VETBIONET

Veterinary Biocontained facility Network for excellence in animal infectiology research and experimentation

Deliverable 15.1

Quantity of access provided over the duration of the project to APHA

Due date of deliverable: M72

Actual submission date: M72

Start date of the project: March 1st, 2017 **Duration:** 72 months

Organisation name of lead contractor: APHA

Revision: V1

Dissemination level	
Public	**X**
Confidential, only for members of the consortium (including Commission Services)	
Classified, as referred to in Commission Decision 2001/844/EC	

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

1. TNA Provided

Name of the TNA project	Name of TNA user	Organisation of TNA user	Country of TNA user	Installation from the RI	Start date	End date	Actual costs (€)
Assessment of the immunogenicity of bovine herpes virus 4-based vectors delivering Nipah virus glycoproteins in swine	Gaetano Donofrio	Università di Parma	Italy	Cat2	Feb 2019	Apr 2019	40,878.21
Susceptibility of camelids to influenza C and D viruses	Gilles Meyer	Ecole Nationale Veterinaire de Toulouse	France	268	Aug 2021	Sep 2021	106,962.82
Pathogenesis/immunology of AFSV	Dolores Gavier-Widen	National Veterinary Institute	Sweden	265	June 2020	Aug 2020	78,299.41
Assessment of the immunogenicity of a bivalent porcine reproductive and respiratory syndrome / Nipah virus vaccine	Nanchaya Wanasen	National Center for Genetic Engineering and Biotechnology (BIOTEC)	Thailand	268	June 2021	Aug 2021	62,740.43
Harnessing local immunity for protection against influenza	Matthias Tenbusch	University Hospital Erlangen, Institute of Virology	Denmark	Cat2	Jan 2021	Feb 2021	14,139.85
Pathogenesis of Rift valley fever virus in an intranasal-challenged ferret model	Ignacio Garcia	University of Cordoba	Spain	265	Jan 2023	Feb 2023	71,623.98
Investigating transmissibility of moose chronic wasting disease to sheep	Sylvie Benestad	Norwegian Veterinary Institute	Norway	Cat 2	Sep 2021	Feb 2023	91,729.07
Transmission and pathogenesis of High Pathogenic Avian Influenza in wild bird galliform species	Lars Larsen	DTU Copenhagen	Denmark	265	Aug 2021	Aug 2021	91,729.07

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The role of MPB70, a Mycobacterium bovis secreted protein, in multinucleated giant cell formation and bovine tuberculosis (bTB) pathology	Ed Lavelle	Trinity College Dublin	Ireland	268	Jan 2022	Mar 2022	180,765.49

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2. Final reports of each TNA provided

1. TNA 1

Assessment of the immunogenicity of bovine herpes virus 4-based vectors delivering Nipah virus glycoproteins in swine

Aim:

Nipah virus (NiV) is an emergent pathogen capable of causing acute respiratory illness and fatal encephalitis in pigs and humans. A high fatality rate and broad host tropism makes NiV a serious public and animal health concern. There is therefore an urgent need for a NiV vaccines to protect animals and humans.

The objective of this project was to assess the immunogenicity of bovine herpesvirus (BoHV-4) vectors expressing either NiV attachment (G) or fusion (F) glycoproteins in pigs. The vaccines were benchmarked against a canarypox (ALVAC) vector expressing NiV G, previously demonstrated to induce protective immunity in pigs.

Experimental procedures:

Eighteen 8-10-week-old, weaned, female [48], large white-landrace- Hampshire cross-bred pigs were randomly allocated to three treatment groups (n=6) that were immunized with either ALVAC NiV G (1 × 108 PFU/dose in 1 mL; Boehringer Ingelheim Animal Health, Lyon France [26]. BoHV-4-A-CMV-NiV-GΔTK (1 × 106 TCID50/dose in 5 mL) or BoHV-4-A-CMV-NiV- FΔTK (1 × 106 TCID50/dose in 5 mL). The identity of the treatment groups was blinded to the laboratory investigators until the immune response data was collected and analyzed. Vaccines were delivered by intramuscular injection and animals received identical immunizations on days 0 (prime) and 21 (boost). Animals were monitored daily post-vaccination (dpv) and clinical signs and rectal temperature were recorded.

Nasal and rectal swabs were collected from BoHV-4 immunized pigs on -3, 1, 4, 7 and 11 dpv. Blood samples were taken from all pigs on a weekly basis at 0, 7, 14, 21, 28, 35, 42 dpv by venipuncture of the external jugular vein. 8 mL/pig in BD SST vacutainer tubes for serum collection and 30 mL/pig in BD heparin vacutainer tubes for peripheral blood mononuclear cell (PBMC) isolation. Pigs were euthanized at the end of the study (42 dpv) by pentobarbital overdose. Broncho- alveolar lavage (BAL) was collected from the left lung lobes with 100 ml of transport medium supplemented with field antibiotics [100 U/mL penicillin, 80 µg/mL neomycin, 160 µg/mL polymixin B, 3 µg/mL amphotericin B. BAL samples were centrifuged at 500 x g for 7 min and supernatant was collected and frozen for further antibody analysis. One pig from the BoHV-4-A-CMV-NiV-FΔTK group was removed from the study 0 dpv due to an underlying health condition unconnected to the study.

Collected samples were processed for further analysis including T cell IFN-γ ELISpot, surface and intracellular labelling assays, ELISA assay to quantify the presence of NiV-sG specific antibodies and quantitative RT-PCR.

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Conclusion

Both BoHV-4 vectors expressing either NiV attachment (G) or fusion (F) glycoproteins (BoHV-4-A-CMV-NiV- GΔTK and BoHV-4-A-CMV-NiV-FΔTK, respectively) induced robust antigen-specific antibody responses. BoHV-4- A-CMV-NiV-GΔTK stimulated NiV-neutralizing antibody titers comparable to ALVAC NiV G and greater than those induced by BoHV-4-A-CMV-NiV-FΔTK. In contrast, only BoHV-4-A-CMV-NiV- FΔTK immunized pigs had antibodies capable of significantly neutralizing NiV G and F-mediated cell fusion. All three vectored vaccines evoked antigen-specific CD4 and CD8 T cell responses, which were particularly strong in BoHV-4-A-CMV-NiV-GΔTK immunized pigs and to a lesser extent BoHV-4-A- CMV-NiV-FΔTK.

These findings emphasize the potential of BoHV-4 vectors for inducing antibody and cell-mediated immunity in pigs and provide a solid basis for the further evaluation of these vectored NiV vaccine candidates.

2. TNA 2

Susceptibility of camelids to influenza C and D viruses

Aim:

Influenza C and D viruses (ICV and IDV), in addition to influenza A virus, have non-human maintenance hosts and likely circulate in several mammalian species. Camelids, as a reservoir for zoonotic viruses, were not properly studied until the emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012.
The applicant recently identified anti-ICV and/or anti-IDV antibodies in Kenyan dromedary camels [3]. It was however impossible to distinguish between anti-ICV and IDV antibodies due to their cross-reactivity. In addition, screening of a few hundred Middle Eastern camelid nasal swabs for the presence of ICV and IDV genetic material did not detect RNA of either virus in these clinical samples.

The general aim of the project was therefore to assess the putative role of camelids in the IDV ecology, by investigating IDV replication in camelids in an experimental setting.

The specific questions addressed in the study were:
1. Are ICV and/or IDV able to successfully replicate in camelids?
2. With or without viral replication, are anti-ICV and/or anti-ICV antibodies generated post virus inoculation?
3. If ICV and/or IDV replicate in camelids:
- are they associated with any clinical signs?
- what is their tissue tropism?
- what is the duration of virus shedding?
- which is the induced host immune response?

We therefore experimentally inoculated seven camelids with ICV or IDV to assess: (i) clinical outcome with daily scoring; (ii) virus shedding in nasal swabs and in bronchoalveolar lavages

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(BALs), (iii) tissue tropism of the two viruses at necropsy on days 4 and 8 post infection, (iv) immune responses (in BALs), and (v) seroconversion at the end of the study.

Experimental procedures & Results:

Seventeen 1year-old healthy alpacas were purchased from a UK farm. The restrictive condition that the Alpacas included in this study should not have been previously infected with an influenza virus made the sourcing of animals difficult.

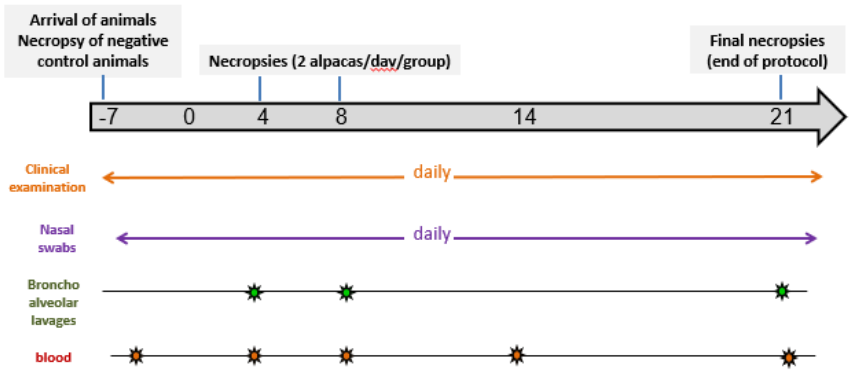
The animals were screened to be negative for anti-ICV and anti-IDV antibodies, as well as for their general health status before enrolment in the study. Animals were divided into three groups:

(i) negative controls (group 1, n=3) were euthanized and necropsied prior to the start of the study to obtain background and control samples (bronchoalveolar lavages (BALs), blood, tissues (nasal mucosa, trachea, right and left cranial lung lobes, accessory lobes, right and left caudal lung lobes, mediastinal and tracheobronchial lymph nodes, olfactory bulbs, kidneys, liver, spleen, mesenteric lymphatic nodes, duodenum, jejunum, ileum, and all organs showing macroscopic lesions)), and nasal swabs,

(ii) alpacas in group 2 (n=7) were inoculated with ICV; and (iii) alpacas in group 3 (n= 7) were accommodated in a separate room and were inoculated with IDV. All animals from groups 2 and 3 had a 7-days period of adaptation inside the ABSL-2 facilities before starting the experiment. BALs were collected on euthanized animals.

The following ICV and IDV strains were used: the Australian strain C/Victoria/1/2011 ICV and the French strain D/bovine/France/5920/2014 IDV. Both strains were already characterized and titrated at the ENVT and produced with a minimum number of passages in cell culture to avoid loss of virulence for a putative natural host. Alpacas were inoculated intranasally with 10^6 and 10^7 TCID50 in a 4ml volume (2ml/nostril) for ICV and IDV, respectively.

Alpacas were monitored for the development of any clinical signs (sneezing, coughing, nasal discharge, tachypnea, dyspnea, watery eyes, weakness, loss of appetite). Unintended clinical signs were notified. Respiratory frequencies and temperatures were taken daily and recorded during the whole experiment. Sampling of alpacas was performed following the experimental design shown below.



Study of the pathology induced by ICV and IDV in alpacas and study of virus distribution after experimental infection

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The pathogenicity of ICV and IDV in alpacas was assessed according to standardized methods currently used by the two partners. Clinical signs remained mild for most animals, and similarly the gross pathology and histology findings were unremarkable. Elevated temperature and slight diarrhea were observed in some animals in the ICV group. Nasal shedding of viral RNA was detected in nasal swabs between 1-7dpi for both viruses and peaked between 2-4dpi. Assessment of tissues confirmed the presence of RNA for both viruses in the upper respiratory tract at 3 and 7dpi. Additional RNAScope experiments - that were not included in the initial TNA application program- were carried out on site in 2022. They confirmed presence of viral RNA in the upper respiratory tract of inoculated animals.

Study of ICV and IDV induced immune response in alpacas

Serology was carried out on day 21 sera. Hemagglutination inhibition assay were used to assay anti-ICV antibodies and seroneutralization tests were used to assay anti-IDV antibodies. The tests confirmed seroconversion of ICV inoculated animals with the presence of anti-ICV antibodies and seroconversion of IDV inoculated animals with the presence of anti-IDV antibodies. Results were specific, as specifically non-inoculated animals were seronegative for both viruses, ICV inoculated alpacas did not harbor anti-IDV antibodies nor did IDV inoculated alpacas harbor anti-ICV antibodies.

The nature of the host immune response will be investigated at the applicant's laboratory through transcriptomic analyses by RT-qPCR. They have already designed primer pairs specific for more than 60 bovine genes of the innate and adaptive immune responses (IFN-I, PRRs, Interleukins, Chemokines…). Primers for 40 llama innate immune response genes used for previous MERS-CoV studies will be assessed on alpaca samples. RT-qPCR will be performed on nasal turbinate tissue and, if possible, on cells isolated from bronchoalveolar lavage fluid collected during necropsies (days 0, 4, and 8). These analyses are planned for 2023.

Conclusion

This study demonstrated that alpacas can be experimentally infected with both ICV and IDV with subclinical infection of the upper respiratory tract, suggesting that virus transmission could potentially occur. These findings, in agreement with previous serology results obtained for camelids, indicate a putative role for these species in ICV and IDV ecology. Further studies are needed to assess whether they may represent asymptomatic reservoirs for IDV.

A poster with some of the results was presented at the "OPTIONS XI for the control of influenza" congress (Belfast, sept 2022; https://www.optionsxi2022.org.uk/).

3. TNA 3

Pathogenesis/immunology of African Swine Fever Virus

Aim:

The overall objective of this TNA project was to perform a comparative analysis of the pathogenesis of ASF and the antiviral immunity developed in domestic pigs and wild boars during the early stages of viral infection.

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African swine fever (ASF) is a devastating hemorrhagic infectious disease caused by ASFV virus (ASFV), a large and complex enveloped DNA virus, which affects domestic and wild suids (all *Sus scrofa*) and constitutes the biggest threat faced by the world pork industry in decades. There is no treatment or effective vaccine commercially available.

Wild boars have been shown to be as susceptible as domestic pigs. In Europe, the disease established self-sustaining cycles within the wild boar population, which may contribute to its spread and maintenance by representing a major reservoir and serving as a source for arthropod vectors of the disease.

To date, experimental results in domestic pigs and wild boar have highlighted the variability in experimental outcome and severity of signs after experimental infections with genotype II isolates of African swine fever virus currently circulating in Europe. Furthermore, little is known about the pathogenic mechanisms or immune response induced by these genotype II isolates in domestic pigs and, to a lesser extent, in wild boars, despite their high susceptibility to the disease.

The specific objective of this TNA project consisted in performing an experimental infection with a highly virulent isolate of African swine fever virus in domestic pigs and wild boars and to compare the evolution of clinical signs, lesions, viremia levels, viral excretion, viral load in target organs and immune responses.

Experimental procedures:

The highly virulent ASFV isolate used (Armenia 07) was grown in porcine blood monocytes (PBM). Viral titres were determined as the amount of virus causing hemadsorption in 50% of infected cultures (HAD_{50}/ml).

Nineteen commercial domestic pigs of both sexes aged 10-12 weeks at the start of the experiment, and nineteen wild boars of both sexes, aged 16-18 weeks, were used. After the acclimatization period at BSL-3 facilities, thirty-two animals (16 domestic pigs and 16 wild boars) were sedated and then experimentally inoculated by intranasal route to mimic natural infection with a dose of 10^4 HAD_{50}/ml (1 ml per nostril). Pigs were randomly distributed in 4 rooms (8 animals per room). To avoid potential aggressive behaviour, domestic pigs and wild boars were not mixed in the same pen. Animals were randomly allocated before challenge to the time points they were euthanized. Six animals (3 pigs and 3 wild boar) were kept in separate accommodation as negative non-inoculated controls. These animals were mock infected with cell culture medium used to grow the virus.

During the experiment, clinical signs and temperatures were monitored daily. After intranasal inoculation, animals were euthanized in batches of 6 (3 pigs and 3 wild boars) at day 1, 2, 3 and 5 post- infection (pi). The remaining inoculated animals (4 pigs and 4 wild boars) were euthanized once they reached the clinical endpoint at day 6 (wild boars) and day 9 (domestic pigs) respectively. Control animals were euthanized at the end of the experiment (day 12 pi) once all inoculated animals had been euthanized.

EDTA blood and serum samples were taken from all animals twice prior to the inoculation (day -3 and 0 pi) to provide a baseline for leukopenia and other immunological parameters. After inoculation, EDTA blood and serum samples, along with swabs (nasal and fecal) were taken from inoculated animals at the time of euthanasia. Blood and serum samples were also taken from all control animals and from the remaining inoculated domestic pigs at day 8 pi.

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During necropsies, macroscopic evaluations were carried. A large range of tissue samples from all animals (infected and control non-infected) were fixed in 10% buffered formalin solution and routinely processed for subsequent histopathological and immunohistochemical studies. Tissue samples from tonsils, lungs, spleen, retropharyngeal, tracheobronchial and gastro-hepatic lymph nodes were also taken and frozen at -80ºC for subsequent ASFV genome detection by quantitative PCR (qPCR). Fresh tissue samples from tonsils were collected for cell phenotyping and characterization of local immune response and some were frozen at -80ºC for subsequent genomic and proteomic analyses.

Conclusion:

Infected wild boars showed an earlier increase in clinical signs and temperatures and shorter clinical courses than infected domestic pigs. In addition, wild boars that were euthanized once they reached a humane endpoint (day 6 dpi) displayed higher clinical scores and temperatures than those observed in domestic pigs euthanized once they reached a humane endpoint (day 9 dpi). However, in wild boars, the number and severity of macroscopic lesions characteristic of acute ASF were lower than those observed in domestic pigs throughout the experiment.

These results demonstrated that wild boars have a higher sensitivity and are less resilient to infection with a highly virulent isolate of ASFV. This set of data will fill important gaps in the knowledge of the differences in the pathogenesis of ASF between domestic pigs and wild boars and will contribute to a better understanding of the epidemiology of current ASF outbreaks in Europe and Asia.

4. TNA 4

Assessment of the immunogenicity of a bivalent porcine reproductive and respiratory syndrome / Nipah virus vaccine

Aim:

The overall objective of this project was to develop a bivalent vaccine targeting Nipah virus (NiV) using Porcine Reproductive and Respiratory Syndrome Virus 2 (PRRSV-2) as a vector to deliver the NiV antigen

NiVcauses acute respiratory disease and fatal encephalitis in humans. It also infects pigs, in which it causes a less severe disease. The development of a vaccine against NiV in pigs would help reduce the risk of future epidemics. Furthermore, since a PRRSV-2 live attenuated vaccine is already widely used and pigs in affected areas are exposed to both viruses most of the time, the objective was to develop a bivalent vaccine targeting these two economically important diseases in a single product. The applicant has therefore generated a live attenuated recombinant vaccine against PRRS-2 that expresses the protective antigen of NiV (G protein).

The specific objective of this TNA project was to compare an attenuated PRRSV-2 vector expressing NiV-sG (rMLV- NiV-sG) to a recombinant PRRSV-2 MLV (rMLV)

- to explore the immunogenicity of the recombinant vaccine vector and its ability to elicit immune responses against NiV in pigs, and
- to evaluate whether the inclusion of the NiV transgene has an impact on the ability of the vaccine to induce PRRSV-specific immune responses.

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Experimental procedures:

PRRSV-2 rMLV and bivalent rMLV-NiV-sG vaccines were previously constructed and virus stocks were provided by the applicant.
Seven days prior to vaccination, 12 PRRSV naïve, Large White-Landrace-Hampshire cross, female pigs, 6-8 weeks of age, were randomly assigned to 2 groups of 6 pigs housed in separate rooms within a SAPO level 3 biocontainment facility at APHA. Pigs were allowed to acclimatise for 7 days. Clinical scores, body weight and rectal temperatures were monitored throughout the study to assess vaccine tolerability.
On 0 days post vaccination (dpv) blood samples (one 8 mL SST Vacutainer of blood for serum, and three 8 mL Heparin Vacutainers of blood for PBMCs per animal per timepoint) and nasal swab samples (1 swab used in each nostril and placed in 2 mL of virus transport medium per animal per timepoint) were collected from each animal by Animal Services staff at APHA Weybridge and transported to The Pirbright Institute.
Vaccines were administered in The Pirbright Institute. One group received rMLV-NiV-sG and the other group received rMLV, both by intramuscular injection of 105 TCID50 in a volume of 1 mL. At 3, 10 and 17 dpv nasal swab samples were collected. At 7, 14, 21, 28, 35 and 42 dpv blood samples and nasal swabs were collected. At 42 dpv animals were culled, and bronchoalveolar lavage fluid (BALF) was collected.
Collected samples were processed for further analysis including T cell IFN-γ ELISpot, surface and intracellular labelling assays, ELISA assays (presence of PRRSV-2 specific antibodies and of NiV-sG specific antibodies) and quantitative RT-PCR.

Conclusion:

This study showed that the rMLV-NiV-sG bivalent vaccine candidate did not induce sufficient humoral or cellular responses to either NiV-sG or PRRSV-2 up to 42 dpv. Both rMLV and rMLV-NiV-sG showed poor immunogenicity with no robust B cell or T cell response specific to PRRSV-2 or NiV-sG. The animals had low to non- detectable levels of vaccine RNA. Further analysis showed that the vaccine titres were as intended, and that a different PRRSV-2 strain (VR-2332) was able to elicit a T cell response. We hypothesize that the low immunogenicity of rMLV-NiV-sG is due to the fact that the live attenuated PRRSV-2 vaccine used as a vector is too attenuated and replicates too weakly in pigs. Future studies should include the evaluation of a bivalent PRRSV-NiV with higher efficacy and better replication in pigs.

5. TNA 5

Harnessing local immunity for protection against influenza

This project was done in collaboration with TPI (see D14.1). The low containment vaccination work (immunogenicity studies) was done at the APHA, the animals were later transferred to Pirbright for challenge experiments (efficacy studies).

Aim:

This study addressed whether mucosal vaccination with adenoviral vectors encoding for influenza A Virus (IAV) antigen and the immunomodulatory molecule Interleukin- 1beta (IL-1b)

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can provide protection against non-vaccine related IAV in the large natural host animal pig model.

The immunisation approach using with adenoviral vectors encoding for influenza A Virus (IAV) antigen and the immunomodulatory molecule Interleukin- 1beta (IL-1b) was previously shown to induce strong local T-cell responses and demonstrated high vaccine efficacy in mice. In the highly relevant large natural host animal pig model however, the beneficial effects of IL-1b on the generation of mucosal T-cells was confirmed, but this did not translate into protection against heterologous IAV infection. Furthermore, it was also shown that a previous infection with H1N1 virus did not provide protection against infection with heterologous H3N2 virus. This sharp contrast between the two animal models supported the notion that vaccine candidates must be validated in more than one animal model and certainly in at least one large animal model.
The previous study in pig model also generated crucial information on the prerequisites for mucosal vaccines to induce local T cell resident memory responses. Tissue-resident memory T-cells (TRM) were shown to be essential for the control of influenza viral replication and disease progression in the absence of strain-specific neutralizing antibodies in mice and ferrets. The hypothesis was that pre-exposure with pH1N1 virus or pulmonary immunisation with Ad-HA/NP and Ad-IL-1ß generate lung TRM which are capable of mediating heterosubtypic protection against H3N2 virus.

The specific aims of this TNA project were to address the following questions:
1. Are the lung TRM responsible for heterosubtypic protection?
2: Are the TRM generated by Ad-HA/NP immunisation and pH1N1 infection different?
3: Does IL-1β enhances the generation of TRM and what factors potentiate their induction?

Experimental procedures:
Due to difficulties in breeding of the Babraham pigs, insufficient numbers of pigs were available and our initial plan had to be adapted and separated for the immunogenicity and the efficacy parts of the study.
- The immunogenicity study with the detailed T-and B-cell analyses was performed as initially intended in inbred Babraham pigs allowing the measurement of antigen-specific TRM-cells by tetramers.
- The efficacy study was performed in outbred pigs which for translational reasons is even better suited than the inbred model mimicking the higher genetic diversity in humans.
The immunological data obtained confirmed the adjuvant properties of IL-1b in the large natural host animal model. In addition, in-depth analyses on antigen-specific memory B-cells were performed, exceeding the original plan. The challenge infection was performed as described in the original proposal and viral load in tissues was analysed by plaque assay and qRT-PCR. Lung gross- and histo- pathology was evaluated including immunohistochemistry for NP.

Conclusion:

This study confirmed the adjuvant effect of adenovirus encoded IL-1b on the induction of antigen-specific TRM in the lungs. The TRM responses against the H1N1-derived epitopes were significantly higher in the Ad-IL-1b treated animals than in the non-adjuvanted group and in the H1N1 pre-exposed animals. Furthermore, higher frequencies of HA-specific antibodies and memory B-cell responses in Ad-IL-1b immunised animals were observed.

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Surprisingly, the strong T-cell responses against the conserved NP-epitopes and enhanced Ab responses were insufficient to control viral replication after heterologous H3N2 infection. The lung gross and histopathology showed enhanced pathology in Ad- IL-1b treated animals following H3N2 challenge. Prior infection with H1N1 did not induce sufficient heterotypic immunity either, which supports the notion that the correlates of protection differ between the small rodent and the large pig animal model.
This new information will be very important for the further development of universal flu vaccines. In addition, this study raised new questions about the suitability of different animal model for predicting translational outcomes in human clinical trials.

The results of this study are presented in a manuscript which is currently under review at *Mucosal Immunology.*

6. **TNA 6**:

Pathogenesis of Rift valley fever virus in an intranasal-challenged ferret model

This access was completed in the final two months of the VetBioNet project due to a high workload of the facility and time lag/difficulties sourcing the number of ferrets required. The ferrets finally used had to be imported from the United States as only remaining UK breeder licenced to supply ferrets for experimentation could not supply in time. Therefore, the final results of this access will only be available after the end of the VetBioNet project.

Aim:

Rift Valley fever is a high consequence zoonotic disease affecting livestock in Africa. Infection is primarily by mosquito bite and contact with material contaminated with Rift Valley fever virus (RVFV). Human infection can result in neurological disease signs and severe morbidity.

The aims of this project were
- to develop an intranasal challenge model for Rift Valley fever virus in ferrets (*Mustela putorius*), in order to investigate of the inhalation infection mechanism in a model with strong parallels to the human respiratory tract.
- to create a range of swab, blood and tissue samples that can be shared with VetBioNet collaborative institutions for further studies on Rift Valley fever virus.

Experimental procedures:

Intranasal inoculation of ferrets has been proposed as a suitable method to study inhalation infection with viruses (Barbeau et al., 2020, mSphere 5, e00789-20). Eighteen female ferrets (4 months) were sourced from the United States due to supplier shortages within the United Kingdom. Each was acclimatized to specialist containment accommodation then microchipped. The microchip numbers were used to randomize ferrets into 6 groups of 3. Temperature and weight measurements were taken for 7 days to establish baseline levels prior to experimental infection. The first group (control) received an intranasal inoculation of tissue culture medium, the remaining 5 groups received 1mL of RVFV (strain ZH501, 10^7 pfu/mL,

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0.5mL per nostral) under general anaesthetic. Pre-blood samples (200 μL) and oral/rectal swabs were taken.

Ferrets were monitored twice daily and assessed using a 10-point clinical scoring sheet. Any animal showing initial disease signs and given a clinical score >10 was humanly killed. An intermediate time point (day 3) was included to enable investigation of the infection process where three animals were humanely killed. The control group were humanely killed at day 10. For each animal a terminal clotted blood sample was taken, post-mortem swabs (oral, nasal and rectal) and a full necropsy was conducted. Fresh tissue samples were divided into formalin-fixed samples and unfixed fresh tissue for cryostorage (-80°C) as listed below.

Blood sample	**Swabs**	**Tissue samples**
Clotted blood for serum	Oral	Olfactory bulb
	Rectal	Frontal cortext
	Nasal	Cerebellum
		Cervical spine C1
		Tonsil
		Spinal cord (C6-7)
		Heart
		Cervical trachae
		Lung
		Liver (right medial lobe)
		Kidney (right)
		Spleen
		Inguinal lymph node
		Stomach (pylorus)
		Small intestine (duodenum)
		Large intestine
		Mesenteric lymph nodes
		Bladder
		Urine (1mL)

Conclusion:

With the exception of the control and day 3 animals, initial clinical signs were detected on day 6, all virus-infected animals developing moderate signs by day 8. Intranasal infection with a high RVFV dose produces a very consistent disease pattern.

Further investigation of samples will include:

1. Temperature and clinical scoring data collation and analysis.
2. Histopathological and immunohistopathological investigation of pathological changes and RVFV distribution in response to infection.
3. Serological testing for RVFV seroconversion.
4. Viral load in blood and tissue.

Serum and tissue samples will be shared with VetBioNet collaborators on request.

7. TNA 7:

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Investigating transmissibility of moose chronic wasting disease to sheep

This experiment is ongoing. Despite intracerebral inoculation the chronic wasting disease prion has not developed into clinical disease in the sheep as quickly as expected so under its ethical obligations APHA will support this experiment once the VetBioNet funding finishes until completion.

Aim:

This study aimed at determining whether chronic wasting disease (CWD) found in moose in Norway was transmissible to sheep and to describe and characterize the resulting disease if it transmits. CWD is a novel disease in Scandinavia, which is different to the North American disease, and its origin is unknown. This study in sheep would further help to assess whether sheep sharing pastures with wildlife are at risk of infection and whether the produced disease is different to naturally occurring prion diseases in sheep and thus not linked to a sheep prion strain.

Experimental procedures:

Sheep were inoculated intracerebrally because it would by-pass the gut and brain barrier and thus more likely result in infection with a shorter incubation time. Two groups of 5 sheep each were inoculated, which represented different prion protein genotypes (AHQ/AHQ and VRQ/AHQ or ARQ/AHQ at codons 136, 154 and 171) because of their different susceptibility to various transmissible spongiform encephalopathy (TSE) agents. After inoculation, sheep were monitored clinically (clinical examinations at least quarterly, weekly checks by video observations) for signs of TSEs. Rectal biopsies were taken annually to determine TSE status by immunohistochemical examination for prion protein as well as fecal and blood samples for further tests. The clinical end-point would trigger euthanasia after collection of a final blood sample and saliva sample for further tests, and a range of central nervous and peripheral tissues would be collected for TSE diagnosis (Western immunoblot, immunohistochemistry, other ultrasensitive prion detection tests) and further investigation (e.g. mouse bioassay to characterise the isolate).

Conclusion:

Inoculation was performed without complications. Sheep are currently alive at 18 months post inoculation (mpi). Clinical examinations and camera observations have so far not detected any abnormalities suggestive of a TSE. A rectal biopsy taken at 12 mpi did not present with detectable disease-associated prion protein. There is currently no evidence that CWD from moose transmits to sheep with a prion protein genotype that is associated with susceptibility to TSEs, but further monitoring is needed, which will be funded under a different project.

The findings are not completely unexpected:

1) Transmission of a TSE isolate from one species (moose) to another (sheep) may be delayed because of the species barrier, thus clinical disease may take longer to develop.

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2) Clinical disease may not develop at all, even if the animal is infected. A brain examination should be able to determine infection status, but this would only be possible in a dead animal. There is a possibility that statutory diagnostic tests do not confirm disease, even if the animal is infected, but disease confirmation may be achieved by ultrasensitive prion detection tests or mouse bioassay. The likelihood of statutory tests to confirm disease in infected animals is greater with increased survival time of the animal.

3) Prions may not spread to the periphery, particularly if the inoculation route is via the brain, and the agent does not accumulate in lymphoid tissues in a concentration high enough to be detectable by the current statutory tests. Hence, the rectal biopsy would not be useful to provide a diagnosis. This is already the case in the original host (moose) where the prion was confined to the central nervous system.

Termination of this study is not recommended at this stage as it may be too premature to detect infection as outlined above. Disease characterisation requires brain as this is the material used for statutory diagnosis and differentiation from other naturally occurring TSEs. It is thus desirable to maintain the sheep for longer (usually a minimum of 60 mpi) to have more confidence that transmission did not occur.

8. TNA 8

Transmission and pathogenesis of High Pathogenic Avian Influenza in wild bird galliform species

Aim:

High pathogenicity avian influenza viruses (HPAI) have spread among wild birds worldwide during recent years causing annual die-offs among wild birds and outbreaks in poultry in multiple European countries. The outbreaks significantly impact the poultry and game bird sectors. Infected game birds may act as a bridging species potentially enabling spread of virus into commercial and backyard premises.

The aim of the present project was to assess the potential role of gamebirds as a bridging host for the introduction of the current circulating HPAIVs.

Experimental procedures:

Intra- and inter-species transmission of H5N8-2021 and H5N1-2021 HPAIVs isolated from pheasants in the UK were studied by experimental inoculation in pheasants and partridges. Clade 2.3.4.4b HPAIVs used in the study were A/pheasant/Wales/9383/2021 (H5N8-2021) and A/pheasant/Scotland/11039/2021 (H5N1-2021). Eight-week-old pheasants (Phasianus colchicus) and partridges (Alectoris rufa) were directly inoculated via the intraocular and intranasal route with three different doses of H5N8-2021 and H5N1-2021. Infectivity, mortality, and pathogenesis were investigated and compared. Intra-species transmission among pheasants and onward transmission to contact chickens were assessed. Pheasant to partridge transmission and onward transmission to partridges were also investigated. Buccal and cloacal

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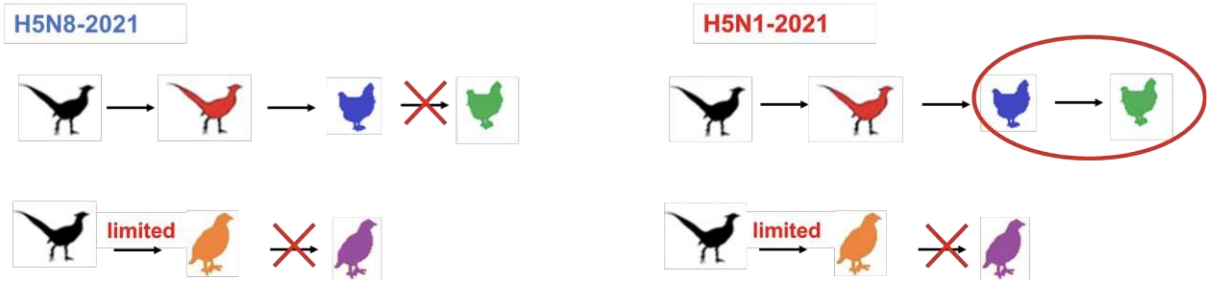
swabs, environmental, and tissue samples were collected and viral RNA detected by M gene RRT-PCR.

Conclusion:

Overall, partridges were less susceptible to H5Nx HPAIV infection compared to pheasants and chickens, which is in accordance with observations from the field in that partridges are seldom found positive for HPAI even when co-housed with infected pheasants.



The study confirms that the viruses currently circulating in Europe are highly virulent in pheasants and partridges and emphases the role of these game birds as a bridging host for the infection of commercial poultry.

These findings will inform poultry disease policy for European (and other) countries, particularly as the commercial rearing of pheasants and partridges continues to be a key sector in the poultry industry, which includes the movement of birds as part of trade within the EU internal market that extends to neighboring non-EU states. These outputs will also guide interventions and mitigation strategies of direct relevance to the gamebird sector and its interface with the extensive European commercial chicken sector.

9. TNA 9:

Role of MPB70, a Mycobacterium bovis (M.bovis) secreted protein, in multinucleated giant cell formation and bovine tuberculosis (bTB) pathology

Aim:

The *Mycobacterium tuberculosis* complex (MTBC) are the group of bacteria that cause TB in mammals. Although *M. tuberculosis* and *M. bovis* are 99.9% identical at the nucleotide level, they have distinct abilities to sustain in human and animal populations, respectively. However, the precise genetic mechanisms which define MTBC species host preference are yet to be defined. Amongst key differences between *M. tuberculosis* and *M. bovis* is the expression and secretion of MPB70. In *M. tuberculosis* MPB70 is expressed at low, inducible levels, while in *M. bovis* it is expressed at high, constitutive levels. Furthermore, comparative analysis of *ex vivo* macrophage infections with *M. tuberculosis* and *M. bovis* revealed that only *M. bovis* could trigger the formation of bovine multinucleated giant cells (MNGC) macrophages, a hallmark of the TB granuloma. In contrast, in vitro macrophage infections using an *M. bovis* MPB70 mutant

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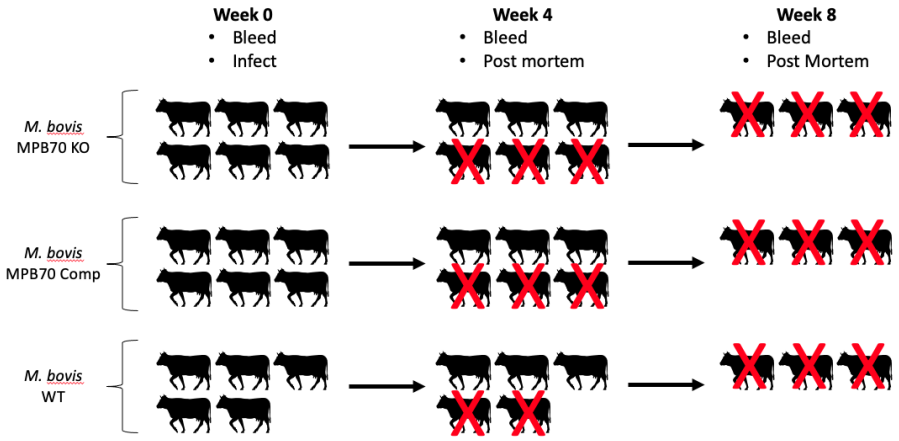
could not trigger the formation of bovine MNGCs, whereas the complemented mutant restored the MNGC phenotype.

The objective of this project was to address the role of MPB70 in the formation of MNGC in the pathology induced by *M. bovis* following *in vivo* infection of cattle.

Experimental procedures:

Three *M. bovis* strains were used that were all derived from *M. bovis* AF2122/97, a genome sequenced strain. These three strains were: (a) *M. bovis* AF2122/97 (WT), (b) *M. bovis* Δmpb70 mutant and (c) *M. bovis* Δmpb70::mpb70 complemented mutant. Animals were assigned to one of three groups (6 animals per group) and each group was infected with one of the three *M. bovis* strains via the endo-tracheal route. Peripheral immune responses were followed over the course of infection. Animals were killed at either 4 or 8 weeks post infection for post-mortem examination, where the severity of the gross pathological changes in the lymph nodes of the head and respiratory tract and in the lungs was scored using a semi-quantitative scoring system. Samples from these tissues were then preserved in 10% phosphate-buffered formalin for 7 days. Sections were cut and stained with H&E or ZN staining, and scanned for microscopic examination to assess the number, developmental stage, and distribution of each granuloma (types I-IV), the number of MNGCs, as well as assessing the quantity and location of acid-fast bacilli for each granuloma within the tissue section.



Conclusion:

Similar peripheral immune responses were seen across all three groups of infected animals, with responses detected at 4 weeks post infection and maintained until the end of the experiment. Similarly, all three *M. bovis* strains induced gross pathological changes in lymph nodes and lung. These changes were detected as early as 4 weeks post infection, but with a trend for higher pathology scores 8 weeks post infection. Histological examination of tissue samples revealed differences in the numbers of MNGCs in granulomas from animals infected with the three different *M. bovis* strains. However, no defect in the generation of MNGCs was evidenced in the group infected with the *M. bovis* Δmpb70 mutant as compared to the *M. bovis* wild type or *M. bovis* Δmpb70::mpb70 complemented mutant.

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The discrepancy between these *in vivo* findings and the previous *ex vivo* data in terms of MNGC formation may be due to several factors, including the animal-to-animal variation in infecting dose or pathogenesis, as observed during the experiment. The applicant plans to further explore the *ex vivo* responses of macrophages to a range of *M. bovis* mutants lacking MPB70 and other components of the SigK regulon, such as the related MPB83.

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