infected. In this study, the overall OD mean values obtained in the IFN-γ using PPDs and the ESAT-6/CFP-10 peptides were 0.446 (0.281–0.610, 95% C.I.) and 0.246 (0.099–0.394, 95% C.I.), respectively, for both reagents although the values were more scattered for PPDs. Se of the IFN-γ assay using PPDs and ESAT-6/CFP-10 was similar in both flocks but the overall Se was very low in the recently infected flock (54.5% and 50% using PPDs and ESAT-6/CFP-10 respectively versus 80.9% in the flock with a long history of tuberculosis). In general, IFN-γ assay showed the highest Se, although in flock 1 it was similar to that observed using severe interpretation of the intradermal test. Regarding the overall correlation between lesions and the IFN-γ production, in both flocks correlation between lesion score and IFN-γ production using ESAT-6/CFP-10 was not significant (flock 1: Spearman´s rho=0.080, p>0.05; flock 2: 0.140, p>0.05) (Bezos *et al.* 2011). The third study was included to evaluate **two antigenic cocktails** containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the IFN-γ assay for diagnosis of bovine tuberculosis. The biological potency of ESAT-6, CFP-10 and Rv-3615c presented as peptide or recombinant protein cocktails in comparison with the standard bovine PPD used routinely in Spanish eradication campaigns was evaluated. The study was performed in 23 cattle from a herd with natural *M. bovis* infection. Animals were simultaneously injected with PPD and the peptide and protein cocktails. The percentages of cattle reacting positively to single intradermal test were 60.9% (bovine PPD), 47.8% (peptide cocktail) and 60.9% (protein cocktail), with no significant difference between the actual skin fold thickness increases (p>0.05). The IFN-γ assay detected 60.9% of animals when stimulation was performed with bovine PPD, but decreased to 52.2% when stimulation was performed with the peptide cocktail and to 47.8% when stimulation was performed with the protein cocktail. However, no significant differences were found between IFN-γ responder frequencies (p>0.05). These results demonstrated that protein and peptide cocktails consisting of antigens such as ESAT-6, CFP-10 and Rv-3615c were capable to induce detectable skin reactions thus permitting an animal to be classified as reactor (Casal *et al.* 2012).

Partner 1 (UCM) and Partner 9 (CAO) investigated the **impact of non-tuberculous mycobacteria** on the ante mortem diagnosis of bovine tuberculosis (Deliverable 4b2.). Partner 1 carried out a first study in a TB-free dairy herd formed by approximately 600 cattle where **false positive skin reactions** had been described in the last years and IFN-γ assay positivity rates ranged between 5-10%. Specificity (Sp) of the IFN-γ assay using a cocktail containing synthetic peptides derived from the sequences of ESAT-6, CFP-10 and Rv3615c was evaluated and compared to that obtained using avian and bovine PPDs. Tests were performed in young males (age between 8 and 12 months) before they were sent to the slaughterhouse. Blood samples were collected for six months every 30-60 days and single intradermal comparative cervical tuberculin (SICCT) test was performed every two months. While skin positive reactions were observed in only one animal (after avian PPD inoculation), 23 animals showed positive responses in the IFN-γ test at least once and, in six cases, in more than one occasion. A certain seasonal pattern was identified: higher

responses were observed on spring, while in the last sampling period all animals became negative. A similar pattern was identified regarding response to stimulation with avian PPD, thus highlighting a possible seasonal factor on the occurrence of false positive reactions, probably due to a higher exposure of animals to environmental mycobacteria. However, as all animals had nearly the same age, a possible age-effect could not be discarded. The Sp of the IFN-γ test was 96.9% while using the antigenic cocktail decreased to 84.8%. Several animals showed high OD values in bovine PPD stimulation, but on the classic IFN-γ interpretation this was compensated by the higher OD results obtained after avian PPD stimulation. This could indicate that usefulness of antigenic cocktail for discrimination of false positive reactors might be limited if high exposure to mycobacterial antigens (indicated by high OD values after avian PPD stimulation) are suspected.

In a second study, **Sp of single intradermal tuberculin (SIT) test, SICCT test and IFN-γ** assay was evaluated in caprine flocks under different epidemiological situations (vaccination against paratuberculosis-PTB and *Corynebacterium pseudotuberculosis* infection). The study was performed in a total of 937 goats from 8 different TB-free caprine flocks from Castilla y León (Spain). Maximum Sp was found using SCIT test (99.4-100% depending on the interpretation criteria) while SIT test and IFN-γ assay showed a slightly lower overall specificity (97.6-99.2% and 96.4-98.4% respectively). Sp of the SIT test in a *C. pseudotuberculosis* infected flock was significantly ($p<0.05$) lower if a severe interpretation criterion was applied. Similarly, Sp values of SIT test and particularly IFN-γ assay in a PTB-vaccinated flock were lower than those observed in non-vaccinated flocks. Higher proportion of false positive reactors to TB tests (SIT and IFN-γ assay) was observed among animals positive in the PTB-ELISA in PTB vaccinated flock. These results demonstrated that TB diagnostic tests show an adequate Sp when performed in goats from TB-free flocks in most situations. However, certain factors such as *C. pseudotuberculosis* infection and paratuberculosis vaccination can have a negative impact in the most sensitive tests (Bezos *et al.* 2012).

Partner 9 (CAO) investigated the **impact of non-tuberculous mycobacteria** on the ante mortem diagnosis of bovine tuberculosis. 2,574 tuberculin-test reactor bovine animals without any visible lesions nor confirmed MTBC infection were culture and in 17% of the sample non-tuberculous mycobacteria (NTM) were isolated. The most frequent cause of non-specific interference reactions in Hungary is *Mycobacterium avium* complex (MAC) being *M. avium* subsp. *paratuberculosis* the predominant subspecies. The role of other non-MAC NTM species in these interference reactions is moderate in numbers (25% of all NTMs). The most frequent species of non-MAC NTMs were *M. nonchromogenicum*, *M. kansasii*, *M. thermoresistible*, *M. intermedium* and *M. gordonae* in 35%, 17%, 17%, 10% and 10%, respectively. Single and comparative intradermal cervical skin tests (ICTT) have been hampered by cross reactions caused by non-tuberculous mycobacteria. Although comparative ICTT specificity is higher than single ICTT is still not acceptable in MAC-infected cattle. A slight decrease in response in time for bovine PPD was measured in MAC-infected cattle. The evaluation of ICTT should be especially critical in herds with any suspect of MTBC-infection, because single ICTT has enough sensitivity for TB by *M. caprae* just with strict interpretation (>2mm).

WP5. Control of populations.

Surveillance and descriptive studies on tuberculosis are valuable in regions and species that have received less attention or are (at least apparently) emerging. Nonetheless, limiting the research effort to the mere reporting of tuberculosis outbreaks is of limited value if management recommendations are not given at the same time. Thus, more empirical and experimental approaches are needed to produce substantial knowledge that enables authorities to make targeted management recommendations. Among the most intriguing aspects of this new scientific branch is the link between wildlife pathogens, environment, and human activities, further complicated due to the increased risk of

contact (Gortázar *et al.*, 2006). These situations require the integration of veterinary, ecology and wildlife management expertise through multidisciplinary teams. Understanding the risk factors associated with tuberculosis infection at the domestic livestock/wildlife interface is fundamental to the development of effective control policies. In this context, the main aim of this WP was to identify the major management and environmental factors associated with the occurrence and transmission of tuberculosis at the domestic livestock/wildlife interface.

Specifically, this work package was divided into the following objectives: 1) Development of new management tools to identify wildlife overabundance and to monitor the effect of management changes on both population and disease indicators; 2) Risk factor analysis of current wildlife management practices in Mediterranean habitats, including specifically the risks associated to carrion consumption and hunting carcass remains consumption; 3) Analysis of the ecology of wildlife feeding and watering in Mediterranean ecosystems, and the possibilities of reducing the associated disease transmission risks; and 4) Development and characterization of oral bait delivery methods to European wild boar and Iberian red deer under Mediterranean field conditions.

Regarding the first objective, the main results in management tools to identify wildlife overabundance and to monitor the effect of management changes are listed below.

1. Estimating roe deer abundance from **pellet group counts** in Spain. Partner 2 (UCLM-IREC) evaluated different methods of estimating deer abundance in Mediterranean woodlands based on pellet group counts. Distance sampling applied to pellet counts and a new easier and cost-effective method based on strip-variable transect counts (FST) were assessed comparing their estimates (pellet group density) with the abundance indices obtained from traditionally used reference methods (faecal standing crop) in 61 localities (n = 183 surveys). Distance Sampling may be used when human resources and skills are enough but FST is a rapid and efficient alternative to estimate pellet group density when they are not (Acevedo *et al.*, 2010).

2. Effects of **density and supplementary forage** on body mass and pregnancy rates of female red deer in Spain. Partner 2 examined the short- and long-term effects of rainfall and absolute density on hinds in two of the southernmost populations of red deer in Europe. One population received supplementary forage. Hinds in the population that received supplementary forage were heavier and more likely to become pregnant than were the hinds in the unsupplemented population. The likelihood of pregnancy occurring was strongly influenced by hind body mass; the proportion of yearlings that became pregnant was consequently lower in the unsupplemented population than in the population that received supplementary forage. The management of Iberian red deer populations should consider supplemental feeding practices, which can buffer density- and climate-dependent effects and reduce natural selection pressures (Rodríguez-Hidalgo *et al.*, 2010).

3. Progress in the use of **fat indexes** to identify red deer overabundance. Body condition is a useful measure of the nutritional status and performance in ungulates. The most widely used indicators of body condition are based on fat reserves, mainly those surrounding the kidneys. UCLM-IREC used red deer (*Cervus elaphus*) as a model – both under experimental and field conditions – to analyze the patterns of response of the body condition indicators (calculated from left and right kidneys) to supplementary feeding, sex, age class and season.

4. Relationships between **faecal parasitism and diet quality** with habitat and population abundance in red deer (*Cervus elaphus*). Monitoring diet quality and parasite loads helps in characterizing population heath status in a broad sense. The goals of this research were (i) to describe the spatio-temporal variation on diet quality (fecal nitrogen levels) and parasite load (fecal excretion of parasite propagules of the nematode *Elaphostrongylus cervi*) in red deer in South Central Spain), (ii) to determine baseline values, and (iii) the relationships between such indices and local characteristics of habitat and deer

population abundance. Thirty plots were selected in a typical game hunting area from South Central Spain (6700 ha), and stratified according to habitat characteristics. Fresh fecal pellets were collected every 2 months during one year period (2011). A negative association between diet quality (measured as fecal nitrogen %) and *E. cervi* L1 excretion levels was observed. This association could be mediated by the relationship between the diet quality and the immune system. When attending to the relationship between the number of fecal pellet groups (as a local measure of red deer abundance) and the excretion of *E. cervi* L1, separately for each sampling season, in September, coinciding with the rutting, the relationship was statistically positive. This result may indicate a denso-dependent relationship at a very local scale, in particular during a period when deer are nutritionally constrained in Mediterranean habitats and stressed due to breeding, conveying a high excretion of parasite propagules. The study of parasite excretion levels and diet quality indicators are potential tools to evaluate and monitor population health of deer populations.

5. **Lesion distribution and sampling strategies** for bTB surveillance in red and fallow deer. Partner 2 compared the pattern of lesions present in wild red and fallow deer that were naturally infected with *M. bovis*, and used this information to develop a sampling strategy for the isolation of *M. bovis* from the lymphoid tissues of the head of these animals. Approximately 30% of each deer population had no gross lesions. Fallow deer were significantly more likely to have thoracic lesions than red deer. Lesions were observed in the retropharyngeal lymph nodes of 64% of the culture-positive red deer and 43% of the culture-positive fallow deer. One third of the red deer, but none of the fallow deer, had well-encapsulated abscess lesions. Bacteriological culture from both the tonsil and retropharyngeal lymph nodes increased the rate of isolation of *M. bovis* by 22% over culture of the retropharyngeal lymph nodes alone in both species. These findings indicate that investigation of wild deer for bTB-compatible lesions should include examination of the medial retropharyngeal, left tracheobronchial, mediastinal, mesenteric and ileocaecal lymph nodes. These protocols may prove useful in bTB surveillance and control in regions where wild deer contribute to the circulation of *M. bovis* (Martín-Hernando *et al.*, 2010).

6. Improved ELISA for the detection of **serum antibodies** against *M. bovis* in **wild boar**. Partner 2 tested sera from 96 culture confirmed *M. bovis* infected Eurasian wild boar from several sites in southern Spain, and 104 culture negative and lesion negative wild boar from sites in northern Spain with no bTB history. Serum samples were tested for anti-bPPD IgG antibodies by means of an ELISA using bovine tuberculin purified protein derivative (bovine PPD) (CZ Veterinaria S.L., Spain), as antigen and Protein G horseradish peroxidase (Sigma Chemical, St. Louis, MO, USA) as a conjugate. By introducing slight modifications to the existing protocol, the ELISA sensitivity and specificity was improved to 79.2% (76/96) and 100% (104/104) respectively. Given its high specificity, this ELISA constitutes a valuable tool for TB monitoring in wild boar (Boadella *et al.*, 2011a).

The knowledge derived from the risk factor analysis of current wildlife management practices in Mediterranean habitats (objective 2) and the ecology of wildlife feeding and watering in the Mediterranean ecosystems (objective 3) was acquired thanks to the following studies:

1. Use of **ELISA to study the factors** determining Eurasian wild boar contact with *Mycobacterium bovis* in the Iberian Peninsula. An indirect ELISA using bovine-purified protein derivative was applied to determine the spatial and temporal distribution of wild boar contact with MTBC in the Iberian Peninsula and to model and identify the associated risk factors. Wild boar apparent seroprevalence was 22%. Seropositives were detected in 71% of 81 sites, including 23 sites where wildlife was thought to be bTB free. The results described a new geographic range of wild boar contact with MTBC and a stable prevalence in this wildlife reservoir that contrasts with the success of bTB control in cattle. Inference of which host (wild boar or cattle) is driving bTB maintenance was not possible with these correlational results. The possibility of a wild boarbTB emergence in non-

endemic regions should urgently be taken into account to avoid a future scenario resembling the current situation in south-central Spain (Boadella *et al.*, 2011b).

2. Carrion and hunting gutpile consumption and TB transmission risk. Partner 2 studied the factors affecting the activity of **scavengers** to infer relative exposure to disease agents and potential for spread in ungulate carcasses disposed in three areas from South Central Spain, aiming: i) to describe the guild of vertebrate scavengers, ii) to quantify carcass use by scavengers, and iii) the species-specific, carrion and habitat related factors involved in the usage. Wild boar and red fox accounted for a relevant proportion of the scavenging activity, the suid even becoming locally the predominant scavenger in absence of vultures (locally accounting for up to 90% of scavenging units). Contaminated carrion feeding by facultative scavengers could facilitate intraspecific (cannibalism in wild boar) and interspecific TB transmission. Management agencies should consider removal of harvested animals and viscera to limit potential tuberculosis deposition near a kill site where mammals, and specially wild boar, can access. Obligate scavengers and other birds effectively remove infectious tissue, and some of them are of special conservation interest. It should be assessed which disposal regime is more beneficial to obligate scavengers, guarantying them food supply while reducing exposure of disease susceptible mammals.

Regarding hunting gutpile consumption, in spite that hunting is regulated by different laws (specifically in the study area where burying them in situ, processing them in authorised plants or disposing them into a legal vulture restaurant is allowed), gut piles are frequently left in situ (Vicente *et al.*, 2011). Work in central Spain [18 ungulate gut piles (wild boar and red deer) were monitored by photo and video recording in 7 hunting estates] determines the sanitary relevance of big game remains, specially attending to the persistence and spread of TB in facultative scavengers, namely the wild boar, which play a key role in the epidemiology of the disease. The specific aims are: i) to describe the guild of vertebrate scavengers, ii) to study the species-specific, carrion and habitat related factors involved in the usage, and iii) the potential for disease spread in a bTB-endemic wildlife area. Wild boar and red fox accounted for a relevant proportion of the scavenging activity on hunting remains, both becoming the predominant scavenger in habitats covered by dense vegetation. Therefore, feeding on contaminated remains is facilitating intraspecific (cannibalism in wild boar) and interspecific TB transmission. These results reinforce the idea that management agencies should consider the removal or disposal of harvested animal remains in order to avoid that wild boar gain access to them.

3. Analysis of the ecology of wildlife **feeding and watering** in Mediterranean ecosystems, and the possibilities of reducing the associated disease transmission risks. Partner 2 focused on 10 extensive farms (South Central Spain), and risk points for direct or indirect disease transmission risk were monitored by photo recording. The relative occurrence of different wild ungulates, their behaviour and their potential for facilitating disease transmission are described. Finally, correlates of risk point use (specific farm-based resources, big game demography and environmental variables) were examined. In addition, seasonal variation was investigated since two critical periods and risk points were identified: watering points in summer and the fall of acorns in autumn. Preliminary results show that big game frequently use watering points in summer, potentially allowing for indirect disease transmission between them and livestock. Big game exhibited close 'nose-to-nose' contact with themselves and excreted/marked on and around water. The fine examination of these observations allow prescribing management options for disease prevention to reduce opportunities for direct or indirect contact between big game and cattle. Efforts to exclude wild ungulates from watering points shared with cattle are therefore recommended.

4. Use of **extensive farm resources** by big game and interaction with livestock in a Tuberculosis endemic area from South Central Spain. Increased interactions at the domestic livestock-wildlife interface, especially in open air livestock breeding situation is favoured by territorial expansion and population growth of wild ungulates. The

aggregation of resources that are commonly used by both domestic and wild individuals results in spatial and/or temporal overlap among them. Partner 2 described and quantified the presence of wild ungulates in food and water sources used by extensive livestock in South Central Spain (province of Ciudad Real) by using infrared camera trapping in 9 open air livestock herds. In summer season, visits to farms by red deer (67%), red fox (16.3%), wild boar (15.3%) and roe deer (1.4%) were documented. 90.4% were detected in water sources (troughs or water holes), 2.7% in outdoor feeders (troughs or on the ground) and 6.9% in control points. In autumn survey, 81.1% of visits were due to red deer, 13.5% wild boar, 3.8% red fox and 2.2% roe deer. No visitations were detected in farm buildings. Although domestic and wild ungulates shared the same drinking and food areas, no documentation exits regarding both in the same picture (just one case series in a control point involving pigs and red deer). They partitioned use temporally, especially wild boar, more frequent in nocturnal hours. UCLM-IREC detected an important proportion of days with red deer and wild boar presence (1 every 4 days and 5 days, respectively, in summer) were detected. Increased concentration of animals visiting the same locations seeking drinking water could lead to indirect transmission of the disease within and between both wildlife and livestock. Excluding large wildlife species and reducing the attractiveness of the farm to them and providing alternative water sources to limiting access to wildlife could reduce disease transmission.

5. Habitat use and risk factors related to wild boar - livestock interactions by integrating **GPS-GSM monitoring technology**. Results coming from 22 GPS-GSM collared wild boar during 2009 and 2011 evidenced the capacity of wild boar to undercross fences. Maximum distances travelled in a day were up to 14 km. Ranging by wild boar coming from hunting areas in cattle grazing areas was detected. This multidisciplinary study is providing knowledge on the role of wild boar as potential disease reservoirs for diseases that can seriously compromise livestock health, but also regarding the sustainable use of wild ungulates by hunting, and regarding public health. The objectives of this type of studies are (i) to describe and quantify the local patterns of contacts (connectivity among individuals) between domestic livestock and wild ungulates, and among each species in an area from South Central Spain; (ii) to determine the individuals or groups that present the highest risk for disease transmission within and between species; and (iii) to evidence the factors (including habitat use) that determine the local pattern of contacts between livestock and wild ungulates. Cattle, pigs, wild boar and red deer were collared with proximity contact data logger devices. Finally, interviews were carried out on more than 70 cattle farms in a bTB endemic area of Ciudad Real. These interviews aimed at characterising cattle management (specifically including feeding and watering) and the interactions with wildlife.

6. Is **wild boar culling** a suitable TB-control strategy?. A 50% Eurasian wild boar abundance reduction was achieved through increased culling. Wild boar TB prevalence remained stable in control sites, whereas it decreased by 21-48% in treatment sites. In one treatment site, the annual wild boar abundance was positively correlated with the annual percentage of skin test reactor cattle. In another treatment site, red deer *M. bovis* infection prevalence decreased after culling wild boar. The reduction in wild boar TB was achieved despite no alternative *M. bovis* host being included in the culling strategy. Culling could become a part of integrated control strategies including habitat and game management changes and vaccination, contributing to increase their success likelihood, or reducing the total expenses.

Regarding objective 4, Development and characterization of **oral bait delivery** methods to European wild boar and Iberian red deer under Mediterranean field conditions, studies were carried out in wild boar and red deer.

1. Oral bait development and delivery to Eurasian wild boar. The effective and efficient field vaccination of wildlife requires the development of baits that are stable under field conditions, safe for target and non-target species as well as the environment, and effective in reaching the target species. For free-ranging *Sus scrofa*, different baits have

been developed. All of them are made with a cereal-based matrix containing a capsule or blister to deliver the vaccine or pharmaceutical. The palatable ingredients used for the bait matrix composition stimulate chewing to open the capsules contained in the baits and releasing their content inside the oral cavity. For TB vaccination, wild boar piglets are the main target. Marking agents are incorporated into baits to enable identification of consuming individuals. In the case of wild boar, ethyl and propyl- iophenoxic acids could be detected in animal serum up to 18 months after their consumption when doses of 5 and 15 mg/kg were delivered. Bait consumption rate and host specificity depend directly on the delivery method employed. Selective feeders were used in order to reduce bait consumption by non-target species in southern Spain. These feeders allow only access of young wild boar, although occasionally small animals such as badgers can enter inside the feeders and have access to the baits. The success of vaccination programs is also determined by the timing of bait delivery. For example, early summer would be the best timing for TB vaccine bait delivery to wild boar piglets in south-central Spain. In addition, bait consumption by wild boar or feral pigs is better if the pre-baiting period lasts longer so that animals get accustomed to feed in the place where baits will be delivered. Other factors such as baiting and/or free-ranging *Sus scrofa* densities can affect bait consumption by target species. Marked-bait consumption was up to 73% wild boar piglets at a bait density of 30 baits/km2 and using one piglet feeder per 2 km2. These baiting densities were lower than those used in previous studies in other countries. Therefore, in future TB vaccination experiments it would be desirable to use higher baiting densities (Beltrán-Beck *et al.* 2012).

2. Oral bait development and delivery to red deer. Selective red deer feeders randomly distributed along one hunting estate were used to supplement feed to free-ranging red deer. These feeders are selective for red deer according to age and sex. Calf feeders and hind feeders are lower than stag feeders. Stag feeders are open while calf and hind feeders have a roof limiting access to antlered deer. Calf feeders have vertical crosspieces impeding hind access. All feeders were too high for wild boar. This experiment was conducted in summer. The number of deer in the site was estimated to be approximately 300 animals. Three groups of three selective feeders each (designed for stags, hinds, and calves; n = 9) distributed along the estate were used for the bait delivery. Twenty-five baits were placed inside each feeder. Bait consumption was checked every morning during 4 days. All baits placed inside stag and hind feeders (25 baits per feeder) were consumed after two days of their delivery. However, calves needed more than 4 days to consume the baits. Only 3%, 7% and 11% of the capsules contained in the baits distributed inside stag, hind, and calf feeders were found, respectively. All of them were chewed and empty in the case of stags whereas most of the capsules found inside hind and calf feeders were intact and had liquid inside (Ballesteros *et al.* 2011).

WP6. Molecular typing of *Mycobacterium bovis* and *M. caprae* isolates: focused epidemiological investigation.

Work devoted to Work-Package 6 has focused on the exploitation of molecular epidemiology to understand the distribution of *Mycobacterium bovis* and *M. caprae* in animal populations. Accordingly, research has been organised at different levels: (1) EU level, (2) national level, and (3) study of tuberculosis outbreaks in domestic and wild animals and tracking outbreaks caused by specific strains. Molecular characterization have used PCR-based fingerprinting techniques such as the direct variable repeat (DVR)-spoligotyping and mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing. The degree of discrimination of each locus, which depends on the locus but also on the geographical origin of the samples, has also been studied. The specific objectives for this workpackage were: a) to determine the effect of agricultural system and farm management practices on the infection in domestic livestock; b) to apply the new PCR-based techniques, such as the second-generation spoligotyping membrane and MIRU-VNTR to concise objectives; c) to evaluate the impact

of mycobacterial diseases in domestic animals (other than cattle) and wildlife; d) to evaluate the risk of infected wild animals for domestic population; e) to select the most appropriate combination of VNTR panels for molecular epidemiological studies of *M. bovis* in Italy, Spain and Hungary; f) to assess the new spacer oligonucleotides for DVR-spoligotyping of *M. bovis* strains; and g) to understand the role of *M. caprae*. This has been achieved by contribution to two complementary tasks:

Task 6.1. To determine the relative contribution of each factor to the maintenance of *M. bovis* transmission. The main purpose of this work-package is to find out the **most relevant sources of infection** under the studied epidemiological situations. The improved molecular characterization techniques have been applied to a representative number of isolates from specific outbreaks in order to determine the relative contribution of the animal species and management conditions in the transmission of the infection. These molecular tools were directed to the study of spatio-temporal association between sub-types in domesticated and wild animal species; to investigate evidence for dispersal of TB via long- and short-range movement of domestic animals; investigate evidence for within-herd (ie. cattle-cattle) spread by sub-typing isolates derived from multi-reactor herds, at same and subsequent tuberculosis tests; to propose source(s) of infection and route(s) of transmission for regional outbreaks and evaluate intervention retrospectively; and finally to investigate *M. bovis* lineage population structure within regions and between member states.

Task 6.2. To understand the role of ***M. caprae* in domestic animals and wildlife**. This research has been performed in Hungary (partner 9), Italy (partner 8) and Spain (partner 1). These countries have been selected because in Spain, goats seem to be the main species affected, but in Italy, and in Hungary (as in other countries of central Europe) the infection is found in other animals. These tasks have been approached by collecting a representative number of isolates from known outbreaks from wild animal species (wild boar, deer, others) and livestock (cattle, goats, pigs) from different geographic areas. A systematic and structured collection of *M. bovis* and *M. caprae* isolates per participant (RD9-deleted lineage), representative of domesticated and wild animals per region, was used. Genotyping of the *M. bovis* and *M. caprae* isolates using spoligotyping and MIRU/VNTR methods was carried out according standardised methods to perform epidemiological investigation of the outbreaks in bovine and wildlife. This methodology also enabled the comparison of the *M. caprae* strains to each other and to other European *M. caprae* strains.

The results from these tasks revealed differences in the diversity within *M. bovis* populations. A high diversity was found in Spain. In this country, while most spoligotypes were distributed throughout the territory, a small number of patterns were restricted to determined regions. In general, a higher discriminatory index in the Spanish northern regions, which started eradication of bovine tuberculosis first and present lower annual herd period prevalence, was found. However, some exceptions existed, likely reflecting animal species, cattle breeds and herd management. The analysis of diversity within each species offered insight into the relative contribution of animal species to the epidemiology of bovine tuberculosis in Spain. The finding of 207 spoligotypes exclusive to cattle gave evidence that cattle-specific spoligotypes are responsible for much of the diversity of the Spanish *M. bovis* population.

Spoligotyping results showed common traits among western continental EU countries and to a limited extent also to the British Isles. As an example, the frequent type, SB0121, was also the most frequent strain in Portugal, the second most predominant type in France after SB0120 and one of the five most frequent Italian types. The similarities between Spain, France, Portugal and Italy can be due to the geographical nearness and trade relationship. SB0121 had also been found with a very low frequency (<1%) in mainland Great Britain. Furthermore, type SB0140, the most common spoligotype of the British Isles, has occasionally been isolated in the Iberian Peninsula.

The presence of tuberculosis in a population of wild animals may interfere with the eradication scheme of bovine tuberculosis and raises problems of wildlife management and public health. The reports from partners corroborated the role of wildlife using molecular characterisation techniques to track epidemiological links among *M. bovis* isolated from livestock and wild animals sharing an ecosystem. Thus, the percentage of exclusive types from wild boar and red deer in Spain was notably lower than that of cattle, but the involvement of wildlife in the epidemiology of the infection is highlighted by the fact that 12 out of the 15 most frequent types are present both in cattle and at least in one wild *Artiodactyla* species. Furthermore, a study carried out in Doñana National Park using a combination of spoligotyping and MIRU-VNTR analysis corroborates that wildlife (*artiodactyla*, carnivores) were infected with the *M. bovis* strains which were more prevalent in cattle (SB1230 and SB1232) and were maintained in the same area over time. This *M. bovis* population was persistent and underwent micro-evolutionary steps over long period of time. The likelihood of inter-species pathogen transmission should be considered before introducing animals in areas that must be preserved from infectious diseases that have detrimental effects in both livestock and wildlife.

Badgers (*Meles meles*) have been implicated in the transmission of *M. bovis* infection to cattle in Ireland and the UK. Recent studies in Ireland have shown that although the disease is endemic in badgers, the prevalence of disease is not uniform throughout the country and can vary among sub-populations. The extent to which the prevalence levels in badgers impacts on the prevalence in cattle is not known as the precise interactions that result in transmission of infection between the species have not been established. Previously, DNA fingerprinting has shown that *M. bovis* strain types are shared between badgers and cattle, and that there are a large number of strain types circulating in the two species. In this study, Partner 3 (NUiD UCD-CVRL) carried out spoligotyping and variable number tandem repeat (VNTR) analysis of *M. bovis* isolates from two groups of badgers, representing a wide geographic area, with different tuberculosis prevalence levels. The results of the typing shows there is no geographic clustering of strain types associated with prevalence. However, two VNTR profiles were identified that appear to be associated with high- and low- prevalence *M. bovis* infection levels, respectively. In addition, spoligotyping and VNTR analysis has provided evidence, for the first time, of multiple infections of individual badgers with different *M. bovis* strains.

The Italian National Reference Centre for Bovine Tuberculosis performed genotyping of 31 *M. bovis* isolates from pigs by spoligotyping and by a panel of 12 MIRU-VNTR markers. In the same period 234 *M. bovis* isolates were collected from cattle coming from Sicily Region. This activity was the result of the compulsory national eradication program based on single intradermal skin test and removing of positive animals. Obtained genetic profiles were compared to a database collection of *M. bovis* genotypes of isolates of cattle origin from Italy. This database comprises more than 100 spoligotyping patterns from about 4,000 Italian *M. bovis* isolates representative of more than 2,000 outbreaks. Geographic information has also been collected and it will be used to define the epidemiological situation of the Region under study. These data have suggested a potential transmission of mycobacteria from domestic animals to black pigs and vice versa. These findings, along with ethological, ecological, and management considerations, suggest that the black pig might act as a reservoir of bovine tuberculosis in the ecosystem under study.

Mycobacterium caprae, as other members of the *M. tuberculosis* complex, has a broad range of potential hosts. The routine application of molecular diagnosis and typing techniques in clinical laboratories has allowed the recognition of its real role as a pathogen. Studies have been based on culture of animal samples from different areas and characterization by spoligotyping and VNTR as an attempt to understand the epidemiology of *M. caprae* infection in EU. The epidemiology is different regarding the main animal species affected in countries where the pathogen has been found. Overall, results show that *M. caprae* infects both domestic and wild animals. *M. caprae* isolates were identified by spoligotyping because they perfectly matched the typical signature

(lack of spacers 3 to 16) common to all *M. caprae* isolates described so far. Besides this, based on the presence or absence of spacers 30-33 two main groups can be further identified: the central European cluster and the Iberian cluster.

M. caprae infection was widespread in Spain and affected goats and also other six domestic and wild animal species. Diversity based on the number of patterns and discrimination index was lower compared to *M. bovis.* Often, there was a geographical link with caprine flocks infected with the same spoligotype pattern. Unlike results from reports from other EU countries, the epidemiology of *M. caprae* in Spain is driven by caprine infections. The microorganism seems to be highly pathogenic for the Spanish goats, based on the disseminated tuberculous lesions that it produces and the fast transmission within a flock. Furthermore, caprine flocks have not been included in the national eradication campaign (except when coexistence with cattle is given or as part of some regional programmes). Therefore, *M. caprae* infection can spread easily through animal movements between flocks. However, *M. caprae* infection is also relevant in cattle and an emergence of this pathogen in cattle has been observed in recent years. *M. caprae* seems to be the dominant species in cattle in Central Europe. *M. caprae* has been the exclusive pathogen of typical tuberculosis both in domestic and wild animal species in Hungary for last two decades. With spoligotyping and MIRU-VNTR genotyping of the *M. caprae* strains isolated from cattle, wild boar, red deer, fallow deer, roe deer and red foxes from Hungary in 2006-2012 the existence of epidemiological contact between farm and wild animals were proved. Wildlife (especially wild boar and red deer) should be considered as the natural reservoir of bovine tuberculosis caused by *M. caprae* in Hungary. Two closely related spoligotypes (SB0418 and SB0835) are prevalent in wildlife in some regions and cause sporadic outbreaks in cattle. Based on the available data wildlife independent cattle outbreaks were caused by a special VNTR-type of SB0418 and the unique SB1199 strains. The data of genotyping and the cattle registration database demonstrated spreading of *M. caprae* from cattle to cattle between and inside herds.

In conclusion, there is compelling evidence which shows that *M. caprae* is a pathogen that poses a serious health risk not only for goats but also to other domestic and wild animal species. Molecular epidemiology of *M. caprae* infection and the relative contribution of each animal host to the maintenance of animal tuberculosis vary amongst European countries. Considering the role of *M. caprae* in animal tuberculosis, relevant legislation should be adapted to address the infection as it is done with *M. bovis.*

WP7. TB risk and control in EU systems.

1. Potential use of a bTB vaccine to control infection in badger populations: A spatial stochastic simulation model was used to evaluate the effectiveness of vaccination in reducing bTB infection in badgers across a range of population densities, and hence contribute to an understanding of the requirements for a successful vaccination campaign to reduce or eradicate bTB in badgers. Because of the multispecies nature of the bTB host community, in which some bTB spread is likely to occur among the different livestock and wildlife components, partner 5 also account for some background infection from these external sources. Specifically, three components were addressed that can influence the success of a vaccination strategy: (1) the efficacy of the vaccine, which incorporates both uptake and effectiveness (protection) at the individual level; (2) the duration of any vaccination campaign; and (3) the potential influence of external sources of infection. The modelling results showed that in the absence of external sources of infection (e.g. from sympatric livestock), vaccination over short time periods has lasting effects and can reduce disease or even eradicate it from the badger population. However, the presence of external sources of infection amplifies disease and hinders control. The effect of this amplification of disease caused by trickle-type infection from external sources is greatest for smaller badger group sizes close to the threshold population level for disease persistence, since external infection permits disease to persist within

populations where it would otherwise go extinct. Where the badger population experiences a significant amount of infection from external sources, vaccination with limited duration is ineffective. In these circumstances, vaccination needs to be continued for a long time to reduce or eradicate infection in the badger population. Thus, whilst vaccination does offer some potential for contributing to the control of bTB, it must be viewed as a long-term strategy, and one which must be accompanied by effective management of disease across the entire host community.

2. External sources of infection hamper bTB control in badger populations: The modelling results showed that the presence of external sources of infection reduced the threshold group size for disease persistence but only when external infection was occurring at relatively high levels. External infection at low levels had a negligible impact on disease persistence. Overall, external infection showed a much lower correlation with disease prevalence compared with internal disease-related factors such as badger group size and intra- and inter-group infection rates. External infection can clearly contribute to the persistence of bTB in badgers, especially when it occurs at a relatively high level. In these situations, external sources of infection will reduce the effectiveness of bTB control operations and may allow the disease to persist or re-emerge in badger populations that have been reduced following culling. In such situations, an increase in contact rates as a result of culling-induced perturbation may further encourage re-emergence of disease. Effective wildlife disease control strategies need to be underpinned by management to minimise external sources of infection to the wildlife.

3. Host population structure is a key determinant of disease persistence: Partner 5 carried out an investigation into the joint effects of spatial and social heterogeneity on demography and disease dynamics. SAC used a stochastic description of births, deaths, social and geographic migration, and disease transmission within a spatially structured population. In turn, each group is split into social hierarchical levels, where rank correlates with fecundity. A range of realistic hierarchical structures emerge as a result of a limited set of simple rules and parameters which govern the rates of births and migration. These simulations confirm the well established observation that division of a population into identical unconnected subpopulations significantly reduces disease prevalence by removing connections in the disease transmission network. When subpopulations are not identical, e.g. when the distribution of individuals is controlled by a hierarchical structure, this leads to an increase in disease prevalence compared to the case where all sub-populations are identical. This effect is greatest when disease prevalence is low. There is ample evidence in nature for the types of hierarchies emerging from the model, and the simulations demonstrate that the effects of spatial and social heterogeneity are of similar magnitude. The largest effect occurs when combining both types of heterogeneity. Clearly, the effect of both spatial and social structure on disease dynamics needs to be considered when examining the impact of disease control strategies, particularly in situations where low disease prevalence is critical. This model provides a way to incorporate both spatial and hierarchical effects and the simulations show that including both may be required if we are to accurately capture persistence and spread of infection. The spatial organisation of many wildlife species into stable groups occupying discrete territories (e.g. badgers) is well documented and often there are also strong indications of hierarchical structure within such groups. It has been suggested that social structure plays a role in the spread of bTB among of badgers (Vicente *et al.* 2007; Shirley *et al.* 2003). Given the evidence in nature for the types of hierarchies emerging from the model, the effect of such structures on disease dynamics needs to be considered when developing epidemiological models, particularly in situations where low disease prevalence is critical, for example comparing disease control strategies.

4. Numerical description of the perturbation effect: Population reduction is often used as a disease control strategy when managing infectious diseases in wildlife populations; however, in certain host-disease systems it has been associated with increased disease levels, contrary to predictions made by standard disease models. This unexpected

increase in disease following population reduction is often called "the perturbation effect". Partner 5 use an *SI* model of infection to demonstrate how the perturbation effect can be an emergent property of the underlying population and disease dynamics. Both non-spatial and spatial versions of this model were examined; in the former, it was shown how the inclusion of an explicit behavior change as a result of population reduction gives rise to a perturbation effect, while in the spatial case it is shown to emerge as a result of the combined action of density dependent dispersal and spatial heterogeneity. Partner 5 found that the perturbation effect can be both transient and persistent, and can occur in a spatial context with no explicit behaviour change mechanisms. It is most likely to occur in spatially heterogeneous disease systems with low overall disease prevalence, and subject to a change in host behaviour or tendency to increase in host mixing following population reduction, particularly when the disease has been recently introduced. All of which are characteristic of bTB in badgers.

5. bTB risk mapping for the UK: SAC considered the distribution of bTB hosts (badgers, deer and cattle) in the UK and synthesised this information with the current evidence for the role of wildlife in the persistence of bTB in cattle. Partner 5 concluded that at the landscape level, risk-mapping based on host community composition and distribution can provide the basis for targeted surveillance. The management of bovine TB remains a controversial problem in Britain, which is made all the more intractable by the high number of factors actually or potentially contributing to its persistence in certain areas. To manage the disease more effectively, there is a need to understand the potential role of all these factors, including any synergistic effects. It is also necessary to recognize that their relative importance is likely to vary between different areas due to differences in host community composition and abundance, husbandry practices, landscape factors and climatic conditions which may affect the survival of bacilli in the environment. Vaccination may provide some assistance in the control of bTB in host communities in Britain in the future but this, as yet untested approach, should not be seen as the answer to the bTB problem. Whilst a badger vaccine may well contribute significantly to the ability to control bTB in the future, history has taught us that there are no 'silver bullets' when it comes to bTB eradication. Despite bTB being one of the best-studied disease systems in Britain, it is clear that we still have much to understand. Given this uncertainty, when developing bTB control strategies (e.g. through the use of epidemiological modelling frameworks), we should not assume levels of precision that are not supported by the available data. In particular, we should be wary of predictions based on mean field approaches when new research is increasingly showing us just how significant heterogeneity can be, from individual behaviour to landscape-level patterns of transmission risk. A systems-based approach that builds on a mechanistic understanding of behaviour within all components of the system and considers all the control options, seems a pragmatic starting point. This will require the cooperation of all stakeholders to ensure bTB risk is managed at all levels from farm management practices to bTB testing regimes, livestock movements and wildlife host population reduction. Based on the scientific evidence and means of management available at present, the recommendations are as follows:

a. Prevention of bTB spread to traditional bTB areas must be a priority: transport of cattle, surveillance of the host community and improvements to the cattle bTB testing. Investment in this surveillance now will reduce the likelihood of future costs due to bTB occurrence.

b. Proactive badger culling should not be completely discounted, and it may be the most appropriate technique for specific, spatially distinct bTB hotspots. However, if carried out, it must not be selective, the efficiency must be high, and it must be done in conjunction with a range of cattle control methods. The effects of badger culling should always be monitored and related to cattle bTB prevalence, and any badger culling operation should be evidence-based with well-defined end points relating to expected disease control targets.

c. There needs to be a focus on minimizing the reproductive capacity of the disease. This should be done through a twin-level approach, based on the continued development of strategic assessments of risk at the landscape level alongside tactical approaches to reduce the opportunities for transmission at the farm level. In particular, greater effort should be put into understanding the links between these different levels in terms of how local contact processes contribute to disease persistence at the landscape level, and how and why their relative importance changes in different situations.

d. Bovine TB control strategies should include changes to husbandry and farm management practices. Management to reduce the risk of transmission needs to be applied by farmers in a collaborative manner, so that it is applied consistently over wide areas of the landscape.

6. bTB risk mapping for the EU: The UK sysnthesis was extended to the EU. The resultant bTB hazard maps suggest that there are a number of currently bTB-free host communities at risk of bTB spread. As such the maps may be used to inform targeted surveillance strategies to reduce the risk of introducing bTB in currently disease free areas of the EU. By following a precautionary approach, further spread of bTB in Europe could be reduced. The paucity of data on the distribution of wildlife species and their bTB status in the EU was noted during the study. As such the EU maps should be seen as a relatively crude indicator until better data is available.

7. Identification of high risk individuals within a bTB host contact network: The difficulty of studying transmission networks in free-living populations means that the relative opportunities for intra- versus inter-specific disease transmission have not previously been demonstrated empirically within any wildlife-livestock disease system. Using recently-developed proximity data loggers, partner 5 quantified both intra-and inter-specific contacts in a wildlife-livestock disease system, using bovine tuberculosis (bTB) in badgers and cattle in the UK as our example. SAC assessed the connectedness of individuals within the networks in order to identify whether there are certain 'high-risk' individuals or groups of individuals for disease transmission within and between species. The results show that contact patterns in both badger and cattle populations vary widely, both between individuals and over time. They recorded only infrequent interactions between badger social groups, although all badgers fitted with data loggers were involved in these inter-group contacts. Contacts between badgers and cattle occurred more frequently than contacts between different badger groups. Moreover, these inter-specific contacts involved those individual cows, which were highly connected within the cattle herd. This work represents the first continuous time record of wildlife-host contacts for any free-living wildlife-livestock disease system. The results highlight the existence of specific individuals with relatively high contact rates in both livestock and wildlife populations, which have the potential to act as hubs in the spread of disease through complex contact networks. Targeting testing or preventive measures at high-contact groups and individuals within livestock populations would enhance the effectiveness and efficiency of disease management strategies (Böhm *et al.* 2009).

8. Cattle grazing behaviour and inter- versus intra-specific bTB risk via the faecal-oral route: Livestock herbivores are at risk of bTB exposure from livestock and wild mammal faeces during grazing. Livestock exposure to bTB will be dependent on the behavioural contact processes between grazing livestock and host animal (both livestock and wild mammal) excreta at the bite scale. Partner 5 used grazing experiments to determine the affect of faeces from different species (badger, cattle, fallow deer and rabbit faeces; and non-contaminated control patches) and in different defecation patterns on the grazing response of cattle. The cattle's grazing response was determined by measuring sward depletion at each of the treatment patches. Cattle-grazed control and rabbit faecal–contaminated patches the most, whereas badger contaminated patches were grazed the least. Cattle grazed the treatment with the most disperse faeces distribution the most. The results suggest that as the number of faecal deposits in the environment increase and the concentration of faeces at each defecation site decrease, cattle increase their

exposure to faeces during grazing. Thus, the behaviour of animals such as badgers, which generally exhibit strong latrining behaviour (e.g., for scent marking), may act to reduce livestock exposure to their faeces and pathogens. However, wildlife host species that deposit a greater number of faecal defecations across the pasture (e.g., deer) may lead to increased cattle exposure to their faeces and pathogens. Additionally, in wildlife host species that display flexible defecation behaviour (e.g., badgers at lower densities), those individuals which deposit a greater number of single defecations in the environment may increase cattle's exposure to their faeces compared with individuals that deposit their faeces at single defecation sites. These results support Hutchings, Service, and Harris (2001) who proposed that the more dispersed faecal patterns from low-density badger populations may increase cattle contact rates with pasture contaminated with badger faeces and thus increase the risk of cattle exposure to bTB; an effect that could offset the benefits of badger population reduction in response to TB in cattle. This is also consistent with the recent findings of perturbation effects of badger population reduction on bTB risk to cattle, that is, the risk of bTB to cattle can increase after badger culling.

9. Grazing management of livestock affects their exposure to bTB via the faecal-oral route: In grazing systems, heterogeneous distributions of forage resources and faeces result in localised accumulations of nutrients and parasites, creating trade-offs between the costs of exposure to infection and the benefits of nutrient intake. Each contact between livestock and faeces in the environment is a potential transmission event. Thus, livestock must make foraging decisions in complex environments which will affect their intake of both nutrients and pathogens. However, the pattern of forage and faecal resources in agricultural environments will also be affected by the grazing management system in place. To investigate the effect of grazing management on the risk of infection to livestock partner 5 used a spatially explicit individual based stochastic foraging model to simulate livestock contact (both grazing and investigative) with faeces in the environment. The model was parameterised to simulate cattle grazing under three types of grazing management: set stock (i.e. where sward growth and cattle intake are in equilibrium in a single field); a two pasture rotation grazing system with increasing number of rotations; and a rotational grazing system with two rotations and increasing subdivisions of the pasture. Overall the amount of cattle contact with faecal-contaminated patches was similar in both set stocking and rotational grazing scenarios, suggesting no difference in the risk of infection or infestation between the different systems. However, the timing and absolute amounts of peak contact varied greatly indicating that different grazing management systems expose livestock to risks of different types of pathogens at different times of the grazing season. Intensive rotational systems with small pasture blocks (especially the first grazing period) maximised livestock contact with fresh faeces, and thus exposure to microparasites (e.g. bacterial pathogens such as bTB). Livestock reentering pasture blocks in rotational systems and set stocked livestock had the highest contact with old faeces and thus have a greater risk of macroparasite transmission (gastrointestinal nematodes). This study highlights how livestock management affects the highly dynamic interaction between livestock and distributions of parasites in the environment and thus the levels of livestock exposure to parasites and pathogens via the faecal–oral route.

10. Inter- and intra-specific exposure to bTB via the faecal-oral route: SAC used a foraging model to simulate the grazing behaviour of beef cattle in two grazing systems to compare the relative inter-specific and intra-specific exposure risks to parasites/pathogens. The results of the simulations, in combination with the often greater amounts of livestock *vs.* wildlife faeces in the agricultural systems, highlight the far greater risk of intra- *vs*. inter-specific parasite/pathogen exposure risk via the faecal–oral route. However, under certain conditions, particularly for microparasite infections such as bTB in badgers, wildlife faeces can also pose a significant risk of exposure to pathogens. Our model has quantified how this risk can be modified by different patterns of cattle avoidance behaviour, wildlife faecal deposition in the environment and agricultural grazing systems, with rotation grazing systems posing a greater parasite/pathogen exposure risk to grazing cattle compared to set-stock grazing systems. These risks can be

enhanced when cattle are first turned out onto pasture and in situations where intra-specific variations in wildlife behaviour result in more dispersed defecation patterns.

11. Identification of risk factors for the wildlife-livestock transmission of bovine tuberculosis in south central Spain: In Spain, cattle test-and-slaughter schemes have dramatically reduced bTB levels, but a wildlife reservoir of the disease is thought to be preventing its total eradication in certain regions. Identifying the risk factors for transmission between wildlife and livestock will help to inform the development of improved management strategies to combat the disease. In a case control study, SAC used a mixed-methods approach to identify key risk factors for bTB. Partner 5 combined a farmer-based questionnaire and participatory mapping with government records from bTB testing schemes in Almodovar, Spain. Questionnaire data were collected from a mixture of bTB-free and infected farms, yielding a total sample size of 73 farms based on a 94% response rate. Generalised linear modelling was used to identify the risk factors most strongly associated with the presence of bTB on a farm, and farmers were also asked their opinions on bTB and wildlife management. The main risk factors were the cattle testing procedure used, the number of streams per hectare on the farm and the presence of wildlife. Farmers' opinions about wildlife and bTB were influenced by their experience of the disease on their farm, whether they were a hunter, and the presence of wildlife on their farm. As wildlife appear to play such an important role in bTB risk, excluding large game wildlife species and reducing the attractiveness of the farm to them could reduce disease transmission. Strict and independent cattle bTB testing will reduce any bias, improving the accuracy of surveillance, which is important for well-informed management decisions. Changes in farm management such as improving biosecurity, providing alternative water sources and/or limiting access to streams, and improvements to pre-testing of cattle before movement between farms are recommended.

D. THE POTENTIAL IMPACT (INCLUDING THE SOCIO-ECONOMIC IMPACT AND THE WIDER SOCIETAL IMPLICATIONS OF THE PROJECT SO FAR) AND THE MAIN DISSEMINATION ACTIVITIES AND EXPLOTATION OF RESULTS.

D.1. POTENTIAL IMPACT

WP1. Vaccination of domestic animals (cattle and goats).

Tuberculosis in livestock (e.g. cattle and goats) is prevalent in UK, Republic of Ireland, Portugal, Spain, Italy and Greece, with sporadic outbreaks in other European countries. Livestock tuberculosis causes significant economic losses and is also of zoonotic importance. Its effective control and eradication is therefore important for animal and human health and for sustaining dairy and meat production industries. The data generated in this work package has provided information that could inform control policy decisions. Vaccination in general is a cost-effective way to impact on disease control and improved diagnosis could also have a direct impact on disease control. Whilst vaccination of cattle against tuberculosis is currently prohibited under EU and National legislation. The data generated in this work package on cattle vaccination and DIVA assays could be instrumental in allowing an informed discussion and review of this legislation. Vaccination of other ruminant against tuberculosis is not prohibited and the data generated in goats can therefore have a direct impact on disease control discussions. Vaccination will be more acceptable by wider strata of society than slaughter of animals, in particular when culling of wildlife species is involved. Therefore a wider societal implication of livestock vaccines could be the easier acceptance of wildlife control measures based on culling.

WP2. Evaluation of vaccines for use in wildlife populations in the EU.

Mycobacterium bovis is the causative agent of tuberculosis in domestic and wild animals. Across the world *M. bovis* infection is found in many wildlife species. The principal wildlife species involved include European badgers (*Meles meles*) in Ireland and the United Kingdom (UK), white-tailed deer, (*Odocoileus virginianus)*, bison (*Bison bison*) and elk (*Cervus canadensis*) in North America, wild boar (*Sus scrofa)* and deer species in mainland Europe, and the African buffalo (*Syncerus caffer*) in southern Africa and the brushtail possum (*Trichosurus vulpecula*) in New Zealand.

The research conducted in WP2 related to different aspects of wildlife vaccination and the results have made significant advances towards development of a wildlife vaccine. The studies conducted by Partner 3 (NUiD) have shown that oral vaccination with BCG is effective in badgers and that baits can be developed to deliver the vaccine to large populations. Such a study is currently being conducted in Ireland in a large scale badger vaccine field trial and it hoped to introduce strategic oral vaccination into the national control program in years to come. The BCG is also very effective in wild boar in Spain (Partner 2, UCM/UCLM), they have also shown that a heat-inactivated *M. bovis* is also an effective vaccine in wild boar. Diagnostic biomarkers have also been evaluated that can potentially be used as markers for immune protection. Subject to results of ongoing studies on biosafety aspects, the vaccine studies on the wild-boar will lead to delivery strategies to target large populations. Future research in alternative vaccines will be required either to replace or improve the performance of the BCG vaccine. Rapid progress has been constrained by the lack of model systems to evaluate vaccines. This has been addressed by Partner 6 (QUB) who have developed a ferret model vaccine/challenge model to test vaccines. This could be invaluable as it provides a means for rapidly indentifying vaccine candidates that can be frought forward for field-testing. With the large numbers of species affected in the game reserves in South-Africa, the problem of non-specific reactions to different diagnostic tests caused by environmental bacteria serves as an impediment to implementing control programs. The studies conducted by Partner 12 (UP) have investigated the underlying immunological processes that influence reactivity and have identified gene expression biomarkers that appear to distinguish between specifc and non-specific reactions. This is likely to be very useful for assessing the effect of BCG vaccination in the national parks in South Africa.

The results of the WP2 studies provide scientific support for the incorporation of BCG vaccination into the national tuberculosis control and eradication programmes. The BCG vaccine is currently the only vaccine licensed for use against tuberculosis. It is a very safe vaccine with a low prevalence of significant adverse reactions where it has been tested. The BCG has certain properties that make it attractive as a potential wildlife vaccine: it is inexpensive to produce, can be administered via a number of routes, including oral delivery, is effective after a single dose, is safe and has efficacy in reducing haematogenous spread of virulent mycobacteria. From the scientific data generated in WP2, there is reason to be optimistic that wildlife vaccination can be implemented on a large scale in the near future and that it will contribute to the control and ultimately the eradication of tuberculosis from cattle wherever there is a wildlife reservoir of *M. bovis* infection. Acceptance of wildlife vaccination as a realistic and feasible control strategy will directly animal and human health (particularly in developing countries) and will benefit society efforts to preserve wildlife diversity.

WP3. Development of new glycolipid antigens of *M. bovis*.

The use of previously neglected (glyco)lipid antigens for the use of diagnostics for *M. bovis* infections in cattle and wildlife has been investigated in this work package. For *M. avium subsp. paratuberculosis* and *M. avium* subsp. *avium* promising antigens have been found that could be used for diagnostics of mentioned mycobacterial infections or as complementary diagnostics to existing *M. bovis* serodiagnostics. However, no *M. bovis* (glyco)lipids have been isolated that could be used as a specific antigen in *M. bovis* serology.

Neverthless, in this project a large number of *M. bovis* derived (glyco)lipids have been examined and characterized for their potential use in diagnostic assays. The development of a highly sensitive immune-TLC protocol in this project will facilitate further studies expanding the number of detectable antigenic (glyco)lipid candidates. In this workpackage a number of lipids were characterized as having a significant T-cell antigenicity. Even though, they were shown thus far to have a limited value in diagnostics, they remain potential candidates for the development of a (DIVA) vaccine against tuberculosis. The conditions for the CMI assays will need to be optimized further, especially with respect to repeatability. In particular, for to be able to study the correlates of protection needed to be provoked by potential vaccine candidates in further studies.

WP4. Improvement of immunology-based diagnosis in wildlife and livestock.

Since antibody responses against *M. bovis* may be important in natural infections of wildlife species and may be used for tuberculosis surveillance and treatment monitoring one objective for this WP was the development of an affordable serological test for use in wild animals. In this sense, Partner 10 (Ingenasa) has developed an immunological assay able to detect specific antibodies to *M. bovis* in different species. Initially, several assays were tested (competition assays, double recognition enzyme-linked immunosorbent assay-DR/ELISA and Lateral flow assay-FLA) although the DR-ELISA expressing the MPB83 recombinant protein was finally selected since it is a rapid and sensitive assay to recognize not only IgGs but also other immunoglobulins, such as IgMs.

Nowadays, the tuberculosis diagnostic tests are based on the detection of the cellular immunity mechanisms. The skin tests rely on intradermal testing with the purified protein derivative (PPD) of *M. bovis*, often compared to that of *M. avium*. The IFN-γ detection test is based in the same principle, but the stimulation with PPDs is performed on whole blood cultures. The target of this WP was the evaluation of the performance of the antigens regarding specificity and sensitivity in livestock. Partner 1 (UCM) performed field studies to evaluate the sensitivity of the IFN-γ assay for tuberculosis diagnosis using PPDs and a peptide cocktail (ESAT-6 and CFP-10) in caprine flocks as well as to evaluate two antigenic cocktails containing ESAT-6, CFP-10 and Rv-3615c in the intradermal test and the IFN-γ assay for diagnosis of bovine tuberculosis. Moreover, partner 1 and 9 (CAO) performed studies to determine the effect of other mycobacterial infection (non-tuberculous

mycobacteria, vaccination against paratuberculosis-PTB, *Corynebacterium pseudotuberculosis* infection) in diagnosis.

An improved version of BOVIGAM®, BOVIGAM® 2G was developed by partner 11 (Prionics) with better reproducibility, repeatability and robustness. Lelystad tuberculin PPD antigens (avian and bovine) (Prionics, Switzerland) or a synthetic peptide cocktail, developed by Prionics AG, were used for stimulation of whole blood cultures. Diagnostic sensitivity of the peptide cocktail PC-HP was not significantly different from PPD whereas peptide cocktail PC-HP resulted in diagnostic specificity that was significantly higher than stimulation with PPD. These studies show that peptide cocktails have superior performance compared to tuberculin PPD in the BOVIGAM® 2G assay and open up new possibilities for highly specific diagnostic tools in the eradication of bovine TB. Moreover, partner 11 designed a transportation device which ensures stable temperature of 37°C inside the box for 120 hours when the environmental temperature is between -10°C and 42°C therefore this transport device is suitable to be used as a complete in tube stimulation solution for facilitating the collection of blood, transportation and stimulation in order to detect TB infected cattle. Within this WP, Partner 8 (ISS) developed a pig IFN- γ test for the detection of TB infected animals.

WP5. Control of populations.

The findings in this WP have significantly contributed to: (1) improved wildlife population monitoring and disease monitoring; (2) better understanding of wildlife TB epidemiology and identification of wildlife-related risk factors for bTB maintenance; and (3) development of wildlife disease control tools. This workpackage 5 has significantly contributed to confirm the role of wildlife hosts in bTB maintenance, as well as in the development of tools suitable for TB control in wildlife. One new test (ELISA TB test for wild boar) has been transferred to a SME, and three tools for wild boar TB vaccination (oral bait, selective delivery cage, and MdR vaccine) have been send out for patenting. These tools are ready for transfer to private companies. Moreover, this research has significantly contributed to the view that diseases are shared between wildlife and livestock, and that disease control strategies need to be integrated and consider both compartments.

WP6. Molecular typing of *M. bovis* and *M. caprae* isolates: focused epidemiological investigation.

The impact obtained with the results of this WP is related to the improved knowledge of epidemiology of animal tuberculosis that can be applied to the design and monitoring of the eradication campaigns; this would result in better use of resources and would reduce public health risks.

A technical summary report on *Mycobacterium caprae* was submitted to the EU Commission. The report intended to present an update of the current knowledge on this pathogen including main topics (Introduction: *Mycobacterium caprae*; Taxonomy; Role as an animal pathogen; role as zoonotic agent; Epidemiology; Relevant legislation, and a list of scientific references). The target of the report was to offer the knowledge background that could help to solve the EU concern about the legal problem associated to the identification of the pathogen in a herd because taxonomically is now considered to be independent from *M. bovis*. The report offers compelling evidences that show that *M. caprae* is a pathogen that poses a serious problem for Animal Health not only for goats but also to other domestic and wild animal species, and it has been demonstrated to be a human pathogen. According to the role of *M. caprae* in animal tuberculosis, relevant legislation should be adapted to address the infection, and notification and management of outbreaks caused by this pathogen should be performed as with *M. bovis.* The conclusion may be taken into account within the current EU and national legislation in the eradication programmes and reporting system. Information described in the report was based on results obtained during the implementation of research described in this project.

This workpackage has delivered progress in the tuberculosis eradication campaigns in the EU affected countries where the disease is present in both domestic and wildlife populations. Knowledge of the transmission of the infection between domestic animals and wildlife resulted in adequate ways to control infection. Thus, the epidemiological situation of bovine and wildlife tuberculosis were communicated inside the Hungarian veterinary authority and for broader professional public. Being fully aware of the role of wildlife tuberculosis on bovine outbreaks are very important to prevent spreading of the pathogen to cattle with making relevant actions like fencing the pastures of cattle herds. Strict pre-movement and annual testing is important to prevent spreading between and inside herds. Increasing awareness of tuberculosis helped to realize this by veterinarians. The actions taken by the veterinary authority and farmers resulted significantly decreasing incidence of bovine tuberculosis last years.

In Italy, acquired information shed new light on the possible role of the pig in the diffusion of *M. bovis* especially in areas where there tuberculosis has epidemiological overlaps between ruminant and pig populations. In addition, partner 8 validated a diagnostic tool to diagnose pigs infected with *M. bovis* and this tool could practically contribute to the control of tuberculosis in endemic areas where pig population plays a role as true reservoir. Moreover, genotyping by spoligotyping and MIRU-VNTR has become a valuable tool in the study of *M. bovis* epidemiology, allowing investigators to better identify the sources of infection and achieve a wider knowledge of TB transmission routes. The data obtained allowed to understand the importance of the Sicilian black pig in the transmission and maintenance of TB in this region. On the basis of this knowledge, specific eradication plans could be implemented.

WP7. TB risk and control in EU systems.

The potential impact of WP7 is through improved predictive power of epidemiological and risk models of bTB that can be used to improve bTB control.

(1) Risk based bTB surveillance to protect and reduce the incursion risk in bTB-free areas. The risk maps produced in WP7 are the starting point for the development of targeted surveillance strategies. Research from the WP7 team has recently shown that in the UK the risk of bTB in low risk areas is largely determined by cattle movements whereas in high risk areas it is largely determined by the history of disease and the probability of badger occurrence. The risk maps of WP7 highlight the locations of rich bTB host communities and thus those that are currently infected and those at risk of developing a persistent bTB problem following an initial introduction event (e.g. via between farm movements of infected cattle). As such they can inform more targeted use of surveillance resources to monitor bTB in infected areas and protect bTB free areas from bTB introduction.

(2) Increased efficiency of bTB control through improved predictive power of epidemiological models used to inform the development of bTB control strategies. The detailed badger bTB epidemiological modelling work suggests that bTB vaccines could improve bTB control in badger populations and highlights the high sensitivity of control in badgers to sources of external infection (e.g. from another host such as cattle). However, the more generic modelling in WP7 has highlighted a number of key modelling components and approaches that could improve the predictive power of the bTB specific models and that should be included in future bTB modelling frameworks used to inform disease control strategies. The numerical description of the perturbation effect is a useful step in improving the predictive power of models as it indicates why previous bTB models failed to predict the perturbation effect and characterised the effect along with the likely knock-on consequences for control. The perturbation effect highlights the complex nature of disease systems and the role of ecology in the persistence and spread of wildlife disease. The generic modelling approaches used in WP7 to understand the role of ecology in disease persistence and the perturbation effect will inform future bTB modelling but also how other wildlife diseases are modelled. Future bTB epidemiological models that

incorporate the findings of the TB-STEP project will be better placed to predict the disease control benefits of the new epidemiological tools (e.g. vaccines & diagnostics) and to inform the development of future bTB control strategies that are likely to be based on combinations of the control options considered in this project.

D.2. DISSEMINATION ACTIVITIES AND EXPLOTATION OF RESULTS

A) Dissemination activities.

1. Bovine Tuberculosis: Analyzing the parameters of the interferon gamma assay and improved diagnosis with new antigens. Oral Presentation. Greensboro, North Carolina, US 22-27 October 2008. PRIONICS; **2.** The role of wild boars in sustaining bovine TB in the Zselic region of Hungary. Presentation. Budapest (Hungary) 27/January/2009. CAO; **3.** Annual TB-STEP report. Annual report to Dutch Ministry of Agriculture. March 2009. The Hague. CVI; **4.** Enfermedades compartidas entre el Ganado bovino y la fauna Silvestre. Conference, A Coruña, Spain, 6-8/05/2009. IREC-UCLM; **5.** Caracterización molecular de aislados del complejo *M. tuberculosis* mediante espoligotipado y MIRU-VNTR. Madrid. 01/06/2009 - Oral communication. UCM; **6.** Wildlife reservoirs of intracellular pathogens. Conference, Murcia, Spain 14-16/06/2009. IREC-UCLM; **7.** Importancia de la identificacion de los agentes causantes de micobacteriosis en animales. Madrid. 17/06/2009 - Poster communication. UCM; **8.** Puesta a punto y resultados preliminares del empleo de la citometría de flujo para la medición del IFN-gamma intracelular producido por los linfocitos CD4+ y CD8+ en animales con tuberculosis y/o paratuberculosis. Madrid. 17/06/2009 - Poster communication. UCM; **9.** Factores técnicos que afectan a los resultados de la prueba de detección del IFN-y utilizada en el diagnóstico de la tuberculosis bovina y caprina. Madrid. 17/06/2009 - Oral communication. UCM; **10.** Characterization of a new antigenic lipopeptide of Mycobacterium paratuberculosis. Conference. Inverness 26,27/5/2010. CVI; **11.** Tuberculose Bovina em ungulados selvagens em Espanha. Relação com os animais domésticos. Diagnóstico necessário e sua limitação. Portugal, Faro. 04/07/2009 - Oral communication. UCM; **12.** Wildlife and the epidemiology of bovine TB in the Iberian Peninsula. Conference, Portugal 04/07/2009. IREC-UCLM; **13.** Programas de erradicación en tuberculosis. Badajoz. 17/07/2009 - Oral communication. UCM; **14.** Tuberculosis in goats: diagnostic test results and correlation with macroscopic lesions. M.bovis V International Conference, Nueva Zelanda, Wellington. 25/08/2009 - Poster communication. UCM; **15.** Proper identification of the causative agents of animal micobacteriosis for the Tuberculosis Eradication Programme. M.bovis V International Conference. 25/08/2009 - Oral communication. UCM; **16.** High discrimination of MIRU-VNTR technique for the most frequent spoligotype in Spain. M.bovis V International Conference, Nueva Zelanda, Wellington. 25/08/2009 - Oral communication. UCM; **17.** Flow cytometry for the study of tuberculosis and paratuberculosis infection in goats. M.bovis V International Conference, Nueva Zelanda, Wellington. 25/08/2009 - Oral communication. UCM; 1**8.** OmpATb: A novel antigen for the diagnosis of bovine tuberculosis. Poster. Wellington, New Zealand 25-28 August 2009. PRIONICS; **19.** Improved performance of the interferon gamma (IFN-γ) assay with alternative antigens for stimulation of whole blood. Oral Presentation. Wellington, New Zealand 25-28/08/2009. PRIONICS; **20.** Development of a second generation BOVIGAM® interferon gamma (IFN-γ) assay. Oral Presentation. Wellington, New Zealand 25-28 August 2009. PRIONICS; **21.** Progress in the Control of Bovine Tuberculosis in Spanish Wildlife. Conference, Wellington, New Zeland, 25/08/2009. IREC-UCLM; **22.** Mycobacterial infections in wild ungulates in Doñana national park, Spain. Conference, Wellington, New Zeland 25/08/2009. IREC-UCLM; **23.** TB-STEP.Strategies for the eradication of bovine tuberculosis. VLA International Conference Animal Diseases 2009, Veterinary Laboratories Agency. Department for Environment, Food and Rural Affairs, United Kingdom, London. 02/09/2009 - Poster communication. UCM; **24.** Improvements to the BOVIGAM® interferon gamma (IFN-G) assay for use with alternative antigens as stimulants of whole blood cultures. Oral Presentation. London, UK 2-4 September 2009. PRIONICS; **25.** Papel del diagnóstico molecular en la investigación epidemiológica de los focos de tuberculosis

bovina. Aspectos practicos de los programas de Sanidad Animal, Cantabria. 01/10/2009 - Oral communication. UCM; **26.** Evaluation of a second generation BOVIGAM® interferon gamma (IFN-γ) assay with alternative antigens for stimulation of whole blood cultures. Oral Presentation. San Diego, California, US 7-14/10/2009. PRIONICS; **27.** Tuberculosis in cattle and other animals in Hungary: actual data and risk for humans . Annual Congress of Hungarian Society on Zoonoses, Hungarian Society on Zoonoses, Hungary, Tiszafüred. 16/10/2009 - Oral communication. UCM; **28.** Tuberculosis bovina: una revision de los agentes etiologicos, patogenesis e implicaciones de la infeccion. Procedimientos para la toma de muestras para el cultivo microbiologico de tuberculosis bovina, Valencia. 19/10/2009 - Oral communication. UCM; **29.** Técnicas de diagnóstico in vivo: prueba de la tuberculina y test de interferon-gamma. Procedimientos para la toma de muestras para el cultivo microbiologico de tuberculosis bovina, Universidad CEU Cardenal Herrera, España, Valencia. 19/10/2009 - Oral communication. UCM; **30.** Inspeccion post-mortem: localizacion de las lesiones, recogida y envio de muestras. Normativa. Procedimientos para la toma de muestras para el cultivo microbiologico de tuberculosis bovina, Valencia. 19/10/2009 - Oral communication. UCM; **31.** Herramientas de diagnostico en el laboratorio: cultivo e identificacion. Aplicacion de la caracterizacion molecular para fines epidemiologicos. España, Valencia. 19/10/2009 - Oral communication. UCM; **32.** Diagnóstico de la tuberculosis bovina: interferencias diagnosticas con otras micobacterias. Actualizacion en tuberculosis bovina, Madrid. 29/10/2009 - Oral communication. UCM; **33.** Efecto de la alimentación suplementaria sobre las características de la historia natural den crías de ciervo ibérico en zonas mediterráneas. Conference, Bilbao, Spain 4-7/12/2009. IREC-UCLM; **34.** Avanzando hacia el control de la tuberculosis en el jabalí. Conference, Bilbao, Spain 4-7/12/2009. IREC-UCLM; **35.** Contribución relativa al uso de carroñas de ungulados por la comunidad de vertebrados, factores determinantes y riesgos asociados a la transmisión de enfermedades en áreas mediterráneas. Conference, Bilbao, Spain 4-7/12/2009. IREC-UCLM; **36.** Evolución temporal de la prevalencia de la tuberculosis bovina en el jabalí de la Península Ibérica. Conference, Bilbao, Spain 4-7/12/2009. IREC-UCLM; **37.** Diagnóstico de la tuberculosis bovina: prevalencia, diagnóstico y epidemiología. Curso de actualización para ganaderos. Piedrahita, Avila. 10/12/2009. UCM; **38.** bTB in the UK. Conference, 14/01/2010. London, UK. : Scientific community (Society for Applied Microbiology): UK. SAC; **39.** Atypical Mycobacterium infections of the Hungarian Cattle Herds, diagnostic difficulties. Presentation. Budapest (Hungary) 26/January/2010. CAO; **40.** TB-STEP, WP2/WP6 vaccine sub-group. Presentation, Madrid 22/2/2010. NUiD; **41.** TB-STEP. Presentation, Madrid 21/5/2012. QUB; **42.** Annual TB-STEP report. Annual report to Dutch Ministry of Agriculture. March 2010. The Hague. CVI; **43.** Diagnóstico de la tuberculosis bovina. Spain, Zaragoza. 28/04/2010 - Oral communication. UCM; **44.** Characterization of a new antigenic lipopeptide of Mycobacterium paratuberculosis. Workshop. Torino, 17-20/6/2009. CVI; **45.** Experiences and challenges in wildlife disease monitoring: examples regarding mycobacterial infections in Spain. Conference. Misiones, Argentina 30/5–4/6/2010. IREC-UCLM; **46.** Diagnóstico laboratorial y nuevas herramientas de epidemiología molecular. Jornadas de debate sobre la erradicación de la Tuberculosis Bovina, Spain, Santander. 29/06/2010 - Oral communication. UCM; **47.** Non-tuberculous Mycobacteria in Animals in Hungary. Conference. Bled (Slovenia). 4-7/July/2010. CAO; **48.** Gamma-inteferon assay for tuberculosis diagnosis in cattle and goats: study of factors affecting results of the assay. Slovenia, Bled. 04/07/2010 - Poster communication. UCM; **49.** Spanish database of animal mycobacteriosis (mycoDB): application in epidemiological studies. Slovenia, Bled. 04/07/2010 - Oral communication. UCM; **50.** Molecular epidemiology underlines the importance of Mycobacterium caprae in livestock and wildlife. Slovenia, Bled. 04/07/2010 - Oral communication. UCM; **51.** Mycobacterium tuberculosis infection in cattle. Slovenia, Bled. 04/07/2010 - Poster communication. UCM; **52.** The role of Mycobacteria in Veterinary Medicine. Slovenia, Bled. 05/07/2010 - Oral communication. UCM; **53.** The role of red deer, fallow deer and roe deer in the epidemiology of bovine TB and paratuberculosis in southwestern Europe: consequences for disease control. Conference, Neltume, Chile 01-06/08/2010. IREC-UCLM; **54.** Tuberculosis and other mycobacterial infections of wildlife in

Hungary. Conference. Vlieland, (The Nederlands) 12-17/September/2010. CAO; **55.** TB in European Wildlife: whats new?. Conference, Vlieland, Holanda 13-16/9/2010. IREC-UCLM; **56.** Field epidemiology of wild boar-liverstock interactions in South Central Spain. Conference, Vlieland, Holanda, 13-16/9/2010. IREC-UCLM; **57.** Comparative study of different antigenic preparations in the development and validation of an enzyme-linked immunosorbent assay for antibodies against Mycobactarium bovis in red deer. Conference, Vlieland, Holanda 13-16/9/2010. IREC-UCLM; **58.** Is the long-term monitoring of E.cervi L1 excretion in red deer a sensitive indicator of changes in population and management?. Conference, Vlieland, Holanda 13-16/9/2010. IREC-UCLM; **59.** Preliminary results of the prevalence and distribution of micobacterial infections in eurasian badgers (meles meles) in Spain. Conference, Vlieland, Holanda 13-16/9/2010. IREC-UCLM; **60.** Capture, chemical immobilization and post-release monitoring of wild boar (Sus scrofa) in South Central Spain. Conference, Vlieland, Holanda 13-16/9/2010. IREC-UCLM; **61.** Problemas (y soluciones) en el diagnóstico de la tuberculosis. Zoonosis: enfermedades clásicas y emergentes, Universidad Internacional del Mar. Universidad de Murcia, Spain, Murcia. 17/09/2010 - Oral communication. UCM; **62.** Actualización en tuberculosis bovina. Curso de actualización para ganaderos. Curso de actualización para ganaderos. Piedrahita, Avila. 02/11/2010. UCM; **63.** Diagnóstico laboratorial de tuberculosis: Gamma interferón y espoligotipado. Spain, Santiago de Compostela. 03/11/2010 - Oral communication. UCM; 6**4.** bTB dynamics and control. Research briefing: 16/11/2010: London, UK: Science & Policy; UK. SAC; **65.** Control Strategies for bovine tuberculosis. Conference, Santiago 16/11/2010. NUiD; **66.** Progress toward an experimental vaccine-challenge model in ferrets. Meeting, VSD, Belfast 1/ 2011. QUB; **67.** Occurrence of TB-suspicious lesions in wild boars between 2008-2010 in the Zselic region of Hungary. Presentation. Budapest (Hungary) 25/January/2011. CAO; **68.** Diagnostic methods at the Central Agricultural Office to prove paratuberculosis infections between 2006-2010. Presentation. Budapest (Hungary). 25/January/2011. CAO; **69.** Diagnóstico de Tuberculosis Bovina. Jornada Técnica sobre Tuberculosis Bovina, Spain, Toledo. 26/01/2011. UCM; **70.** Annual TB-STEP report. Annual report to Dutch Ministry of Agriculture. March 2011. The Hague. CVI; **71.** El proyecto TB-STEP avanza en la erradicación de la tuberculosis. Madrid. 2011. UCM; **72.** Comparative mycobacterial diseases. Symposium, Tampa, USA 16/3/2011. QUB; **73.** Many Hosts of Mycobacteria. Conference, Tampa, Florida 21-23/3/2011. IREC-UCLM; **74.** Epidemiología y su aplicación al diagnóstico de campo de la tuberculosis bovina y caprina. Importancia del uso de pruebas complementarias. Valladolid. 13/04/2011. UCM; **75.** Epidemiología molecular e interferencias diagnósticas de la tuberculosis bovina. Xornada Técnica Galicia 2011, Spain, Santiago de Compostela. 15/06/2011. UCM; **76.** Molecular Characterization of Hungarian Mycobacterium Avium Complex Isolates. Conference. Lübeck (Germany) 25-29/June/2011. CAO; **77.** The Perturbation Effect in wildlife diseases: An emergent behaviour of simple models. *8th European Conference on Mathematical,and Theoretical Biology*, 29/06/2011: Krakow, Poland. SAC; **78.** Vaccination of wildlife against tuberculosis. Presentation, Dublin 9/7/2011. NUiD; **79.** Molecular Methods for the Identification of Mycobacterium Isolates with Animal Origin. Conference. Budapest (Hungary) 20-22/July/2011. CAO; **80.** Tuberculosis en fauna silvestre en España: monitorización y control. Conference, Mexico 28-30/7/2011. IREC-UCLM; **81.** Vaccination of wildlife with BCG to control tuberculosis. Conference, Mexico 29/7/2011. NUiD; **82.** Stochastic modelling in systems biology. Presentation made as discussant at *58th ISI Congress*, Dublin Ireland, 24/08/2011. SAC; **83.** Evaluation of a serological assay for the detection of bovine tuberculosis. AHVLA International Conference 2011, Animal Health and the Veterinary Laboratories Agency, United Kingdom, London. 15/09/2011. UCM; **84.** Use of antigenic cocktails in the skin test for in vivo diagnosis of bovine tuberculosis. AHVLA International Conference 2011, Animal Health and the Veterinary Laboratories Agency, United Kingdom, London. 15/09/2011. UCM; **85.** Training of Ethiopian staff in M. bovis culture, spoligotyping and VNTR. Capacity building. AHVLA Weybridge 30.09.2011. VLA; **86.** Technology transfer/training of methods of bovine macrophage and dendritic cell infection and RT-PCR cytokine profiling. Capacity building. AHVLA Weybridge, Inst. for Pathobiology Univ. Addis Ababa 30.09.2011. VLA; **87.** Control

Strategies for tuberculosis. Conference, Montevideo 3/11/2011. NUiD; **88.** Evaluación de pruebas diagnósticas de tuberculosis bovina mediante análisis bayesiano. Mexico, Merida, Yucatan. 22/11/2011. UCM; **89.** Diagnostics and vaccine development. Meeting, Dublin 22/11/2011. NUiD; **90.** Desarrollo de ensayos serológicos multiespecie para la detección en suero de anticuerpos frente a *Mycobacterium bovis*. Conference. Tenerife (Spain) 24/11/2011. INGENASA; **91.** El papel de la epidemiología molecular en el control de enfermedades endémicas; Tuberculosis. Curso de Formación sobre Epidemiología Molecular, Madrid. 01/12/2011. UCM; **92.** Stochastic modelling of ecology and epidemiology. *Theoretical Ecology Group Seminar*, 01/12/2011 University of Bergen, Norway. SAC; **93.** Ferret infections /immunology. Discussions, Copenhagen 3/12/2011. QUB; **94.** TB Vaccines. Conference, Copenhagen 4-5/12/2012. QUB; **95.** Actualities in the bovine and wildlife tuberculosis situation in Hungary. Presentation. Mór (Hungary) 7/February/2012. CAO; **96.** A new perspective on the perturbation effect: characterisation and emergence in simple models. *Maxwell Institute for Mathematical Sciences Statistics Seminar*, Heriot-Watt University, 2012, Edinburgh. SAC; **97.** Epidemiología molecular de *Mycobacterium bovis* y *Mycobacterium caprae* en España. Thesis. Universidad Complutense de Madrid. Facultad de Veterinaria. Madrid, 9th March 2012. UCM; **98.** Epidemiology of animal tuberculosis in Spain. Molecular characterisation. Seminario Universidad de Berna, Departement of Infectious Diseases and Pathobiology. Vetsuisse-Fakultät. Universität Bern, Switzerland, Bern. 28/03/2012. UCM; **99.** Vaccination of badgers is Ireland; what have we learned so far. Symposium, Belfast 14/5/2012. NUiD; **100.** International vaccine symposium. Organisation of Conference. Belfast 14/05/2012. QUB; **101.** Training course on genome sequence techniques and analysis. Capacity Building. Armauer Hansen Research Inst., Addis Ababa 15.-18.05.2012. VLA; **102.** TB-STEP. Presentations. Madrid, Spain 21/05/12. QUB; **103.** Development of multispecie serological assays to detect antibodies specific of *mycobacterium bovis* in serum samples. Poster. Brighton (UK) 12/06/2012. INGENASA; **104.** Bovine TB vaccine research at the Jenner Institute, Oxford. Strategic advisory board meeting. Oxford, UK 18/06/2012. VLA; **105.** Development of multispecie serological assays to detect antibodies specific of *mycobacterium bovis* in serum samples. Poster. Kazimierz Dolny (Poland) 1/07/2012. INGENASA; **107.** Spoligotyping and VNTR typing of Mycobacterium bovis and Mycobacterium caprae–a review. 33rd Annual Congress of the European Society of Mycobacteriology, European Society of Mycobacteriology, Romania, Brasov. 01/07/2012. UCM; **108.** The population structure of Mycobacterium bovis in Spain. 33rd Annual Congress of the European Society of Mycobacteriology, European Society of Mycobacteriology, Romania, Brasov. 01/07/2012. UCM; **109.** Factors affecting intradermal tuberculin test for the diagnosis of bovine tuberculosis. 33rd Annual Congress of the European Society of Mycobacteriology, European Society of Mycobacteriology, Romania, Brasov. 01/07/2012. UCM; **110.** Bovine TB vaccine development: Biomarkers of protection. International Conference. Merida, Mexico 19-22/08/2012. VLA; **111.** Caprine tuberculosis in Spain: etiology, diagnosis and epidemiology. 1ª Conferencia Regional sobre Mycobacterium bovis para las Américas, Universidad Nacional Autónoma de México, Mexico, Merida, Yucatan. 19/08/2012. UCM; **112.** Spoligotyping: study of different methods of DNA extraction. 1ª Conferencia Regional sobre Mycobacterium bovis para las Américas, Universidad Nacional Autónoma de México, Mexico, Merida, Yucatan. 19/08/2012. UCM; **113.** Diagnosis of tuberculosis in goats with a multiplex serological assay. 1ª Conferencia Regional sobre Mycobacterium bovis para las Américas, Universidad Nacional Autónoma de México, Mexico, Merida, Yucatan. 19/08/2012. UCM; **114.** Applications and limitations of epidemiological tools for the control and eradication of bovine tuberculosis. 1ª Conferencia Regional sobre Mycobacterium bovis para las Américas, Universidad Nacional Autónoma de México, Mexico, Merida, Yucatan. 19/08/2012. UCM; **115.** International vaccine symposium. Conference, Belfast 14-16/5/2012. QUB; **116.** Faculty day 2012, faculty of Veterinary Science, UP. Faculty Day. Pretoria 9/9/2012. UP.

B) Exploitation of resources.

1. Production and labeled of recombinant proteins in E.coli and Baculovirus. Commertial exploitiation of R&D results. INGENASA.

2. Development of an improved IFN-g assay for the detection of Tb infected cattle in terms of robustness, repeatability and reproducibility and Development of two peptide cocktails for the stimulation of whole blood samples for the detection of Tb infected animals. General advancement of knowledge. PRIONICS.

3. Commercial product (BOVIGAM® 2G). Commercial exploitation of R&D results. PRIONICS.

4. Commercial product (Prionics® PC-HP). Commercial exploitation of R&D results. PRIONICS.

5. Commercial product (Prionics® PC-EC). Commercial exploitation of R&D results. PRIONICS.

E. WEBSITE AND RELEVANT CONTACT DETAILS

Project website address: http://www.vigilanciasanitaria.es/tb-step/

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