VETBIONET

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

Deliverable D8.3

Application of newly developed high throughput transcriptomic analytical tools NanoString and Fluidigm to livestock to examine the response to BSL3 pathogens

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Dissemination level	
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Confidential, only for members of the consortium (including Commission Services)	
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1. Summary

Objectives:

This deliverable sought to optimize and apply two novel transcriptomic platforms (NanoString and Fluidigm) to RNA samples from farm animal species infected with pathogens as a route to assess immune response in the host.

Rationale:

To address the objective, a set of nCounter Codesets (NanoString) and primer/probe sets (Fluidigm) for selected immune genes were designed, with a focus on cattle and chicken. The aim was to assess up to 150 such targets, against host tissues or cells that had been stimulated/infected with selected pathogens.

Teams involved:

NUID-UCD, UEDIN, INRAE, WBVR.

2. Introduction

The identification of biomarkers of infection and/or disease progression and remission are needed for multiple infectious diseases. Identification, assessment, and validation of such biomarkers from 'omics approaches often requires the expression of hundreds of genes across multiple samples to be assessed in parallel. This task sought to assess two such high throughout transcriptomics systems, namely Nanostring nCounter system and Fluidigm Dynamic Arrays.

The nCounter Analysis System (NanoString) allows mRNA target molecules to be specifically captured from complex mixtures and directly counted, without the use of amplification or enzymatic steps [1]. Two sequence specific, target-complementary probes (codesets) for each gene of interest are used: the capture probe contains a biotin tag for downstream purification, while the reporter probe contains a fluorophore bar-tag. Probes bind in solution to their targets and following affinity purification and immobilization are imaged. Each unique fluorophore tag identifies the target nucleic acid of interest, allowing direct quantification of target molecules through counting of tags. The nCounter can quantify up to 800 targets from a single sample in 24 hours, detecting as few as 2000 molecules in a sample with a dynamic range of 6 logs.

The Fluidigm system [2] is a microfluidics-based PCR system, with a 96x96 (or 12x12, 48x48) array of quantitative PCR reactions. The microfluidic chip can undergo thermal cycling, with product accumulation assessed at the end of each cycle via imaging. As with standard RT-qPCR, cycle thresholds (Ct values) can be determined that are inversely proportional to the concentration of the target nucleic acid in the starting sample.

While initial set up of the reaction targets, primers and conditions requires greater optimization with Fluidigm as compared to Nanostring, the running costs of Fluidigm are cheaper. Also, as Nanostring does not involve amplification of target, the starting material (usually 100ng) is greater than that for

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Fluidigm. This task set out to assess these two platforms in parallel to weigh up the various pros and cons depending on the research questions being asked.

3. Results

3.1 *Mycobacterium bovis* infections (NUID-UCD and INRAE)

As a first step in the optimization of the Nanostring platform, we designed codesets against a set of differentially expressed genes that had been identified by RNA-seq data from cattle infected with *Mycobacterium bovis* and tested these against the same RNA samples, showing an excellent correlation (Figure 1).

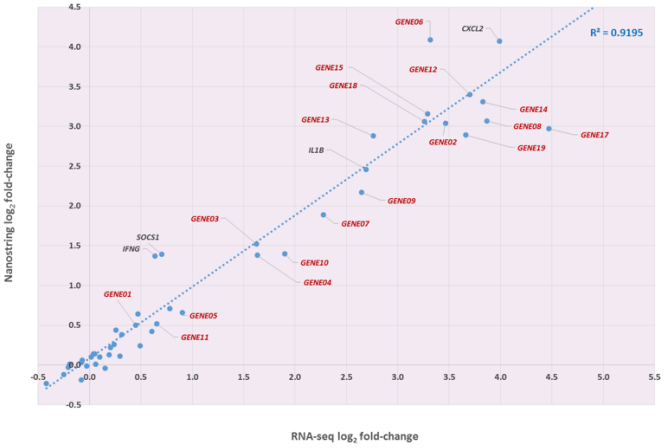

Figure 1: Comparison of RNA-seq vs Nanostring for selected bovine targets.

NUID-UCD initially designed and validated 150 Nanostring codesets that targeted 150 bovine immune and housekeeping genes. To extend this work NUID-UCD developed a collaboration with INRAE on the use of precision cut lung slices (PCLS) as an ex vivo infection model, and to allow 'head-to-head' comparison of Nanostring vs Fluidigm platforms. The NUID-UCD Nanostring codesets were augmented to include another 42 genes, giving a final list of 192 target genes. Our initial PCLS work needed to be repeated as quality of RNA was too low for Nanostring (but sufficient for Fluidigm). RNA extraction was hence optimized for Nanostring (using a bead-method). PCLS from two cattle breeds were infected with either *M. bovis* (x2 strains) or *M. tuberculosis* (x2 strains), and at 0, 24 and 48h post-infection PCLS were processed for RNA extraction. RNA was analyzed using 192 Nanostring codesets and 96 Fluidigm probes, with Fluidigm showing greater sensitivity with the low RNA quantities extracted. The advantage of the Fluidigm platform for amplification of starting material with lower quality and quantity meant it was the preferred option for analysis of PCLS infections. Detailed findings of our PCLS work and comparative infections have been published in Remot et al [3].

Collaborative work between NUID-UCD and INRAE on the role of vitamin D modulation of bovine neutrophils in response to *M. bovis* was also performed using the Fluidigm system. Two neutrophil subsets (MHC-II^{neg} and MHC-II^{pos}) recently characterized by INRAE [4] were sorted, stimulated (or not) with vitamin D and challenged with *M. bovis*. The response to two *M. bovis* strains was compared: BCG (vaccine) and AF2122/97 across 6 biological replicates, with gene expression of 96 genes assessed using the Fluidigm Biomark system. This revealed differential expression of genes encoding host

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defence peptides and pathogen recognition receptors that are known to an important role in microbicidal activity against mycobacteria. Further *ex vivo* analyses revealed that in calves supplemented with vitamin D, after BCG challenge (relative to control animals) an anti-inflammatory profile was observed, with significant downregulation of a cluster of genes including IL1B, IL1R1, CXCL1, CXCL2, CXCL5, MMP9 and COX2 and an upregulation of CXCR1, CX3CR1 and NCF1. The results suggest that dietary vit D3 modulates anti-mycobacterial and innate immune responses.

3.2 Avian immune-related genes (UEDIN and INRAE)

The Fluidigm Biomark system was used at the Roslin Institute to develop a high-throughput quantitative reverse-transcriptase PCR array for avian immune-related genes. Over a hundred genes that were consistently differentially transcribed in response to pathogens, or constituents thereof, were identified and used for array design. For 89 of these genes, primers were designed and validated in qPCR assays. A panel of reference genes that exhibited stable transcription following infection or stimulation with agonists of innate immunity were also selected. Primer pairs were designed, sensitivity and specificity assessed before validation of the Fluidigm 96.96 IFC dynamic array with samples collected from commercial broilers in both low- and high-biosecurity environments. Details of this work are provided in Borowska et al [5].

An improved pipeline for analysis of Fluidigm high-throughput qPCR data relating to avian immune-related gene expression was subsequently developed and employed for all ongoing and future projects. Raw data were pre-processed through a multi-step pipeline including: (i) correction for primer efficiency, (ii) inter-plate calibration to permit analysis of data from multiple plates, (iii) normalisation to geometric means of stably expressed reference genes, (iv) relative quantification to appropriate control and (v) log transformation prior to statistical analysis (all performed in GenEx software). Statistically significant differentially expressed genes (DEGs) were obtained and the relative quantification data was analysed via principal component analysis, heat-maps (ggplot2 and pheatmaps R packages) to permit visualisation and further analysis of experimental data.

The Fluidigm high-throughput qPCR platform was used to analyse innate immune responses to a panel of RNA viruses. Lung macrophages were isolated from *CSF1R*-reporter transgenic chickens infected with (i) avian influenza virus (AIV) H7N1 (ii) Newcastle Disease virus (NDV) vaccine strain clone 30, (iii) infectious bronchitis virus (IBV) strain M41 and (iv) an attenuated derivative IBV vaccine strain H120. Controls that were mock infected or stimulated with the TLR7 agonist, R848, were included for assessment of minimum and maximum responses and data normalisation addressing both intraplate and bird-to-bird variation. Principal component analysis showed a clustering of the transcriptomic response of avian lung macrophages respective of viral infection status and time-point. Stimulation with R848 resulted in different patterns of expression of interferons and interferon-stimulated genes over 6-24-48 hours post-stimulation. AIV infection induced a potent innate response, with the highest number of DEGs showing an innate response of higher magnitude, peaking at 24 hours post-infection with 49

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upregulated and 10 downregulated DEGs. IBV (M41 and H120) and NDV had highest numbers of total DEGs at 6 hpi and 48 hpi, respectively and AIV induced a higher number of unique genes across all time-points.

Similar to the works performed by UEDIN, and integrating their results, INRAE has used the Fluidigm qPCR platform to assess the chicken immune response to infections with Salmonella, Eimeria spp. or AIV in various live animal or alternative models. The Fluidigm qPCR protocols are currently used in various collaborative projects between UEDIN and INRAE (including collaborations in two ongoing TNA projects) and are part of the VetBioNet service portfolio in the ISIDORe infrastructure project.

3.3 Porcine gene expression (NUID-UCD and WBVR)

To extend the target species and collaboration network, NUID-UCD and WBVR collaborated in the analysis of gene expression across from lung, bronchus, trachea, and blood from pigs that had been immune stimulated with bacterial subunits. A custom porcine Nanostring codeset panel was generated by Nanostring and shipped to NUID-UCD who then performed analysis across 48 porcine samples, which after initial quality checks were transferred back to WBVR. This shows the flexibility of the Nanostring system to rapidly move from RNA target to analysis, without the need qPCR optimization that is required for Fluidigm.

4. Conclusions

This deliverable assessed the Nanostring nCounter and Fluidigm Dynamic Array as multiplex platforms to quantify immune gene expression across bovine, avian and porcine samples. Various pros and cons of the two systems were apparent: the Nanostring system did not require initial optimization of amplification as is required by Fluidigm, hence accelerating the ability to assess gene expression across multiple species. The lack of amplification also meant that the Nanostring detected transcripts directly, and ‘digitally’ counting abundance and hence not introducing amplification artefacts. The Fluidigm system was more appropriate when dealing with samples that had low starting quantities of RNA, and assay running costs were cheaper. Hence selection of either system depends on factors that include the requirements of the experimental system, RNA concentration, urgency required in results, and budget available.

5. Annexes

References

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