686. Genomewide transcriptome profiling of milk derived primary bovine mammary epithelial cells after pathogen challenge

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Abstract

A method was developed to collect and cultivate primary bovine mammary epithelial cells (pbMECs) from milk samples of healthy lactating cows. Cell cultures from ten Nordic Red cows were used in a pathogen challenge study with two common pathogens, *Escherichia coli* and *Staphylococcus aureus*. Transcriptome-wide changes in gene expression were monitored at four time points after challenge. We present here preliminary results from two cows, showing that pathogen specific differences in gene expression can be detected in the pbMECs, reflecting differences in the immune responses triggered by each pathogen.

Introduction

Mastitis, inflammation of the mammary gland, is the main cause of early weaning in humans, a major animal welfare concern and the costliest disease in dairy cattle. Mastitis can be expressed as mild sub-clinical, acute clinical or chronic inflammation of the mammary gland; caused by various bacteria, which may activate the mammary immune system in different ways, thereby influencing the severity and outcome of the disease (Taponen *et al.* 2017). Understanding of the molecular mechanisms that activate and regulate this response would be central to the development of effective prevention, prophylactics and treatments.

Previous studies show that the pathogen specific immune response of the mammary epithelial cells (MEC), but not of professional immune cells (e.g. macrophages) correlates best with the pathogen specific immune response of the udder (Petzl *et al.* 2018). For studying MEC function, usually tissue samples obtained by biopsies from live or slaughtered animals are used. We developed an ethical noninvasive method suitable for analysing many animals that would be available for follow-up studies. Therefore, we studied the possibility to extract and cultivate primary bovine mammary epithelial cells (pbMEC) from milk samples.

Our aim is to study the innate immune response by genomewide transcriptome profiling in pbMECs to two common mastitis pathogens known to induce different types of mastitis. The pathogens used include the Gram-negative *Escherichia coli* usually causing a strong acute inflammation and the Gram-positive *Staphylococcus aureus* causing a milder, often sub-clinical inflammation. In this paper we describe preliminary results from a subset of the full experiment, providing evidence that pbMECs extracted from milk samples can be used to identify differentially expressed genes.

Materials & methods

Primary cell culture establishment. Milk samples from two healthy lactating cows (Nordic Red Cattle) were collected by standard milking methodology to sterile collection bottles. A protocol modified from Danowski *et al.* (2012) and Hillreiner *et al.* (2017) was used to extract pbMECs. The epithelial character of the cells was confirmed by immunocytochemistry.

Pathogen challenges with *Escherichia coli* and *Staphylococcus aureus*. Cells (~1×10^5 cells/well, Greiner bio-one, collagen I coated, 392-0029) were challenged *in vitro* with two common heat inactivated mastitis pathogens, *E. coli* and *S. aureus* strains extracted from Nordic Red cows having mastitis (S. Taponen, personal communication) using 10 as multiplicity of infection (MOI). Cells were harvested before and at four different time points (3h, 6h, 12h and 24h) post challenge.

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RNA-Seq. Total RNA was extracted from the harvested cells using Rneasy Plus Micro kit (Qiagen) following manufacturer's protocol. RNA quantity and quality were measured with BioAnalyzer, and 50 ng of total RNA was used for ribosomal RNA depletion using RiboCop V1.3 (Lexogen). Ribosomal depletion allows detection of functionally relevant coding as well as non-coding transcripts through removal of ribosomal RNAs (Choe *et al.* 2021).

CORALL Total RNA-Seq Library Prep Kit (Lexogen) was used for library generation. The quality of pooled libraries was first checked on an Illumina iSeq100 platform at the Natural Resources Institute Finland and paired-end sequencing of three biological/technical replicates per timepoints and treatments was performed on an Illumina NovaSeq platform at the Finnish Functional Genomics Centre.

All samples from above-described challenge plan were sequenced with three technical replicates resulting in total 78 samples. An in-house Snakemake RNA-Seq pipeline following common RNA-Seq practices was applied for quality checks (FastQC, multiQC), trimming (fastp), alignment (STAR) and quantification (featureCounts). The pipeline, including additional features, is available at https://www.github.com/fischuu/Pipeline-RNA-seq.

Differentially expressed genes. The data was aligned against the ARS-UCD1.2 bovine reference genome and the quantification was done with reference to the Ensembl annotation 100. Technical replicates were combined by summing the corresponding read counts per gene. Following this practice, a data matrix with raw count data for 27,607 genes and 26 samples was generated and then split pathogen-wise, using the same control samples resulting in two matrices with 18 samples, each with 10 control and 8 pathogen related samples. Next, genes were filtered based on the sample read counts and only those genes were kept that had at least for eight samples 0.5 counts per million (cpm). This way, 11,891 genes were kept for *E. coli* and 11,674 for *S. aureus*. The differential gene expression (DE) analysis was performed with edgeR, using TMM normalized counts and only DE genes with a fold-change of 1.15 (*E. coli*) respective 1.1 (*S. aureus*) were kept.

Results

Primary cell culture. We were able to extract, cultivate and cryo-preserve pbMECs derived from freshly milked milk. Cells were successfully used in pathogen challenges. The pbMECs extracted from milk samples were shown to have epithelial characters by immunocytochemistry (Figure 1).

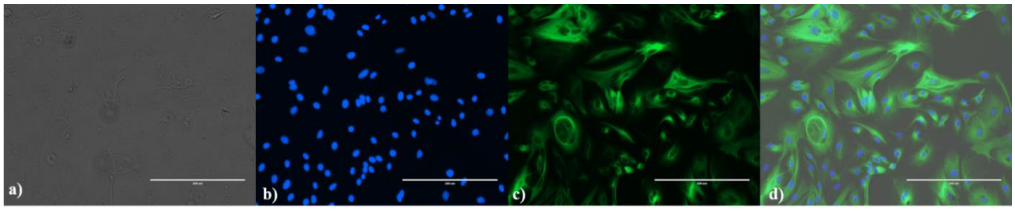

Figure 1. The (a) pbMECs under a light microscope, (b) cells stained with DAPI staining of pbMECs under fluorescence microscopy (c) cytokeratin staining using Monoclonal Anti-Cytokeratin, pan-FITC antibody (Sigma, F3418) d) merged image of light microscope, DAPI and cytokeratin staining images. The bars in the figures are 200 µm.

RNA-Seq analyses. The sequencing depth was between 41 and 87 mio 2×100 bp reads (mean: 62.8) per sample, after quality trimming and filtering the mean dropped only to 62.0, and the R1 (R2) read length to 86 bp (96 bp), indicating high-quality input data.

Prior to the DE analysis basic MDS plots of the raw data (data not shown) indicated a better separation between cases and controls for *E. coli* compared to *S. aureus*. Without fold-change filtering, 2,301 (1,849) downregulated and 2,332 (1,568) upregulated genes were detected after *E. coli* (*S. aureus*) challenge. However, after applying the fold-change filter 1,124 (868) up- and 1,219 (757) downregulated genes remained for *E. coli* (*S. aureus*).

For the top-30 differentially expressed genes, we applied a hierarchical clustering and visualised the results in Figure 2. The heatmaps show clear sample separations, as expected control samples are in a different branch compared to samples exposed to *E. coli*/*S. aureus*.

The specific top-30 up- or down-regulated DE genes differ between the two pathogens (Figure 2). The GO and KEGG analyses (data not shown) of the DE genes after each pathogen challenge show both same affected immune response related pathways (such as cytokine-cytokine receptor interaction, systemic lupus erythematosus) as well as specific affected pathways (such as oxidative phosphorylation and regulation of actin cytoskeleton for *E. coli* and ribosome, protein export and ECM-receptor interaction for *S. aureus*).

Discussion

We show here that pbMECs extracted from milk can be cultured and used for pathogen challenge experiments. The transcriptomic results indicate that we can detect pathogen specific changes in relevant pathways related to immune response, partly similar to those reported before by microarray-based analyses (Günther *et al.* 2017). The results including the full set of animals and timepoints will be published later.

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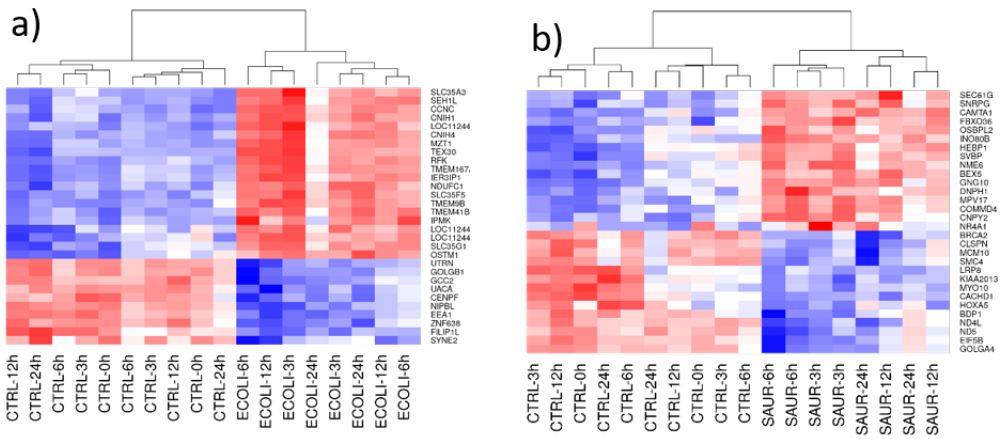

Figure 2. Heatmap visualisation of the clustering of (a) *Escherichia coli* and (b) *Staphylococcus aureus* exposed samples and corresponding control samples, based on the top 30 DE tested genes.

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