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BRIEF COMMUNICATION

The use of microarrays to investigate gene regulation in the bovine mammary gland during *Streptococcus uberis* mastitis

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Keywords: Mastitis, *Streptococcus uberis*, microarrays.

Bovine mastitis is one of the most costly dairy-based diseases worldwide with an estimated $60 M loss to New Zealand per season. Mastitis infections are a result of bacterial or fungal pathogens becoming established in the udder, and are detected by the increase of somatic cells in the mammary gland, bacteriology data and physical signs of infection. In New Zealand, mastitis is usually a result of a gram-positive bacterial infection (Brookbanks, 1966). Of these bacteria, *Streptococcus uberis* is emerging as one of the most prevalent causative organism of mastitis in New Zealand (McDougall, 2002). It resides naturally in the environment making prevention of the disease difficult. Currently the main means of controlling the disease is through administering antibiotics following identification of infection.

The mammary gland is protected from invasion by the activation of non-specific (innate) and specific defence mechanisms. The innate immune response is the predominant defence during the early stages of infection and is induced rapidly at the site of infection (Sordillo *et al*., 1997). Although the local mammary innate immune response has been widely investigated, studies have focussed on known antibacterial compounds and little is known about the novel compounds in bovine milk and how these contribute to the defence system of the mammary gland. The mechanism by which the mammary gland innate immune system is active against *S. uberis* is poorly understood. By developing an understanding into the way *S. uberis* induces an immune response in the mammary gland and the effect of infection on mammary gland metabolism we may identify components which can be utilised to either reduce the symptoms of/or prevent or cure *S. uberis* mastitis.

We have employed, complementary DNA (cDNA) microarrays to evaluate the changes in gene expression accompanying the onset of clinical mastitis caused by *S. uberis*. A healthy, rear quarter of the udder of five Friesian heifers (mid-late lactation) were infused with approximately 1000 colony-forming units of a strain of *S. uberis* following a p.m. milking. The strain of *S. uberis* used had been previously isolated from a cow with clinical mastitis. The cows were observed for visual signs of clinical mastitis, evident as swelling and protein aggregates in the milk. Milk samples were taken for bacteriological examination and somatic cell counts (SCC). Alveolar tissue was taken post mortem from the infected quarter and a control non-infused quarter within 24 hours following the identification of clinical mastitis (Table 1). Total RNA was extracted using TRIzol reagent (Invitrogen, Carisbad, CA, USA) according to the manufacturer's instructions. The RNA was labelled

Table 1: Somatic cell counts (SCC) and bacteriology results for individual quarters. (LF= left front, LB= left back, RF= right front, RB= right back).

Cow	Infection Status	Quarter sampled	Somatic Cell Count (X 10^3 cells/ml)	Bacteriology Comments
1	Non-infected	LF	38	No specific growth
1	Clinical infection	RB	16147	Heavy growth of *Streptococcus uberis*
2	Non-infected	LB	30	No specific growth
2	Clinical infection	RB	1786	Heavy growth of *Streptococcus uberis*
3	Non-infected	RF	219	No specific growth
3	Clinical infection	RB	13865	Heavy growth of *Streptococcus uberis*
4	Non-infected	LB	116	No specific growth
4	Clinical infection	RB	18912	Heavy growth of *Streptococcus uberis*
5	Non-infected	RB	93	No specific growth
5	Clinical infection	LF	17297	Heavy growth of *Streptococcus uberis*

with reactive forms of Cy3 and Cy5 dyes (Ambion amino allyl cDNA labelling kit) and RNA from infected vs. non-infected quarters from the same animal were hybridised to glass slides containing approximately 22,000 expressed sequence tags (ESTs) from bovine immune and mammary tissues libraries. The labelled RNA was hybridised to the microarray slide in a humidified chamber at 50°C for 16-24 hours. The microarray data was read using a GenePix scanner and the images were analysed using GenePix software (Axon Instruments, Inc. CA, USA). The difference in mRNA expression was normalised using REML GenStat (2002, Sixth Edition. VSN International Ltd., Oxford, U.K.) for each microarray slide and log differences were pooled to give the average change in expression for each EST across the five cows.

The microarray slides were an exact replicate of each other, consisting of 21,378 ESTs. Of these, 15,704 ESTs had RefSeq hits and 5,674 had no known RefSeq hit. The ESTs were classified into functional groups using a web-based application, DAVID (Dennis *et al*., 2003). This application enabled the annotation of the dataset based on Gene Ontology organising principles, which grouped the genes based on their biological process. ESTs that were up and down-regulated by greater than 2-fold ($P<0.01$) on the microarray were selected for annotation using DAVID and grouped based on these putative functions. This annotation technique allowed focussing on functional groups of interest and provided clues to the possible role that particular genes within these groups had in *S. uberis* mastitis.

Table 2. Changes in gene expression in the bovine mammary gland for annotated expressed sequence tags (ESTs) following the onset of *Streptococcus uberis* clinical mastitis.

DAVID classification	Up-regulated	Down-regulated
Cell communication	47	33
Signal transduction	16	11
Cell death	8	5
Cell growth and/or maintenance	30	14
Cell homeostasis	38	0
Cell proliferation	19	16
Transport	14	7
Cell motility	20	4
Development	43	18
Metabolism	31	20
Biosynthesis	30	8
Catabolism	22	8
Nucleic acid metabolism	18	20
Reactive oxygen species	8	0
Others	9	5
Immune response	49	9
Unknown/not classified	298	188
Total ESTs	700	366

The ESTs within each functional group were separated depending on their regulation in response to *S. uberis* mastitis (Table 2). The number of ESTs that were up-regulated in response to the mastitis infection was 700 compared with 366 that were down-regulated. There were more genes up-regulated in response to mastitis for all functional categories with the exception of nucleic acid metabolism. It is interesting that there was no increase in nucleic acid metabolism as this category contained all the transcription factors which are required for up- and down-regulation of genes.

The up-regulation in gene expression was most significant in immune response and cell homeostasis. An up-regulation in immune-related genes was expected due to the inflammatory response that is induced following the onset of mastitis. The increase in cell homeostasis genes could be explained by the requirement to maintain a constant internal environment within the mammary gland. These regulatory mechanisms could be up-regulated in response to the internal changes such as heat and swelling which occur as a result of infection.

A quantitative comparison between the numbers of genes up- and down-regulated due to mastitis is not valid for microarray data due to the bias introduced when selecting the ESTs to place on the microarrays. However, assigning functional groups to ESTs enables rapid focussing on particular groups of interest without the need to individually identify each ESTs within the dataset. The expression profile observed for ESTs within immune function category's confirmed the validity of this approach. For instance, lactoferrin, a milk protein with bacteriostatic and anti-bacterial properties, was up-regulated by greater than two-fold, and defensin, a known antibacterial peptide with activity against mastitis pathogens (Cullor *et al*., 1991), increased in expression by greater than four-fold. Serum amyloid A, associated with the acute phase response was also up-regulated by greater than four-fold in the mastitis affected tissue.

This approach may also be applied to the determination of mammary metabolic factors influenced by mastitis and ultimately lead to the identification of genes and pathways which are distinguishing features of mastitis infections. This microarray has demonstrated that milk proteins known to be synthesised within the mammary gland such as caseins, alpha lactalbumin, and beta lactoglobulin are down-regulated by greater than two-fold following the onset of clinical mastitis. Genes involved in fatty acid metabolism and lactose biosynthesis were also significantly reduced ($P<0.01$). These results confirm a down-regulation of genes involved in milk quality and yield, indicative of mastitis infections. Mastitis also appears to up-regulate genes associated with apoptosis or cell survival. This latter process is associated with loss of epithelial cells, and subsequent milk yield, and also degeneration of the alveolar structure (Wilde *et al*., 1997). Identification of mammary factors characteristic of mastitis infections provides further tools for both diagnosis and/or therapeutic restoration of the mammary function.

In summary, the use of microarrays is a valuable tool in the identification of genes and pathways which are altered in response to *S. uberis* mastitis. The ability to classify genes into functional groups allows the rapid identification of components of the non-specific immune response which aid in the defence of the mammary gland and also mammary metabolism factors which are influenced by infection. Many of the immune

components and mammary metabolism factors identified using this approach were known to be associated with mastitis. Future work will focus on the other genes, whose role in this disease are unknown, that may prove to be useful in the development of natural novel mastitis remedies or the selection of mastitis resistant animals.

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