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VetBioNet

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

Deliverable D11.1

Quantity of access provided over the duration of the project to INRA infrastructures

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: INRAE

Revision: V1

Dissemination level	
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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

VETBIONET Veterinary Biocontained research facility Network

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	Number of units of access provided
In vivo imaging: Unraveling Marek's disease virus entry, tropism and tumorigenesis in chickens	Benedikt Kaufer	Freie Universität Berlin	DE	PFIE	Jan 2019 Jan 2022	Dec 2019 Jun 2022	39 AU
CryptO - Selective Chemotherapy against Cryptosporidiosis in Ruminants	Christian Hedberg	Umeå University	SE	PFIE	Sep 2020	Mar 2022	71 AU
Susceptibility of genetically engineered chickens to the infection with highly pathogenic influenza viruses (H7N1)	Hicham Sid	Technische Universität München	DE	PFIE	May 2022	Jan 2023	40 AU
Utilizing αβ and γδ T cell knockout chickens to better understand the role of avian T cells in the immune response against Eimeria infections	Benjamin Schusser	Technische Universität München	DE	PFIE	Dec 2022	Feb 2023	40 AU
Assessing the ferret as a bovine tuberculosis transmission model	Fred Quinn	University of Georgia	US	CIRE (PIXANIM)	Jan 2020	Jul 2022	20 AU
Mucosal vaccination of carp against Spring Viraemia Carp Virus	Maria Forlenza	WUR	NL	IERP	Sep 2019	Jan 2020	60 AU
Genetic determinants of host specificity and pathogenicity in Flavobacterium psychrophilum: a comparative study of strains isolated from ayu, Plecoglossus altivelis and rainbow trout, Onchorynchus mykiss	Erina Nagata	Kindai University	JP	IERP	Jul 2020	Sep 2020	95 AU
Time-course monitoring of immune response and infection for evaluation of DNA vaccine potency	Niels Lorenzen	DTU	DK	IERP	Apr 2021	Jul 2021	79 AU

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Effect of thermic shock on viral infection and specific immune memory in rainbow trout	Daniel Barreda	University of Alberta	CA	IERP	Mar 2021	Sep 2021	92 AU
In vivo determination of virulence of Flavobacterium psychrophilum isolates belonging to rainbow trout-associated clonal complexes, which caused epidemies in Turkish fish farms	Izzet Burcin Saticioglu	Bursa Uludag University	TR	IERP	Jul 2020	Aug 2020	30 AU
Setting out zebrafish as a model to study nodavirus-host interaction	Julia Béjar Alvarado	Institute of Biotechnology and Blue Development (IBYDA), Malaga	ES	IERP	Dec 2022	Feb 2023	40 AU
Oral and injection vaccination of carp against SVCV in combination with two new adjuvants	Maria Forlenza	WUR	NL	IERP	Sep 2022	Feb 2023	240 AU

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2. Final reports of each TNA provided

2.1 TNA 1

In vivo imaging: unravelling Marek's disease virus entry, tropism and tumorigenesis in chickens

A recombinant Marek's disease virus (MDV) expressing firefly luciferase (generated by the TNA user) was used for a chicken infection study including a bioimaging approach (IVIS Spectrum) on the INRAE-PFIE platform. The general objective was to perform a longitudinal study of virus entry, tropism and tumorigenesis in live animals.

Two-week old chickens were infected and the infection process was visualized by IVIS Spectrum in vivo imaging over a 14-day period. The bioluminescence signals were consistent with the known kinetics and life cycle of MDV, and correlated well with viral genome loads measured by qPCR. Strikingly, in vivo imaging also revealed the presence of two unknown MDV-infected organs (confirmed by qPCR). The results highlight the use of in vivo bioimaging to study host-pathogen interactions in the chicken host and shed new light on the pathogenicity and spread of MDV.

An amendment was necessary to generate additional data to the first results in order to publish the results in a manuscript submitted to PLoS Pathogens. This second set of experiments took place in March/April 2022 with 10 chickens (B13) infected with a fluorescent MDV recombinant. Once the animals were euthanized, legs, wings, and beaks were imaged using the IVIS Spectrum technology.

A manuscript co-authored by the User and Access Provider was published in PLoS Pathogens:

In vivo imaging reveals novel replication sites of a highly oncogenic avian herpesvirus in chickens; Published: August 29, 2022 https://doi.org/10.1371/journal.ppat.1010745.

2.2 TNA 2

CryptO - Selective Chemotherapy against Cryptosporidiosis in Ruminants

The project aimed to evaluate new drugs against cryptosporidiosis in a neonatal lamb infection model.

Cryptosporidium parvum is an opportunistic protozoan pathogen whose development is closely linked to the immune system of its host. This disease is a One Health threat. It is known to be the second leading cause of diarrheal disease and death in infants in developing countries and affects immunocompromised adults such as those infected with HIV. It is also the leading cause of diarrheal disease in ruminants before rotavirus infections, which has an additional economic impact and is a source of human contamination. There is no dedicated therapy in humans, the only molecule available in humans (Nitazoxanide) has no efficacy in immunocompromised individuals and new therapies are actively sought. We have generated a molecule against a zoonotic Cryptosporidium parvum strain for which we wanted to evaluate the efficacy in a neonatal lamb infection model.

The animal infection model was coupled to a behavioral monitoring approach using the Tracklab system (collaboration with Noldus, WP9 Task 9.2). A first experiment took place in

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2021 with 3 groups of 8 lambs (infected with Cryptosporidium parvum; treated preventively; treated from day 4 onwards at the onset of diarrheic symptoms).

Both oral treated groups showed a strong reduction in parasite load, up to 2 logs, both by measuring luciferase activity of transgenic parasites and by regular coproscopy. This major improvement was associated with a significant reduction in weight gain during the infection period. Based on these very promising observations, further chemical improvements were made to the drug to improve its efficacy/solubility. This was confirmed first by in vitro tests on intestinal epithelial cell lines (an additional logarithmic decrease in EC50 was obtained) and then by in vivo validation in mouse models (neonatal and adult IFNγ-/- mice). The second experiment was set up on 3 groups of 8 lambs (uninfected, Cp-infected and Cp-infected and treated) to evaluate the new drug derivate and was combined with behavioral analyses with the support by Noldus (Tracklab software). The last experiment took place in January 2022. 3 groups of 8 lambs were infected with Cryptosporidium parvum. One group was treated with the reference drug (Panacur®), one group with the test drug and the last group was not treated.

This experiment ended in March 2022 and the analysis of the last set of data is ongoing.

2.3 TNA 3

Analyzing the role of the duck RIG-I in transgenic chickens infected influenza

This project aimed to explore the role of an innate immune response component in poultry during avian influenza virus infection by studying the duck RIG-I sensor in influenza-infected transgenic chickens.

Eggs resulting from the breeding of heterozygous animals (RNF-135/WT x RIG-I/WT) were produced and transported by the User and then hatched under SPF conditions at the INRAE-PFIE (Access Provider) facility. Right after hatch, blood samples were sent to the User for genotyping. The animals were marked according to their genotype and kept together until the second week of age when they were transferred to the experimental isolators for 1-week adaptation before the beginning of the challenge experiment at 3-weeks of age.

The total duration of the infection trial was 21 days. Homo-/heterozygous transgenic chickens as well as WT chickens were distributed into 4 groups (n=18 per group) corresponding to 5 BSL-3 isolator units. Two additional groups with 9 animals each were kept as a mock-controls.

At 3-weeks of age (day 0 p.i.), the chickens were PBS/mock-treated (groups 5 and 6) or virus-infected (groups 1-4) by intra-tracheal and intra-choanal cleft inoculation of 0.1mL PBS or $5x10^5$ EID_{50}/$1x10^6$ EID_{50} H7N1. During the trial (days 0-21 p.i.), animal behavior and clinical disease signs were monitored twice daily. Birds were sacrificed by intra-occipital pentobarbital injection at days 2, 6 and 21 p.i. (n=6 per group) or once humane endpoints were reached. Samples collected from sacrificed birds included lung, caeca, spleen, kidneys and oropharyngeal or cloacal swabs. Blood samples were collected by occipital sinus puncture prior to pentobarbital injection. Additional swabs and blood samples were taken in the isolator units from 6 live animals per group on days 2, 6, 10 and 21 p.i.

The animal experimental trial terminated in December 2022, and the analyses of all samples collected, including RNA samples that were sent to the Roslin Institute (UEDIN) for transcriptomic analyses (see D29.1) is in progress. The clinical data indicate that the presence of duck RIG-I and/or RNF135 predisposes the animals to more severe infection outcomes.

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2.4 TNA 4

Role of gamma delta T cells in the pathophysiology of Eimeria tenella infection

The aim of this study was to explore the role of αβ and γδ T lymphocytes in the pathophysiology of Eimeria infection. Coccidiosis, caused by parasites of the genus Eimeria, represents the first parasitic plague in poultry farming, associated with massive production losses in broilers that are more or less important depending on the Eimeria species. The most frequently encountered species in poultry farms are Eimeria acervulina, Eimeria maxima and Eimeria tenella, E. tenella being the most virulent species. Eimeria multiply in the gastrointestinal tract (duodenum, jejunum, ileum, or cecum depending on the Eimeria species involved) and/or remain in the intestinal mucosa, causing changes in absorption, mucus, and fluid excretion resulting in dehydration. Eimeria infection is also characterized by a strong inflammatory response that differs among species. The nature of the various cellular and molecular players and the precise mechanisms leading to this inflammation remain largely unknown.

It was previously shown for E. tenella, that neutralization of the inflammation mediator interleukin-17 leads to decreased parasite migration into the submucosa, decreased oocyst (parasite) shedding, and decreased lesion scores in the caeca. As Tγδ lymphocytes are a major pool of IL-17 producing cells, the objective of this project was to investigate the functional role of these cells in the pathophysiology of Eimeria infection by exploiting the knock-out chicken models produced by the team of Benjamin Schusser at the Technical University of Munich (User). These chickens are knocked out for the delta gamma receptor (TCR1-/-) and are therefore free of Tγδ lymphocytes.

The project had 2 specific aims:

- Since the development of the pathophysiology related to infections by E. acervulina, E. maxima or E. tenella is different (site of multiplication, migration or not of the parasite in the submucosa, lesions), our first objective was to determine the involvement of Tγδ lymphocytes in the pathophysiology of infections caused by these three different Eimeria species individually.

- The second objective was to determine the role of these cells on the protective response during reinfection by E. tenella.

Freshly laid chicken eggs were provided by the User and transported to the INRAE-PFIE (Access Provider) facility. The eggs were incubated and hatched in the INRAE-PFIE hatchery. In total, for all experiments - individual infections with the 3 Eimeria species and combined infection experiments, all in duplicates - 700 chickens were produced to obtain the 160 WT and 160 TCR1-/- chickens needed for this study. Indeed, in this model, the chickens being heterozygous, 25% of the animals at hatching are wild type WT and 25% do not express TCR1 (TCR1-/-). At days 1-3 post-hatch, banding followed by blood sampling (10 µl) was performed and the samples were sent to the User team to identify the genotypes of the animals.

For the 3 independent E. tenella, E. maxima, and E. acervulina infection experiments (10 WT and 10 TCR1-/- chickens per group), animal weights, oocyst excretion, hematocrit (control for hemorrhages), serum staining (indicative of intestinal malabsorption), lesion scores, and inflammation parameters (cell phenotype and inflammatory mediators) were monitored daily until day 7. Animals were euthanized at D7 post-infection. This experiment was repeated once for each Eimeria species.

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For infection with E. maxima and subsequent infection with E. tenella (involvement of Tγδ lymphocytes on the protective response during reinfection with E. tenella), chickens (10 WT and 10 TCR1-/-) were infected by E. maxima at 14 days of age and followed for several days (D5-J9) for oocyst shedding kinetics before being infected with E. tenella. After 9 days of infection by E. maxima, chickens (10 WT + 10 TCR1-/- infected and 10 WT and 10 TCR1-/- non-infected) were infected by E. tenella and euthanized at D7 post-infection for determination of oocysts in intestinal contents, oocyst excretion kinetics, oocyst lesions, lesions, hematocrit, serum staining and inflammation. This experiment was repeated once.

The experiments were conducted between December 2022 and February 2023. Analysis of all collected samples is in progress.

2.5 TNA 5

Assessing the ferret as a bovine tuberculosis transmission model

<u>Analysis of M. bovis lesions by MRI in ferrets and badgers</u>

The aim of this TNA project was to use magnetic resonance imaging (MRI) to analyze the extent of lung lesions induced by Mycobacterium bovis in infected ferrets and badgers (post-mortem lung samples).

Bovine tuberculosis (TB) caused by Mycobacterium bovis (M. bovis) remains one of the most important animal diseases in the world and kills at least 12,500 people per year. In Europe, the prevalence of TB can be very high in cattle (as in the United Kingdom and Ireland) and is increasing in parts of France, Spain, and Portugal, with significant financial, social, and environmental impact. The eradication of tuberculosis in cattle is therefore urgent. Wildlife often acts as a reservoir for TB, and the development of oral veterinary vaccines is an international effort to sustainably reduce the long-term risk of transmission to cattle. This goal requires well-defined, physiologically accurate animal models that mimic real-world disease processes in order to most effectively test and measure the protective efficacy of vaccines.

In another closely linked TNA project (ANSES Nancy, TNA 2, D28.1), the user (Fred Quinn, University of Georgia) studied natural transmission of M. bovis between captive ferrets to enable more realistic screening of oral vaccine candidates for mustelids under captive conditions. At the same time, the user was collaborating with a Spanish partner to test the protective effect of oral (heat-inactivated) TB vaccines in badgers. In addition to blood samples collected throughout both animal studies for immunological analysis, post-mortem lungs samples were collected for quantitative analysis of lesion severity by histology, which was complemented by the MRI analysis performed in this TNA.

The specific objective of this TNA was to assess by MRI the size and location of tuberculous lesions in the lungs of M. bovis-infected ferrets and badgers collected post-mortem in the two animal studies.

<u>Experimental procedures:</u>

The formalin-fixed lung from ferrets and badgers were submitted for MRI scanning using a Siemens 3 Teslas (T) Magnetom Verio scanner at the CIRE/PIXANIM platform. Two separate MRI sequences were performed on each lung to obtain two different image contrasts. Both the T1 (spin-lattice relaxation time, in 3D) and the T2 (spin-spin relaxation time, in 2D) contrasts

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were merged for complementary information for quantification. The precise quantification of the lesion volume in the complete lung will be obtained for each animal.

Results:

In the preceding TNA project (ANSES Nancy, TNA 2, D28.1), ferrets infected with M. bovis by the intratracheal route developed a detectable disease in most of the animals at post-mortem 39 weeks post-challenge, with mild histology scores and symptoms, as seen previously in other experimentally infected ferrets (Qureshi et al., 2000) and badgers (Lesellier et al., 2020).

In the few animals with severe lesions, a detection of the lesions by MRI was possible, as in TB infected badgers. An example is shown below (Figure 1).

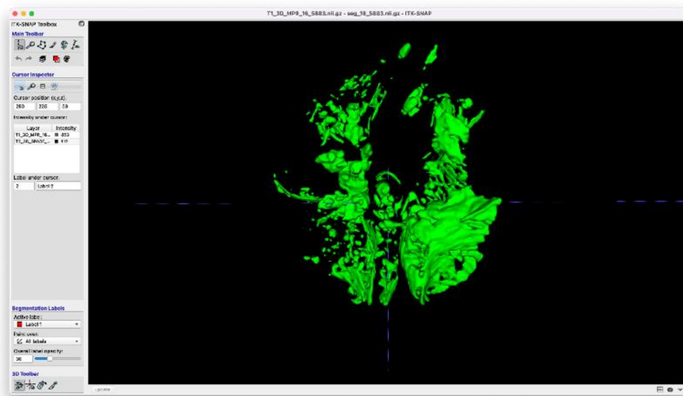

Figure 1: MRI scan of a formalin-fixed TB lesion from a ferret lung using a Siemens 3 Teslas (T) Magnetom Verio scanner.

The experiments were conducted between January 2020 and July 2022. Further analysis of the images is ongoing to quantify the volume of the lesions in all the animals.

2.6 TNA 6

Mucosal vaccination of carp against Spring Viraemia Carp Virus

Aim:

This TNA project concerns the optimization of a mucosal vaccination strategy for carp against spring viraemia virus of carp (SVCV). The applicant (WU) has generated novel antigens that can be administered to carp orally. These antigens are the SVCV glycoprotein (G) produced in insect cells, baculovirus-infected caterpillar or yeast. They were encapsulated and administered orally at different doses and time intervals to carp, in the presence or absence of adjuvants.
The main objective of the project was to identify the best antigen-adjuvant combination for optimal oral vaccination of carp against SVCV.
This general objective was subdivided into 3 specific objectives:
1) to study whether SVCV-G protein produced in insect cells and administered orally as a total cell lysate encapsulated in alginate microparticles elicits protective (mucosal) responses against SVCV
2) to study whether the SVCV-G protein produced in baculovirus-infected caterpillars and administered orally as a total lysate elicits protective (mucosal) responses against SVCV

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3) to study whether SVCV-G protein produced in yeast and administered orally as a total cell lysate encapsulated in alginate microparticles elicits protective (mucosal) responses against SVCV.

Experimental procedures:

Protection conferred by the different vaccine formulations was evaluated using a bath challenge model optimized by INRAE-IERP. A previously optimized i.m. DNA vaccine, previously shown to provide 100% protection, was used as control.

Results:

		RPS
1. unhandled		
2. pcDNA3-empty plasmid, 1ug/g fish	i.m. injection	100%
3. pcDNA3-SVCV-G, 1ug/g fish	i.m. injection	
4. pcDNA3-empty plasmid, 1ug/g fish	Oral gavage (3x with 3d interval)	16%
5. pcDNA3-SVCV-G, 1ug/g fish	Oral gavage (3x with 3d interval)	
6. pcDNA3-empty plasmid, 1ug/g fish	i.m. injection + Oral (3x with 3d interval)	100%
7. pcDNA3-SVCV-G, 1ug/g fish	i.m. injection + Oral (3x with 3d interval)	
8. pcDNA3-empty plasmid, 1ug/g fish	Oral gavage (3x with 3d interval) + i.m. injection	100%
9. pcDNA3-SVCV-G, 1ug/g fish	Oral gavage (3x with 3d interval) + i.m. injection	
10. Unhandled		
11. pcDNA3-empty plasmid, 1ug/g fish	i.m. injection	100%
12. pcDNA3-SVCV-G, 1ug/g fish	i.m. injection	
13. Insect cells (control)	i.m. injection	44 %
14. Insect cells (SVCV-G), (20 ug SVCV-G/fish)	i.m. injection	
15. Insect cells (control)	i.p. injection	59 %
16. Insect cells (SVCV-G), (20 ug SVCV-G/fish)	i.p. injection	

Results are summarized as relative percent survival (RPS) on the right columns of the table below.

As expected, the DNA vaccine showed high efficacy when administered by intramuscular injection. However, when administered orally, protection remains very low (16% PSR), as previously observed (Embregts et al., 2018). By comparing the survival of fish that were first vaccinated by i.m. and then received the oral vaccine (group 6-7), or fish that were first vaccinated with the oral vaccine followed by i.m. vaccination (group 8-9), it could be confirm that the fact that the oral vaccine does not confer sufficient protection is not due to the induction of tolerance (100% PSR in groups 6-9). Indeed, the efficacy of the i.m. vaccine was not reduced when administered after oral vaccination with the same DNA vaccine. This set of data also suggests that the dose, regimen, and conditions of oral vaccination can be optimized without fear of tolerance induction.
The user has previously reported the use of an insect cell-based subunit vaccine. This vaccine did not provide protection when injected orally, intraperitoneally, or intramuscularly (Embregts et al., 2018). However, in the previous study, the insect cells were fixed with formaldehyde and it was hypothesized that this fixation step may have altered the confirmation of the glycoprotein. In the present study, looking at the results of the groups receiving the insect cell lysate expressing the SVCV-G protein in its native conformation and without fixation treatment (groups 13-16), a higher PSR (44-59%) was observed than previously reported (Embregts et al., 2018).

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This suggests that vaccination with subunit vaccines requires preservation of the full native conformation of the SVCV-G antigen. Additional studies (carried out by TNA7) were conducted to test whether unfixed insect cells, presenting antigens in their native conformation, can induce protection during oral vaccination.

2.7 TNA 7

Genetic determinants of host specificity and pathogenicity in Flavobacterium psychrophilum: a comparative study of strains isolated from ayu, Plecoglossus altivelis and rainbow trout, Onchorynchus mykiss.

Flavobacterium psychrophilum, the causative agent of bacterial cold water disease (BCWD) in ayu (Plecoglossus altivelis) is responsible for severe economic losses in Japan. This bacterium is thought to have been imported from overseas, i.e., the pathogen has been spread through global trade with fish and fish eggs. However, as observed by genotyping using MLST, mPCR serotyping and whole genome comparisons, the genotypes of F. psychrophilum are generally specific to one species of salmonid host, e.g., coho salmon, Atlantic salmon, steelhead trout, ayu, etc. Therefore, it is still unclear whether the strains of F. psychrophilum infecting ayu in Japan originate from abroad or are endemic to Japan. In addition, most of the factors involved in host specificity and virulence are still unknown. Variations in the O antigen may play an important role in pathogenesis, contributing to several steps in the infection process, such as the adherence required for host colonization or resistance to host defense mechanisms.

In a previous study, the user showed that all F. psychrophilum strains isolated from ayu had a type 3 O antigen, suggesting that most if not all ayu isolates in Japan are derived from the same ancestor. On the other hand, the sampling of rainbow trout (Oncorhynchus mykiss) during BCWD outbreaks in Japan yielded only one strain with type 3 O antigen and experimental infection challenges showed that this strain is not virulent to ayu (preliminary unpublished data). Outside of Japan, authors found that only 6% of the steelhead isolates had the type 3 O antigen and none of the isolates were from coho salmon. These strains might be virulent to steelhead, but this has not yet been evaluated. Overall, the molecular determinants responsible for F. psychrophilum host specificity are still unclear.

The objective of this TNA was to compare strains isolated from ayu and other fish species, particularly rainbow trout, to understand which gene type(s) are involved in host specificity and virulence when performing infections challenges of ayu or rainbow trout.

The specific objectives were:

- to characterize the genomic diversity and inferring the origin of the F. psychrophilum strains isolated in Japan in order to identify the molecular factors involved in host specificity by comparing the virulence and the complete genomes of strains isolated from ayu and other fish species, especially rainbow trout. The identification of genes encoding virulence and host adaptation factors of F. psychrophilum is of utmost importance to develop epidemiological scenarios and propose relevant countermeasures.

- to identify and characterize the ancestral strain(s) infecting ayu and to understand their evolution by comparing the strains isolated from various fish species in Japan and abroad over a long period of time.

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Experimental procedures and Results:

Bath infection challenge experiments were performed using the rainbow trout host in France and the ayu host in Japan on a selection of seventeen strains isolated from various fish species and possessing either type 0, type 1, type 2 or type 3 O-antigen.

The results showed that F. psychrophilum strains isolated from ayu exhibiting high virulence during experimental infection of ayu were not virulent in rainbow trout. Similarly, all strains isolated from rainbow trout that exhibited significant virulence in the rainbow trout host were not virulent in ayu. Strains isolated from carp and Atlantic salmon had very low virulence in steelhead and no virulence in ayu. In addition, two O antigen type 3 strains originally isolated from rainbow trout had high virulence when experimentally infecting this host. In conclusion, the results indicate a strong association between the strain of F. psychrophilum and the infected host fish species from which the strain was isolated.

Combined with a previous study showing that almost all strains isolated from ayu harbored a type 3 O antigen, the data suggest:

- that the type 3 O antigen is not sufficient to produce disease in ayu, and thus could be considered a fitness factor.

- that the ayu-infecting ancestor of F. psychrophilum, whose origin remains unclear, was therefore likely to possess a 3 O antigen.

Further experiments are needed to clarify the importance of the type 3 O antigen in the F. psychrophilum bacteria-host association.

A publication describing the comparative analyses of genomic characteristics and virulence in the two fish species (ayu and rainbow trout) for all strains characterized in this project is in preparation.

2.8 TNA 8

Time-course monitoring of immune response and infection for evaluation of DNA vaccine potency

Aim:

Control of infectious viral diseases is particularly challenging and viral vaccines are in many cases still at the development stage. However, DNA vaccines against viral disease are particularly efficient and represent a promising future especially since the authorisation by the European Medicines Agency (EMA) of Clynav, the first European commercial DNA vaccine (Collins et al., 2019). Vaccines must be rigorously tested before they are marketed, not only for newly developed vaccines, but also for the manufacture of existing vaccines (batch efficacy/potency testing). A combination of vaccination and experimental infection is usually used to assess protection, involving fairly large numbers of laboratory animals in comparative mortality experiments over time. Viremia is strongly correlated with protection against viruses and can provide strong indications of the efficacy of new vaccines.

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The objective of this TNA project was to take advantage of animal experimentation protocols developed at the INRAE-IERP platform to design and test an improved method for assessing and understanding immunity induced by a promising DNA-based experimental vaccine against one of the most important viral diseases in farmed rainbow trout. This project focused on viral hemorrhagic septicemia virus (VHSV) for which a good experimental DNA-based vaccine is available, based on the user's previous work (Lorenzen et al., 2005).

Experimental procedures:

Based on a previously developed non-lethal infectious model in fish (Collet et al., 2015), this model was combined with the use of a genetically engineered fish cell line, to directly measure viremia, and thus assess vaccine efficacy while reducing the number of animals used.

This approach allowed

- to better understand the pathophysiology of VSHV infection (less severity)

- to analyze the immune response and the viremia in these same animals. Serum and blood cell samples were collected to allow further dissection of immune response profiles and examination of protective correlates. These parameters could be measured with high precision and provided accurate information on the protective capacity of the vaccines tested prior to the onset of the disease.

Blood sampling post vaccination was successfully performed 5 times from all individuals before challenge with virus and also from all live fish after challenge. Since only one of the vaccines provided protection against disease, some of the fish had to be terminated as they developed clinical disease. The challenge experiment was finalized 9 days post inoculation of the fish with VHS virus and at this time muscle tissue from the injection site as well as tissue from liver and head kidney was sampled.

Results:

Initial analysis of viremia and gene expression profiles have been initiated by INRAE. Focus has been on the non-vaccinated controls and the fish receiving the protective vaccine. As shown in Figure 1 (below), an initial strong upregulation of the IFN-regulated viral induced gene 2 (mx gene) was seen in the vaccinated fish along with a more controlled and transient viremia compared to the unvaccinated controls (Table 1). The results suggested that the samples were valid for more in depth analysis and full transcriptome analysis were subsequently performed on 48 selected samples. The results are currently being processed in a collaborative effort between INRAE and DTU.

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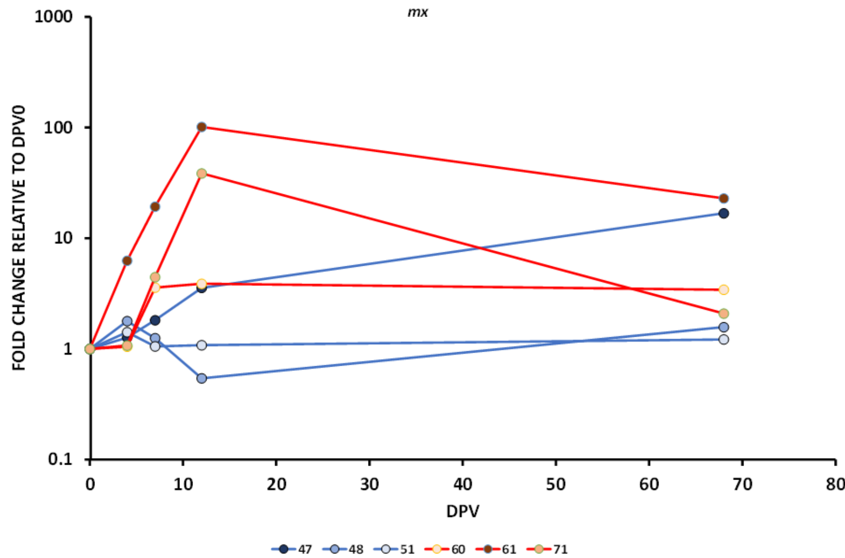

Fig. 1. Time-course development in expression levels of the IFN-induced antiviral gene *mx* in 3 control fish (blue) and 3 fish vaccinated with the protective DNA vaccine (red) determined by qPCR on the blood cells.

Days post infection	Control group			Vaccine group	
	47	48	51	60	71
0	1	1	1	1	1
2	0,68	2,99	2,64	1,10	2,08
5	1,50	3,73	2,91	1,39	2,22
7	1,63	2,81	2,90	0,78	0,95
9	1,37	3,89	3,14	0,66	1,21

Table 1. Comparative levels of viremia after challenge of vaccinated fish.

COVID-19 travel restrictions unfortunately prevented onsite visits by the DTU researchers. However, thanks to an efficient and targeted effort by the INRAE-IERP team, the vaccination/challenge trial with several non-lethal blood samplings post vaccination as well as post challenge was successfully performed.

The initial analyses have revealed that the work has created a very valuable set of samples which will allow to determine key immune response profile elements following DNA vaccination of rainbow trout against VHS along with dissection of host-pathogen interactions related to protective immunity. Further sample analysis is planned to take place at DTU and include comparative expression profiles across all vaccine groups and focusing on immune genes selected based on the initial transcriptomics performed by INRAE.

A manuscript is in preparation.

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2.9 TNA 9

Effect of thermic shock on viral infection and specific immune memory in rainbow trout

Aim:

In contrast to mammals and birds, fish are unable to perform autonomous thermogenesis. Like other ectotherms, their core body temperature is determined by that of their environment. Unfortunately, this has helped to perpetuate the view that fish have a sub-standard immune system compared to mammals. Consistent with this, aquaculture fish are notably susceptible to infections like cold water vibriosis (Vibrio salmonicida), bacterial cold-water disease (Flavobacterium psychrophilum), winter saprolegniosis (Saprolegnia sp), infectious hematopoietic necrosis (IHNV), and others. Mechanistically, this appears to stem from suppression of fish innate and adaptive immune responses at lower temperatures. This includes dampening in the mobilization of white blood cells, functional suppression of antimicrobial phagocytic responses, lower levels of reactive oxygen intermediate production, and a reduction or delay in gene expression for pro-inflammatory molecules that promote antimicrobial defenses (e.g. il1b, tnfa, ifn1, ifng, inos, irf3, mda5 and mx). Also, fish T-lymphocytes are functionally less responsive at low temperature, while B-cells appear less affected. It has been shown that short exposures to high, sublethal temperatures can significantly improve or even cure viral outbreaks in fry in several fish species. However, the mechanisms behind this improved capacity to clear infection remain undefined. It is also unclear whether such treatments induce medium/long-term protection, and if such effects may be specific to the pathogen, thereby corresponding to “immune memory”.

The objectives of this TNA project were

- to determine if repeated heat shocks at sublethal temperatures can protect rainbow trout against rhabdovirus infection (VSHV and IHNV)

- to specify if this procedure leads to a long-term protection (at least 3 months), and if so, if this protection is Ag-specific, which would indicate that surviving fish have developed adaptive responses and an immune memory.

Experimental procedures:

First, the best conditions for hyperthermic treatment in rainbow trout were set up.

Fish (rainbow trout, INRAE “synthetic strain”, about 1 g) were progressively passed from 10°C to 20°C, then subjected to a hyperthermic water at 26°C for 1 hour, and progressively transferred back to 10°C.

In a second step, these treatments were performed on to fish of the same batch (hence, with fish at later stages).

In a third step, challenge infections with VHSV or IHNV were performed on the survivors of the first part of the project, in order to assess their sensitivity to infectious diseases.

Results:

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This set of experiments gave the following results:

1) Small rainbow trout (1 g) tolerated the hyperthermal treatments well. We were able to perform one treatment per day, three days in a row, without induced mortality.

2) Larger fish (2-4 g) did not respond well to hyperthermal treatments, even when these treatments were milder (only two treatments with a 3-day interval). Unexpectedly, high levels of mortality after infection with attenuated HSV were observed.

3) Control experiments showed that in the absence of a parallel viral challenge, hyperthermia alone did not protect against IHNV or VHSV.

4) Second homologous and heterologous tests performed on fish that survived the first test demonstrated specific protection. For fish initially exposed to attenuated VHSV, more survived re-exposure to wild-type VHSV than to IHNV (58% versus 25%, respectively).

5) Fish with initial VHSV infection combined with hyperthermal treatment showed lower susceptibility to VHSV than to IHNV. Unfortunately, the number of survivors did not allow to assess whether hyperthermic treatments protect trout from VHSV or to establish a specific protection.

Therefore, it is planned to repeat these experiments with fish at earlier stages to reduce the negative effects of hyperthermal treatments.

2.10 TNA 10

In vivo determination of virulence of Flavobacterium psychrophilum isolates belonging to rainbow trout-associated clonal complexes, which caused epidemies in Turkish fish farms

Aim:

Rainbow trout (Oncorhynchus mykiss) production has shown rapid development in the last 30 years in Turkey, which is now the largest rainbow trout producer among European countries (FEAP, 2018). Disease outbreaks in rainbow trout caused by Flavobacterium psychrophilum have frequently been reported in Turkey and are associated with high mortality of rainbow trout but also lake trout (Salvelinus namaycush) and Atlantic salmon (Salmo salar).

F. psychrophilum is frequently associated with the early life stages of rainbow trout fry in hatcheries where the rainbow trout fry syndrome also called bacterial cold water disease (BCWD) can result in high mortalities if left untreated. Outbreaks of BCWD are controlled by antibiotics administered in the feed, which can lead to the dispersal of antibiotics into the environment and the emergence of antibiotic-resistant isolates. The lack of effective preventive measures and barriers to the development of protective vaccination strategies against F. psychrophilum (Gomez et al., 2014) are in part due to genetic and serological diversity within the species and poor knowledge of specific virulence factors. Understanding the molecular epidemiology (Bayliss et al., 2017) and bacterial characteristics that contribute to virulence in a susceptible host (Sundell et al., 2019) is critical to designing alternative control strategies (e.g., vaccine development, selection of resistant animals).

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The aims of this TNA project were to determine:

- the in vitro phenotypic properties and genomic characteristics for 18 F. psychrophilum isolates retrieved in Turkey (including the presence of antimicrobial and virulence genes, serotype, sequence type); and

- the virulence of 5 selected F. psychrophilum isolates using in vivo experimental infection challenges in a susceptible host (Oncorhynchus mykiss).

<u>Experimental procedures:</u>

Bath infection challenge experiments were performed using rainbow trout on the 5 selected strains from Turkey. The strain 1882/11 isolated from rainbow trout in France was used in parallel as a positive control. In this experimental infection model, infection with this highly virulent strain resulted in 100% mortality at 11 days post-infection.

<u>Results:</u>

All strains isolated in Turkey from rainbow trout and belonging to CC-ST10 produced significant mortality in the experimental study.

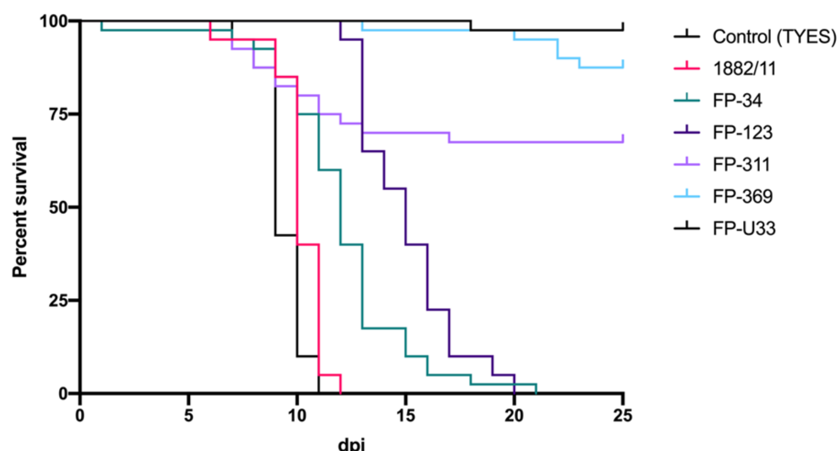

Figure 1. Evaluation of the virulence of F. psychrophilum isolates. Kaplan-Meier survival curves of rainbow trout after bath infection. Each group (n=20) in duplicated tanks were maintained in contact with bacteria for 24 h at 10°C. Each tank was inoculated at a bacterial concentration of 1 (□ 0.6) x 106 CFU/mL.

Strains FP-U33, FP-34 and FP123 were the most virulent based on time to death and cumulative mortality rate. Interestingly, strain FP-311, which showed moderate virulence with a cumulative mortality rate of 30%, harbors a type 3 O antigen, which appears to contribute to bacterial fitness and host specificity (see TNA 2). This serotype was predominant in F. psychrophilum strains isolated from ayu (Plecoglossus altivelis) in Japan (Rochat et al 2017). Consistent with this result, the FP-369 strain that was isolated from Salmo coruhensis did not produce significant mortality in this experimental infection study on the rainbow trout host.

Overall, by combining in vitro phenotypic assays, genomic analyses, and genomic comparisons with in vivo virulence experiments using a relevant bath challenge model in rainbow trout, the study revealed striking heterogeneity among the Turkish strains of F. psychrophilum.

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These results highlight the need for epidemiological surveillance of isolates circulating in Turkey in order to adapt control measures such as vaccine development. Furthermore, they provide a cornerstone for future analysis of virulence traits of this important fish pathogen.

These data will be included in a large-scale GWAS analysis aimed at identifying the genes and SNPs responsible for the observed differences.

2.11 TNA 11

Setting out zebrafish as a model to study nodavirus-host interaction

Aim:

The aim of the project was to establish a zebrafish (Zf) model of betanodavirus infection to determine the susceptibility of Zf larvae to three nodavirus species and their immune responses.

Experimental procedures and results:

Three betanodavirus species were tested, red-spotted grouper- (RGNNV), striped jack- (SJNNV) nervous necrosis virus (corresponding to species detected in the Mediterranean area to date) and a reassortant RGNNV/SJNNV isolated in the experimental facilities of the user from sea bream.

At 3 days post fecundation (dpf), WT zebrafish larvae were injected intracerebrally with 5nL of viral inoculum titrating at 10^7PFU/mL in L-15 medium following Lama et al. 2022. Larvae were incubated at the optimal temperature of growth of each pathogen (22ºC for SJNNV, and at 25ºC for RGNNV and RG/SJ) and daily monitored up to 4 days post infection.

The viral injection did not cause fish death, but clinical signs (edema) were observed from 48hpi for larvae injected with the most virulent species RGNNV and, to a lesser extent, with larvae injected with SJNNV. At the end of the experiment, the larvae were sampled in TRIZOL and sent to the laboratory of the user for viral quantification by RT-qPCR and virus titration experiments. The experiment was repeated twice and similar observations were made in terms of survival rate and clinical signs. Immune responses were assessed by injection of Tg animals, i.e. Tg(mpx:GFP) encoding green fluorescent reporters into neutrophils, which allows quantification of neutrophil recruitment in the brain of infected larvae during infection (at 1, 2 and 4 days) and measurement of the inflammatory response. Image analysis revealed a significant increase in neutrophils in the brains of larvae injected with RGNNV species compared to SJNNV species or control conditions at 4dpi.

Overall, the project allowed the establishment of a zebrafish model of betanodavirus infection that recapitulates the virulence phenotype of three different isolates detected in the Mediterranean region or isolated from sea bream. Intracerebral injections induced viral replication for all three isolates, while the most virulent one activated innate immune responses as was assessed by neutrophil recruitment in the head of infected larvae. These results will be confirmed by RT-qPCR experiments that are being carried out in the user's laboratory in order to determine the expression level of genes involved in the signaling pathways of inflammatory responses (IL1b, TNFa, type I IFN).

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2.12 TNA 12

Oral and injection vaccination of carp against SVCV in combination with two new adjuvants

This TNA represents a follow-up project to TNA 6. The TNA was only terminated at the time of reporting, and the results cannot be presented.

Aim:

The objective of this TNA project was to optimize vaccination strategies against spring viraemia virus of carp (SVCV) based on two prototype vaccines developed by the user:

1 - DNA vaccine against SVCV, for which intramuscular injection of a DNA plasmid encoding the SVCV glycoprotein (G) confers up to 100% protection after a single injection at doses of 0.1 or 1 µg/g fish (Carmen W Embregts et al., 2017).

DNA vaccination via injection, although highly effective, is cumbersome and stressful for fish. In order to perform oral or mucosal immersion vaccination, the user has previously performed work showing that oral administration of the same DNA vaccines encapsulated in alginate microspheres (C. W.E. Embregts et al., 2019) provides highly efficient delivery of plasmid DNA to the mucosa, but does not confer sufficient protection against the pathogenic SVCV challenge in carp (C. W.E. Embregts et al., 2019). On the other hand, it is well known that oral or immersion mucosal vaccination of fish usually results in weak and/or short-lived protection, a phenomenon due to the intrinsic irresponsiveness of mucosal surfaces (skin, gills, intestine) called "mucosal tolerance". To overcome this tolerance, it may be necessary to use vaccines that best mimic a viral infection and thus elicit strong local responses, as well as potent adjuvants and/or higher doses of antigens (Carmen W.E. Embregts & Forlenza, 2016).

In a previous TNA project (see TNA 6), an insect cell subunit vaccine was administered as PFA-fixed whole cells suspension. This vaccine did not confer sufficient protection when administered orally, intraperitoneally, or intramuscularly (C. W.E. Embregts et al., 2019), which may have been due to the fact that PFA attachment may have altered the conformation of the glycoprotein. In a follow-up study, a higher relative percentage of survival (RPS, 44-59%) had been observed when using unfixed whole cells, indicating that preservation of the native conformation of the SVCV-G antigen may be necessary to induce a protective response. The specific objective for the present project was thus to confirm that unfixed insect cells can induce protection during vaccination by i.p. injection, in combination with a new injectable adjuvant or not, and to explore whether they can confer protection by the oral route.

Two parallel approaches were implemented, one to test oral carp vaccination protocols with the two candidate vaccines and the other to test vaccination by i.p. injection of the subunit vaccine, both approaches using novel adjuvants.

This was implemented as follows:

1) delivery of alginate encapsulated SVCV-G-DNA plasmid with or without a new oral adjuvant coated onto feed pellets through a prolonged feeding regime;

2) delivery of insect cells expressing the SVCV-G protein in its native conformation by i.p. injection, in combination with a new injectable adjuvant (assessment of the benefit of the new injectable adjuvant for i.p. vaccination); and

3) delivery of insect cells expressing the SVCV-G protein in its native conformation orally with the same oral adjuvant as in 1, coated onto feed pellets through a prolonged feeding regime.

Experimental procedures:

Table 1: vaccine groups and vaccine antigen formulations

Group	Vaccine	Administration	Nr fish per tank (see sampling below for calculation)
1	pcDNA3-empty plasmid, 1 µg/g fish	i.m. injection	25
2	pcDNA3-SVCV-G, 1 µg/g fish	i.m. injection	25
3	Alginate encapsulated pcDNA3-empty plasmid, 20 µg/g fish, no adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50
4	Alginate encapsulated pcDNA3-SVCV-G plasmid, 20 µg/g fish, no adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50
5	Alginate encapsulated pcDNA3-empty plasmid, 20 µg/g fish, with adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50
6	Alginate encapsulated pcDNA3-SVCV-G plasmid, 20 µg/g fish, with adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50
7	Alginate encapsulated pcDNA3-empty plasmid, 20 µg/g fish, no adjuvant	Feeding regime 2: every three days up to 30 days.	45
8	Alginate encapsulated pcDNA3-SVCV-G plasmid, 20 µg/g fish, no adjuvant	Feeding regime 2: every three days up to 30 days.	45
9	Alginate encapsulated pcDNA3-empty plasmid, 20 µg/g fish, with adjuvant	Feeding regime 2: every three days up to 30 days.	45
10	Alginate encapsulated pcDNA3-SVCV-G plasmid, 20 µg/g fish, with adjuvant	Feeding regime 2: every three days up to 30 days.	45
11	Insect cells (control), no adjuvant	i.p. injection	25
12	Insect cells (SVCV-G), 20 ug SVCV-G/fish, no adjuvant	i.p. injection	25

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13	Insect cells (control), with adjuvant	i.p. injection	25
14	Insect cells (SVCV-G), 20 ug SVCV-G/fish, with adjuvant	i.p. injection	25
15	Insect cells (control), with adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50
16	Insect cells (SVCV-G), 20 ug SVCV-G/fish, with adjuvant	Feeding regime 1: 10 days on, 10 days off, 10 days on	50

Challenge infection experiment (ongoing):

2.5-3 months after vaccination, fish were acclimatized to a temperature of 15°C (2 degree/ 3day) and challenged with 8×10^6 pfu of the SVCV-Kinkelin strain, for 30h. This bath challenge has been optimized by INRA-IERP and has been shown to be very reproducible under these conditions. Fish were monitored daily and survival recorded over a period of 5 weeks, after which the experiment was terminated.

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