VETBIONET

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

Deliverable D29.1

Quantity of access provided over the duration of the project to RI Containment laboratories

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: UEDIN

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	

Table of contents

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	Number of units of access provided
1. The role of inverse autotransporter FdeC in Avian Pathogenic *Escherichia coli* adhesion	Rafał Kolenda	Wrocław University of Environmental and Life Sciences	PL	Containment laboratories	December 2022	January 2023	231 units of access
2. Analysis of the role of the duck RIG-I gene in transgenic chickens during infection with the avian influenza virus	Hicham Sid	Technical University Munich	DE	Containment laboratories	January 2023	February 2023	267 units of access

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

2. Final reports of each TNA provided

2.1 TNA 1

The role of inverse autotransporter FdeC in Avian Pathogenic Escherichia coli adhesion.

Short Summary

Avian Pathogenic Escherichia coli (APEC) is an extra-intestinal pathotype with zoonotic potential and an etiological agent of colibacillosis. APEC infections are common in broiler chickens, laying hens, and turkeys, which leads to decreased weight gain or egg production. APEC is a cause of huge losses in the poultry industry worldwide. Successful establishment of APEC infection depends on the initial step – adhesion to host tissues. The expression of virulence factors during adhesion needs to be controlled to avoid exposition of immunogenic molecules and loss of energy due to production of unnecessary proteins. Dysregulation of virulence factors expression in E. coli might lead to decreased fitness during host colonization and as a result inability to cause disease. We observed that deletion of fdeC gene encoding for adhesin increases adhesion of APEC strain IMT5155 to chicken epithelial cells in vitro. Proteomics analysis indicated that deletion of fdeC increases inorganic ion transport that downregulates YbjN, which normally suppresses motility in WT strain. During animal experiments we noticed that chickens infected with fdeC deletion mutant exhibited altered pattern of blood leucocyte counts in comparison to chicken infected with wild-type strain. Furthermore, fdeC mutant induced a lower immune response compared to WT in chicken lung and spleen. Our data suggest FdeC host-dependent expression and its role in reducing motility, which increases bacterial fitness during host colonization.

Background

The results from in vitro experiments performed by the applicant show the importance of environmental factors on FdeC expression and adhesion of APEC to epithelial cells. This TNA proposal aimed to investigate the role of FdeC in APEC during infection of the host i.e. chicken. Prof. Tina Dalgaard, VetBioNet partner at Aarhus University, is an expert in the use of chicken animal model for investigations of viral and bacterial infections (Andersen et al., 2017; Larsen et al., 2019; Naghizadeh et al., 2019; Wattrang et al., 2020). Therefore, APEC chicken infection experiments were performed at Aarhus University (see D26.1). As deletion of fdeC leads to higher expression of flagella, which might influence the recognition of APEC by immune system, chicken immune response to APEC infection was analysed at The Roslin Institute/UEDIN by VetBioNet partners Professors Lonneke Vervelde and Mark Stevens who are experts in the avian APEC infection model (Alber et al., 2019b, 2019a, 2021).

Experiments at UEDIN

After the animal experiment performed at Aarhus University, selected RNA samples of lung, spleen and blood collected from Wild Type-, Δ*fdeC*- or mock-infected chickens after 6 h, 24 h and 48 h were transported to UEDIN, The Roslin Institute. RNA quality and quantity were assessed using a Nanodrop spectrophotometer and cDNA was synthesised using a High-Capacity Reverse Transcription kit. The cDNA was pre-amplified, ExoI-treated and quantified microfluidic 96.96 Dynamic array (Fluidigm) performed on a BioMark HD instrument as described by Borowska et al (2019 ;

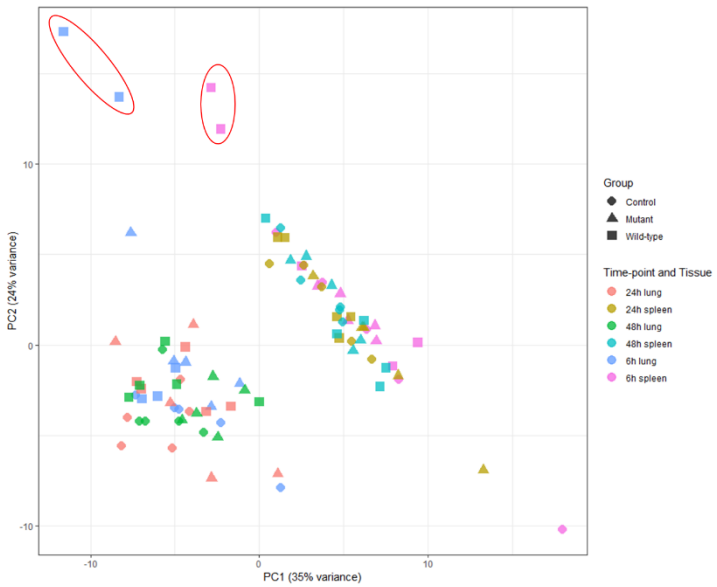
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

doi.org/10.1371/journal.pone.0225658). After the completion of the run, a melting curve of the amplified product was determined. Raw quantitation cycle (Cq) data were collated with the Real-Time PCR Analysis software v 3.1.3 (Fluidigm), setting the parameters of the quality threshold (0.65), baseline correction (derivative) and Cq threshold method to auto (global). The raw Cq values were processed with GenEx.v6 MultiD Analyses AB, with correction for primer efficiency and reference gene normalisation. The stability of the expression of six putative reference genes was evaluated via the NormFinder tool in GenEx. The geometric mean of the most stable genes was used to normalise all samples. Technical replicates were averaged, and the relative quantification values were assessed to the maximum Cq value obtained per gene, transformed to the logarithmic scale. Principal component analysis, assessing the overall clustering of the samples, was performed using ggplot2 in R Studio version 1.1.442. Statistical analysis of the gene expression from the IFC array was conducted to identify significantly differentially expressed genes (DEGs) between treatment groups and was performed using GenEx6, with group means compared with two-way t-tests adjusted for multiple comparisons with post hoc Bonferroni correction, with significant DEGs having a fold change >1 and <-1 For all statistical analyses, p values < 0.05 were considered significant. All statistical analyses were conducted using GraphPad Prism 9 or GenEx v6.

Of note: PhD student Adrianna Aleksandrowicz was awarded a travel grant of the Houghton Trust to visit the Roslin Institute for training purposes, especially the data analysis.

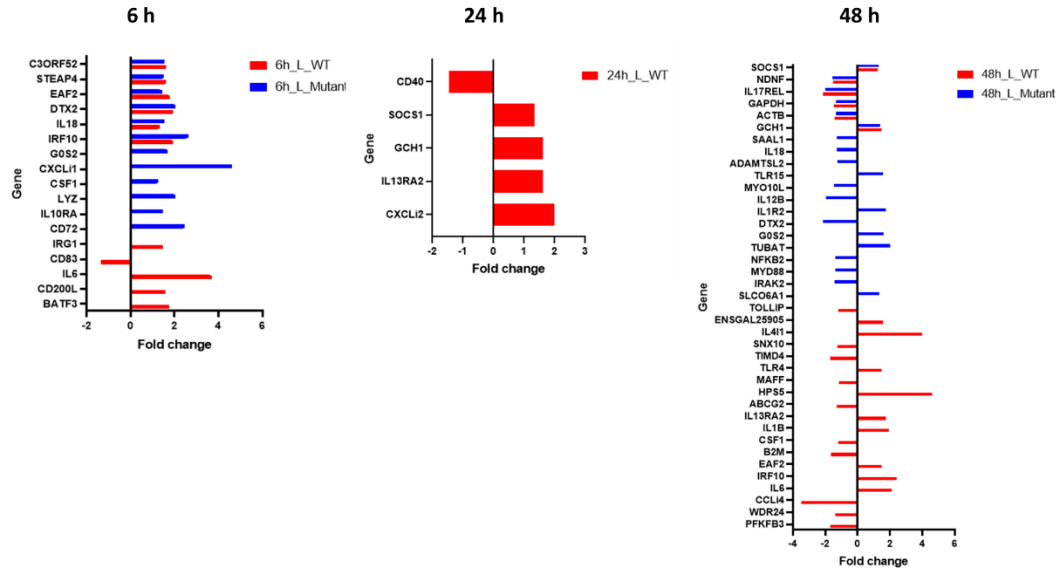
Results

To explore the degree of heterogeneity in the transcriptional profiles, the data were compared using principal component analysis. The tissues with the highest bacterial load clustered separately (encircled) and the samples with no or low bacterial load showed clustering in terms of tissue (lung or spleen).

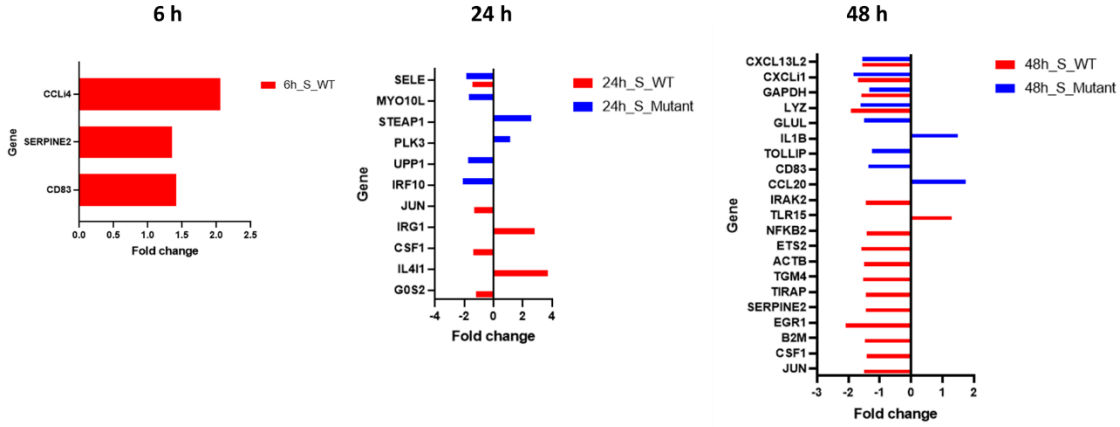


This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°731014

To further investigate the innate responses within the lung over time (6, 24 and 48 h) WT or mutant infected lung was compared to the mock control. The significantly differentially expressed genes are shown as fold change compared to the mock control at each time point.



To further investigate the innate responses within the spleen over time (6, 24 and 48 h) WT or mutant infected lung was compared to the mock control. The significantly differentially expressed genes are shown as fold change compared to the mock control at each time point.



Further in depth analysis of the innate responses is ongoing.

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

2.2 TNA 2

Analysis of the role of the duck RIG-I gene in transgenic chickens during infection with the avian influenza virus

Project description

Avian influenza viruses (AIV) represent a major threat to human and animal health. AIVs are susceptible to mutations in viral proteins, which increases the risk of bird-to-human transmission. Wild waterfowl, including ducks, are the natural reservoir for AIVs. It has been suggested that the genomic characteristics of these avian species are associated with a natural viral resistance against diseases induced by the AIV virus. In ducks, RIG-I (retinoic acid inducible gene 1) is considered a natural resistance gene against avian influenza. RIG-I is involved in triggering the antiviral response characterized by the production of type I and type III interferons (IFN), followed by the production of IFN-stimulated genes. While chickens are highly susceptible to influenza infection and exhibit clinical symptoms, ducks do not develop clinical symptoms upon infection with multiple avian influenza virus subtypes, which may be due to the presence of RIG-I, but may also be related to ring finger protein 135 (RNF-135). RIG-I and RNF-135 are absent from the chicken genome due to evolutionary loss. This project explains for the first time the role of both duck genes during AIV using transgenic chickens. The study of these genes provides basic information concerning their physiological role. This project also helps to understand the interaction of the virus with the host, which will allow the development of new strategies for the control of influenza viruses. The results of this project are very important and will affect different disciplines of science, including virology, immunology and animal welfare.

Experimental plan

Transgenic eggs from the breeding of heterozygous RIG-I with heterozygous RNF-135 (around 300 eggs) from the user/Technical University of Munich (TUM) were incubated and hatched at the INRAE-PFIE facility (see D11.1). The day after hatching, blood samples were collected and sent back to TUM for genotyping. Results of genotyping revealed almost 25% of each line i.e. RIG-I only, RNF only, RIG-I-RNF-135 and WT birds. The chicks were raised up for three weeks and transferred to the dedicated isolators shortly before the start of challenge experiment.

The challenge experiment was conducted to examine the susceptibility of RIG-I/RNF135 chickens to infection with a virulent H7N1 LPAIV. The animals were distributed in the isolators according to their genotype (see table) and were inoculated at 3 weeks of age via intra-tracheal and intra-choanal routes. The H7N1 virus (A/Turkey/Italy/977/1999) is a direct progenitor of highly pathogenic influenza virus and known to induce moderate-severe respiratory symptoms (morbidity approximately 100%) and mortality between 30 and 50% in experimentally infected White Leghorn chickens according to the literature.

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

Table: group distribution of infected and non-infected birds

genotype	RIG-I/RNF135		WT		RIG-I/RIG-I		RNF135/RNF135		WT		RIG-I/RNF135	
inoculation	H7N1		H7N1		H7N1		H7N1		mock		mock	
isolator	402-2 (n=16)		402-3 (n=16)		402-4 (n=18)		402-5 (n=18)		402-6 (n=15)		402-6 (n=15)	
group	A (n=16)		B (n=16)		C (n=18)		D (n=18)		E (n=9)		F (n=6)	
animal # (sex)	204	male	201	female	202	male	205	male	210	male	242	male
animal # (sex)	206	female	209	male	208	female	216	female	233	male	248	female
animal # (sex)	213	male	214	female	211	male	220	male	252	female	260	male
animal # (sex)	227	male	215	female	218	male	235	female	255	male	268	female
animal # (sex)	222	female	226	male	225	male	239	female	264	female	276	female
animal # (sex)	238	male	228	male	230	female	257	male	270	female	311	male
animal # (sex)	246	female	234	female	231	female	273	male	288	female		
animal # (sex)	253	female	237	female	232	female	274	female	298	male		
animal # (sex)	258	female	243	male	240	male	277	male	290	female		
animal # (sex)	262	male	267	female	244	female	279	female				
animal # (sex)	271	male	284	male	247	female	285	female				
animal # (sex)	278	female	301	female	249	male	286	female				
animal # (sex)	280	female	302	female	259	female	287	male				
animal # (sex)	282	male	303	male	265	male	292	female				
animal # (sex)	291	female	306	male	275	female	295	male				
animal # (sex)	294	female	317	male	281	female	307	male				
animal # (sex)					283	male	310	female				
animal # (sex)					305	male	322	male				
total number (m/f)	16 (7/9)		16 (8/8)		18 (9/9)		18 (9/9)		9 (4/5)		6 (3/3)	

Clinical symptoms and gross pathology

Clinical signs were considered as clinical scores 0 (no clinical signs), 1 (mild clinical signs), 2 (severe clinical signs), or 3 (dead/euthanized). Preliminary data revealed that the expression of RIG-I in chickens resulted in a pronounced inflammatory reaction that may have been responsible for high mortality compared to WT-birds. This effect as even greater in RIG-I-RNF-135 co-expressing birds.

Experiments at UEDIN

RNA samples (table) were transferred to the Roslin Institute and processed as described above under TNA 1.

group	exp. condition	animals sampled			animals sampled (total)	RNA samples (total –lungs, ceaca, spleens)
		D2	D6	D21		
A	RIG-I/RNF135, H7N1	6	5	0	11	33
B	WT, H7N1	7	6	3	16	48
C	RIG-I/RIG-I, H7N1	7	6	4	17	51
D	RNF135/RNF135, H7N1	6	6	6	18	54
E	WT, mock	3	3	3	9	27
F	RIG-I/RNF135, mock	3	3	0	6	18
					total number RNA samples	231

The 231 samples passed QC, cDNA was made and pre-amplified and ran on the arrays successfully. Some occasional samples failed and checked by PCR of a reference gene which revealed that the pre-amplification step was unsuccessful. This step was repeated and the samples will be re-run. The subsequent gene normalisation will be similar to described under TNA 1 page 5. The in-depth transcriptomic analysis will then proceed and the innate responses of the transgenic birds compared to the WT birds.

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