Research Article

Distinct MicroRNA Signatures for Mastitis Measured in Milk Following Natural Exposure in Dairy Herds

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Abstract

Mastitis, a global endemic disease in dairy cattle, not only adversely impact milk production/quality leading to increased economic loss to farmers, it poses a consumer health issue as the milk may be unfit for human consumption due to pathogen contamination. Use of Somatic Cell Count (SCC) as an indicator of mastitis may be insufficient for effective diagnosis of disease. MicroRNAs (MiRNAs) are increasingly recognised as promising alternative indicators of mastitis. In this study, we identified circulating miRNAs differentially expressed in milk of mastitic cows after 'natural levels of exposures' and in response to different causative agents 'on farm'. Using a miRNA microarray based approach we found at least 26 miRNAs as generic indicators of clinical mastitis; 7 of which may also be early mastitis indicators. We further identified 27 miRNAs unique to S Uberis-positive (SU) mastitis, including miR-320a/b which has been linked to modulation of trained immune activity. Three differentially expressed miRNAs were unique to mastitis positive for Coagulase Negative Staphylococcus (CNS), and a further 5 miRNAs were unique to SU and CNS mastitis group comparison. Our study design differs from the existing literature which reports the effects of either exogenous dosing with a singular agent or of dosed exposures in the context of single cell types (which individually contribute in only very minor ways to SCC) in an empirical ex vivo setting. Collectively, the differentially expressed miRNAs we have identified are high confidence biomarkers for detection of mastitis (even when asymptomatic), assessment of clinical status and identification of causative agent.

Introduction

Mastitis is a global endemic disease in dairy cattle, is characterised by inflammation of the mammary gland and a cause of considerable economic burden due to loss of production [1]. It usually results from bacterial infection, although algae, viruses, fungi or poor animal husbandry or improper milking procedures also lead to mastitis [1]. Mastitis should be viewed as a complex etiology arising from a variety of agents and resulting in differential pathology, with both subclinical and clinical states of disease. Cases of subclinical mastitis often appear superficially asymptomatic, but may persist for entire periods of lactation or throughout the life course of the animal. Consequently, subclinical mastitis has a profound impact on lifetime performance, as reflected in low production and poor quality of milk [2]. Furthermore, the resulting milk may be unfit for human consumption due to pathogen contamination. In clinical mastitis, the inflammatory responses to infection cause clearly visible abnormal changes in the udder (swelling, redness, pain) and a marked loss of milk consistency/quality. Standard care of clinical mastitis involves antibiotic treatment, although culling may also result if there is a significantly high rate of infection within the herd. Any animals found to be non-responders after antibiotic treatment (and other methods, such as frequently stripping out the milk) would be of major concern as they are likely to act as reservoirs of disease and a source of infection within the herd. As such, prompt determination of infection level and identification of the underlying causative agent are considered vital for effective management at both the individual and herd level.

Currently, 'Somatic Cell Counts' (SCC) in milk samples are used as an indicator of subclinical and clinical mastitis; reviewed in [3]. In response to infection, blood-borne immune cells suffuse the mammary tissue, leading to cell death and sloughing, thus increasing SCC [4]. However, the interpretation of SCC numbers is not without its complications. Whilst a high SCC may directly correlate with low milk quality during mammary gland infection, the status of disease is difficult to accurately determine at lower SCC between 100,000 and 200,000 cells/ml (bulk tank) [5]. Furthermore, the magnitude of SCC change is known to vary with the species of bacteria [3]. Given the obvious limitations of SCC measures, there would be clear benefit in identifying alternative diagnostic indicators. To this end, and leveraging the milk sample as an example of a 'liquid biopsy', we and others have begun profiling the expression of miRNA species in comparison to SCC and in response to different causative agents. MicroRNAs are a class of endogenous, short, non-protein

coding, single-stranded gene products, typically 19–24 nucleotides long [6]. Notably, a change in miRNA expression has been associated with a variety of disease states [6], linked to mammary tissue development and biology, and the regulation of milk composition [7-11].

This study aims to identify miRNAs differentially expressed in mastitis and determine a pathogen-specific miRNA profile that distinguishes between *Streptococcus uberis*-positive (SU) mastitis and mastitis positive for Coagulase Negative Staphylococcus (CNS). Milk from mastitic cows acquired through 'natural levels of exposures' and in response to different pathogens 'on farm'. Using a miRNA microarray-based approach, we found at least 26 miRNAs as generic indicators of clinical mastitis; 7 of which were also significantly differentially expressed in a second independent 'on farm' trial, and as such represent promising candidates of general mastitis indicators. We further identified 27 miRNAs unique to SU mastitis, including miR-320a/b which has been linked to modulation of trained immune activity. Three miRNAs were differentially expressed in mastitis positive for CNS, and a further 5 miRNAs were differentially expressed between SU and CNS mastitis. Our field trials not only provide 'in vivo' validation of earlier observations, but have 1) extended the potential miRNA signature indicative of mastitis infection, 2) 'calibrated' specific miRNA species against SCC measures to discriminate those reflecting both sub-clinical and clinical states, and 3) provide initial insights into miRNA species modulated by specific bacterial agents.

Materials and Methods

All experimental procedures used in this study were approved by the Animal Ethics 107 Committee, New Zealand (Approval Number 12984).

Milk sampling

This study utilised milk samplings from cows that developed mastitis through natural exposure in their environment. Milk samples were collected Friesian-Jersey cows during the early-mid milking season coinciding with early-mid lactation from two independent trials (referred to as Trials 1 and 2) conducted in the farms of Livestock Improvement Centre (LIC) in Hamilton, New Zealand. For mastitic milk, samples were collected upon first detection of mastitis in the cows. Aseptic foremilk samples were taken prior to cups on milking in the farms using standard aseptic technique [12]. These quarter milk samples were put on ice packs for transport. In the laboratory, for each cow, 3ml of milk from each quarter was mixed and pooled. These cow composites were then stored as 1-ml aliquots in -80ºC until ready for nucleic acid extraction. Whole milk somatic cell count for each sampling was measured. Diagnosis of clinical mastitis was recorded based on visual inspection of the cows by the farmer, and later these samples were matched with somatic cell counts recorded in the shed. Trial 1 comprised 19 control and 21 mastitic cows (total of 40). Trial 2 comprised 5 control and 11 mastitic cows (16 in total). Causative bacterial pathogens were also identified for 9 of the 21 mastitic cows in Trial 1.

Bacterial pathogen identification

Bacteriological methods were performed according to recommended procedures of the National Mastitis Council [13]. Briefly, bacterial culture was performed by plating 10μl of quarter samples onto blood-esculin agar plates (Fort Richard Laboratories Ltd, NZ).

Haemolysis-positive cultures were further tested for coagulase positivity. In mastitic samples, we detected either CNS or SU.

RNA extraction and miRNA microarray assay

RNA extraction from milk was performed according to a previous report with minor modifications [14]. Briefly, 1ml of milk was spun at 1500xg for 10 min at 4ºC. The resultant supernatant was again spun at 1500xg for 10min at 4ºC. Following this, the supernatant was spun at 21000xg for 30 min at 4ºC. Chloroform was then added to the final supernatant (whey fraction) at 1:1 ratio, and the sample was vortexed and incubated for 3 min at room temperature. Sample was then spun at 12000xg for 15 min at 4ºC. The resulting supernatant was then mixed with 1.5 volumes of 100% ethanol and loaded onto a miRNeasy column for RNA extraction using the Qiagen® miRNeasy Mini kit according to manufacturer's protocol. RNA integrity was assessed on the Agilent 2100 Bioanalyzer using RNA 6000 Nano kit, and samples showed RIN score ≥8. RNA quantity and quality was assessed with absorbance values at 260 and 280nm using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). All samples showed 260/280 ratio ≥1.8. Reverse transcription was carried out using 200ng total RNA, and cRNA labelling, fragmentation and hybridization onto Affymetrix GeneChip miRNA 3.0 arrays were performed using manufacturer's protocols (Affymetrix Inc., CA, USA).

Differential gene expression analysis

Raw intensity data files (CEL) were read using the publicly available oligo package [15] in R version 3.2.1 (http://www.bioconductor.org/). Background subtraction, quantile normalization and summarization via median-polish, and finally log2 transformed, were performed on the raw data using the R package RMA (Robust Multichip Average) pre-processing methodology [16]. Analysis of differential gene expression using a linear models approach was performed using the R package Limma [17]. Adjusted P≤0.05 using False Discovery Rate (FDR) corrections were considered as statistically significant. Heat map illustration of sample 157 hierarchical clustering was performed using the R package g plots (cran.r-project.org/).
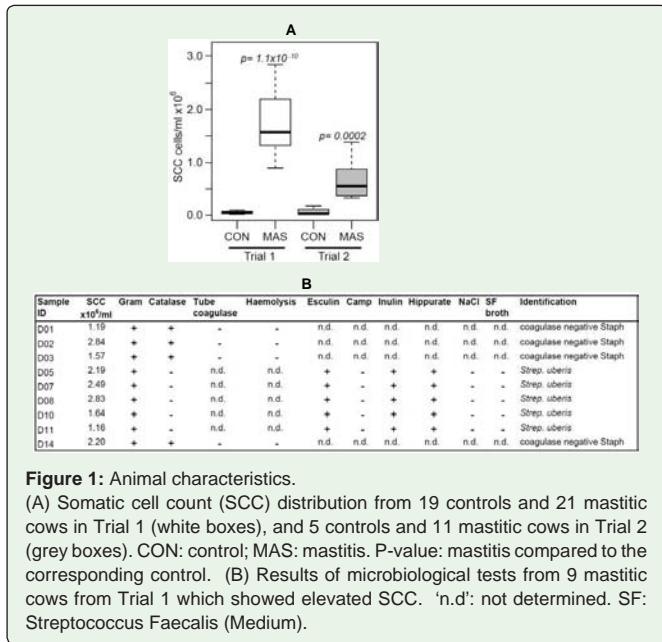
Statistical analysis

Assessment of significant difference in SCC between control and mastitis group was performed using student t-test. Association between level of miRNA of interest (log2 intensity) and SCC was assessed by linear least squares regression. P<0.05 was considered as statistically significant. All tests were performed in R version 3.2.1.
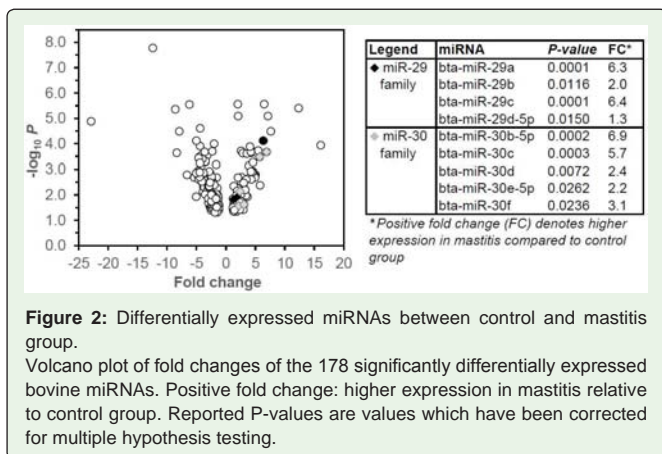
Results

Clinical mastitis phenotype as defined by high somatic cell counts

In Trial 1, SCC in control and mastitic groups were 54.4x10³ ± 26.1x10³ cells/ml and 1728.2x10³ ± 632.6 x10³ cells/ml respectively. SCC significantly increased by 31-fold (P=1.1x10⁻¹⁰) in mastitic group compared to control (Figure 1A). In Trial 2, SCC in control and mastitic groups were 69.6x10³ ± 70.1x10³ cells/ml and 664.2x10³ ± 361.8 x10³ cells/ml respectively. SCC significantly increased by 9.5-

Figure 1: Animal characteristics.
(A) Somatic cell count (SCC) distribution from 19 controls and 21 mastitic cows in Trial 1 (white boxes), and 5 controls and 11 mastitic cows in Trial 2 (grey boxes). CON: control; MAS: mastitis. P-value: mastitis compared to the corresponding control. (B) Results of microbiological tests from 9 mastitic cows from Trial 1 which showed elevated SCC. 'n.d': not determined. SF: Streptococcus Faecalis (Medium).
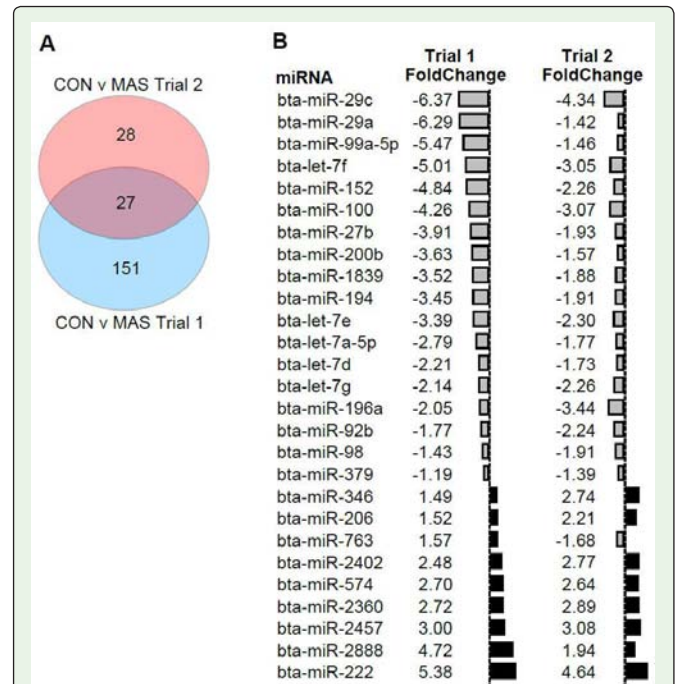
fold (P=0.0002) in mastitic group compared to control (Figure 1A). In addition, whereas SCC in the control group was similar between both trials, SCC in the mastitis group was significantly lower by 3-fold (P=1.28×10^{-6}) in Trial 2 relative to Trial 1. Results of *S uberis* or coagulase negative staphlococcus detection are summarized in Figure 1B.

Differential Mirna Expression Associates with Mastitis Driven By Natural Pathogen Exposure Comparison between mastitic and control groups from Trial 1 yielded 4175 transcripts (P<0.05) which annotated to bovine and non-bovine sequences, and comprised transcripts of various small RNA types. For this study, we have focused our analysis on bovine miRNA transcripts. Of the 4175 transcripts, we found 178 bovine miRNA transcripts with a minimum of 1.2-fold change (up/down) when comparing mastitic to control group (Figure 2). Results of differential expression for this list of miRNAs are found in Supplemental table 1A. Interestingly, the miRNAs which Sun et al. previously reported to be highly enriched in bovine milk exosomes, and which were also differentially expressed in mastitis, significantly overlapped (19 out of 22) with our list of differentially

Figure 2: Differentially expressed miRNAs between control and mastitis group.
Volcano plot of fold changes of the 178 significantly differentially expressed bovine miRNAs. Positive fold change: higher expression in mastitis relative to control group. Reported P-values are values which have been corrected for multiple hypothesis testing.

expressed miRNAs (Supplemental Figure S1). Mir-200a, expression of which we found to be decreased by 4.9 fold (P<0.001) in mastitis (Supplemental Table 1A), was shown in mice *in-vitro* and *in-vivo* to regulate mammary epithelial cell differentiation and production of milk proteins (such as β-case in) [11]. Furthermore, miR-200a may interact with the same target sites (i.e., target genes) as miR-141 based on similarities in their seed sequences. Given the consistent alignment of these miRNAs between previous studies and ours, these miRNAs (which are known to be relevant to mammary gland function) represent promising mastitis indicators.

We also found members of the bta-miR-29 and bta-miR-30 families to be differentially expressed when comparing between mastitis and control (Figure 2). The miR-29 family was shown to epigenetically regulate lactation-related genes in dairy cow mammary epithelial cells [7]. We found that bovine miR-29a, miR-29b, miR-29c and miR-29d-5p levels were lower by 6.3-fold, 2-fold, 6.4 fold and 1.3-fold respectively (P<0.05) in mastitis compared to control group (Figure 2). Similarly, bta-miR-30b-5p, miR-30c, miR-30d, miR-30e-5p and miR-30f significantly decreased by 6.9-fold, 5.7-fold, 2.4- fold, 2.2-fold and 3.1-fold respectively P<0.05) in mastitis group relative to control (Figure 2). Details of the pair wise group comparison are found in Supplemental table 1A. Overall, the differentially expressed miRNAs which we have identified represent miRNAs that potentially relate to perturbed immune response and mammary gland function which are consistent with features of mastitis.

Figure 3: Comparison of differentially expressed bovine miRNAs from Trials 1 and 2.
Numbers within the Venn diagram denotes number of bovine miRNAs corresponding to each region within the Venn diagram. CON: control; MAS: mastitis (defined by high somatic cell count). (B) Fold change in expression of the 27 miRNAs common to both Trials 1 and 2 as illustrated by the overlapping region in (A). With the exception of bta-miR-763, these miRNAs are potential biomarkers of mastitis. Black bar: up-regulated in mastitis (positive fold change); Grey bar: down-regulated in mastitis (negative fold change).

We next questioned whether the differentially expressed miRNAs associated with mastitis in Trial 1 were similarly differentially expressed in Trial 2. Although we found no significantly differentially expressed transcripts at the array-wide level (based on a cut off of P<0.05 after adjusted for multiple hypothesis testing), a total of 1495 transcripts were differentially expressed between control and mastitis group (based on P<0.05). These comprised of sequences of various small RNA types, and annotated to bovine and non-bovine species. Of these, 55 were bovine miRNAs with a minimum of about 1.2-fold change (up/down), and a maximum of about 4-fold change (up/down), when comparing between mastitis and control (Supplemental Table 1B). Among the 55 bovine miRNAs, 27 (50%) were common to those of Trial 1 (Figure 3A). Furthermore, these miRNAs exhibited similar directional change in expression between the two trials, except for bta-miR-763 (Figure 3B). Increasing severity of mastitis is generally associated with increasing SCC [18]. Given that SCC of Trial 2 was significantly lower than that of Trial 1, it is possible that the lack of array-wide level of statistical significance in Trial 2 was attributed to the milder grade of mastitis compared to Trial 1. In spite of this, at least 26 (out of 27) of these miRNAs remained as potentially useful biomarkers of mastitis.

We further performed least square linear regression on log2 intensities (i.e., expression) of the miRNAs which were differentially expressed in both Trials 1 and 2 when comparing between mastitis and control group (Figure 3B), with SCC. Of these miRNAs assessed, 7 miRNAs (miR-27b, miR-152, miR-194, miR-200b, miR-222, miR-379 and miR-1839) were significantly correlated with SCC in both Trials 1 and 2 (P<0.05) (Figure 4), whereas the remaining 18 miRNAs were significantly correlated with Trial 1 (P<0.05) but not Trial 2 (data not shown). That we found a significant association with SCC change in levels of the 7 miRNAs (miR-27b, miR-152, miR-194, miR-200b, miR-222, miR-379 and miR-1839) in both trials strongly suggests utility of these miRNAs as mastitis indicators.

Differential mirna expression associates with pathogen-directed mastitis

The heat map indicates distinct sample clustering between SU-positive, and CNS-positive mastitic cows, and from controls (Figure 5A). This suggests that distinct miRNA expression profiles between different causative agents of mastitis may be present in bovine milk. In light of this, we asked if differential miRNA expression pattern could be used to distinguish mastitis caused by varying bacterial pathogens. Using Trial 1, we found a total of 6859 bovine and non-bovine transcripts that were differentially expressed between SU-positive mastitic and control groups (P<0.05). Of these, we found 223 differentially expressed bovine miRNA transcripts with a minimum of 1.2-fold change (up/down) between SU-positive mastitic and control groups. When comparing between CNS-positive mastitic and control groups, we found a total of 1201 bovine and non-bovine transcripts (P<0.05). Of these, 57 were differentially expressed bovine miRNA transcripts with at least 1.5-fold change (up/down) in CNS-positive mastitic group relative to control. We also assessed differences in miRNA expression pattern between SU-positive and CNS-positive mastitic groups, and found a total of 4073 differentially expressed bovine and non-bovine transcripts (P<0.05). Of these, 144 were bovine miRNAs with at least 1.3-fold change (up/down) between both groups. Results of group comparisons are found in Supplemental tables 1C-1E.

Using miRNA signatures from each pair wise comparison, we next determined the extent of overlap between the various bovine
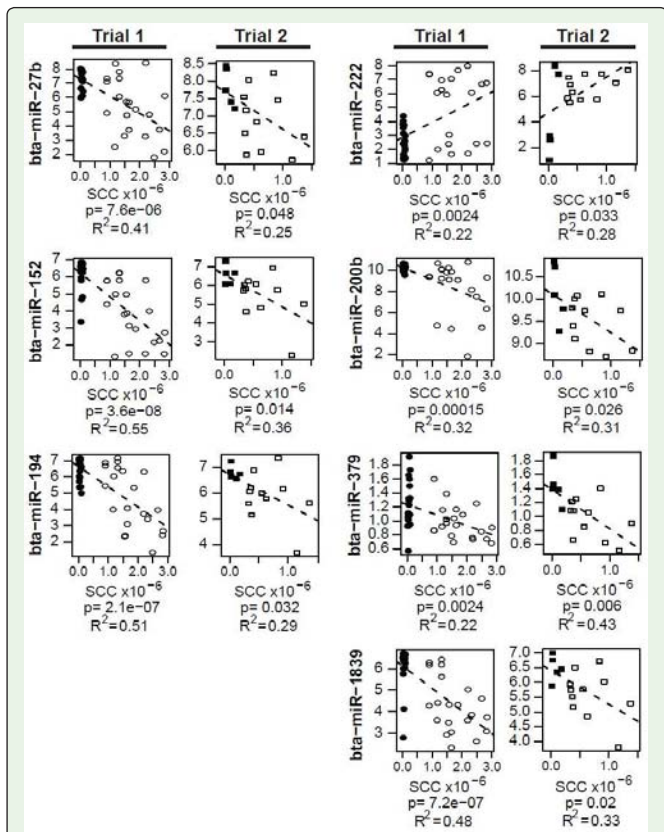

Figure 4: MiRNAs that significantly associate with somatic cell count in Trials 1 and 2.
Y-axis: log2 intensity. Closed and open circles represent control and mastitic cows respectively, from Trial 1 (Columns 1 and 3; left to right). Closed and open squares represent control and mastitic cows respectively, from Trial 2 (Columns 2 and 4).
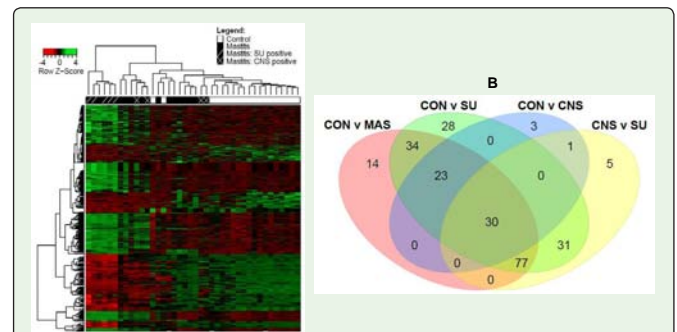

Figure 5: Comparison of bovine miRNA profiles.
(A) Hierarchical clustering of cows in Trial 1 for those significantly differentially expressed bovine miRNA transcripts (P<0.05, FDR method). Agglomeration method used was complete linkage. Distance measure was calculated using Euclidean method. Red-Black-Green colour key denotes low to high expression. SU: S. uberis; CNS: Coagulase negative staphylococcus. (B) Numbers within Venn diagram denote number of bovine miRNAs corresponding to each region in the Venn diagram. CON: control; MAS: mastitis as defined by high somatic cell count; SU: S. uberis-positive mastitis; CNS: coagulase negative staph-positive mastitis.

expression profiles; results are illustrated in Figure 5B. We found that 14 out of 178 (8%) differentially expressed bovine miRNAs were unique to mastitis as defined by high SCC (control versus mastitis group), while the remainder 164 differentially expressed miRNAs (92%) were common to the various pathogen-positive mastitis group comparisons. This is perhaps unsurprising as change in SCC is not obligatory in mastitis. Moreover, mastitis (by definition) reflects inflammation of the mammary gland which can arise from a variety of bacterial and non-bacterial pathogens. The 14 unique miRNAs identified in this study likely reflects mastitis in the absence of SU or CNS. Our results also show that 28 out of 223 (13%) differentially expressed bovine miRNAs were unique to SU-positive mastitis (control versus SU group; Figure 5B) whereas 3 out of 57 (5%) bovine miRNAs were unique to CNS-positive mastitis (control versus CNS group). Fold change in miRNA expression for all group comparisons are found in Supplemental Tables 1A-1E. Log^2 intensity distribution (i.e., expression) of representative miRNAs unique to each group comparison is illustrated as boxplot in Figure 6A-6D. These miRNAs represent those that show potential for the pathogen-specific identification of SU-positive or CNS-positive mastitis given the lack of overlap with those previously reported by Sun et al. [19] for bovine S. aureus-induced intra-mammary infection; the exception being bta-miR-296-5p which we detected as differentially up-regulated specifically in SU-positive mastitis (Supplemental Table 1C; Supplemental Figure S2) but was similarly up-regulated in S. aureus-induced bovine intra-mammary infection as previously reported by Sun et al. Among the 164 differentially expressed miRNAs non-unique to control-versus-mastitis group, these were predominantly (33 out of 164) common to those of control-versus-SU group (Figure 5B) suggesting that SU, compared to CNS bacteria, may be more capable of eliciting substantial changes in circulating levels of bovine milk miRNAs.

In summary, detection of differentially expressed miRNAs which are unique to each pair wise group comparison may be useful to discern between SU and CNS mastitis-causing bacteria in bovine milk. In contrast, miRNAs common to the various pairwise group comparisons may reflect common underlying molecular events associated with mastitis; although they remain as useful indicators of mastitis, they may not be discriminatory between causative agent types.

Discussion

Detection of subclinical mastitis is critical for minimising the economic loss of mastitis to dairy farmers. However, SCC analysis alone may be insufficient to reliably detect mastitis, the magnitude of SCC increase having been shown to vary between pathogen species [3]. Notably, causative agents such as CNS (traditionally classified as a minor pathogen) elicit only a mild increase in SCC following natural intra-mammary infection (clinical mastitis) [20]. CNS infections are prevalent in herds with low bulk milk SCC i.e., below 200,000 cells/ml [21]. Therefore, effective control of CNS infections may be important to manage herd level SCC, especially when the presence of CNS is linked to the host's increased susceptibility to infections caused by major pathogens such as S aureus [22]. However, these findings again question whether single SCC measures for detecting mastitis in both the animal and herd may be insufficient. This has significant implications for the early detection of subclinical mastitis (where the animal is often asymptomatic) before further progression into a clinical state. Of particular note, we have identified seven miRNAs as high confidence indicators of early mastitis infection, based on their significant correlation with the variable SCC levels observed across our two trials. Consequently, we suggest that these specific miRNAs will have particular utility for detection of sub-clinical mastitis, and help to circumvent the obvious limitations of relying solely on SCC measures.

Existing literature reporting on miRNA signatures characteristic of mastitis have largely been conducted by artificially-inducing intra-mammary gland infection of varying pathogen types *in vivo* followed by miRNA profiling in affected mammary tissue [23], or similarly using primary epithelial cells [24] and cell lines *in vitro* [25]. In contrast to this controlled intra-mammary infection approach, cows sampled in our field study acquired mastitis through natural patterns of pathogen exposure and thus representing the natural setting of disease. Although most studies relating to mastitis do not report SCC measures, it has recently been shown that intra-mammary (bovine) gland infection with the bacteria S aureus leads to an average 450-fold increase in SCC (Sun, et al. 2015). In our field trials, SCC measures were seen to similarly increase by 300-fold in Trial 1, but only 10-fold in Trial 2 illustrating the highly stochastic nature of SCC responses in the field following infection. A key point of difference between these singularly analogous studies is our use of whole milk samples as a 'liquid biopsy', rather than an enriched exosomal vesicle fraction, as the starting point for defining miRNA signatures in mastitis. Most encouragingly, despite the differences in bacterial causative agent and technical approach, these two studies have independently identified a common set of presumptive miRNA biomarkers that may constitute a generic signature for mastitis infections (Figure 4 and Supplemental Figure S1). Potentially just as useful are the distinct sets of 'mastitic' miRNAs which differ between these studies (Supplemental Figure S2), and may thus be indicative of responses specific to particular causative agents.
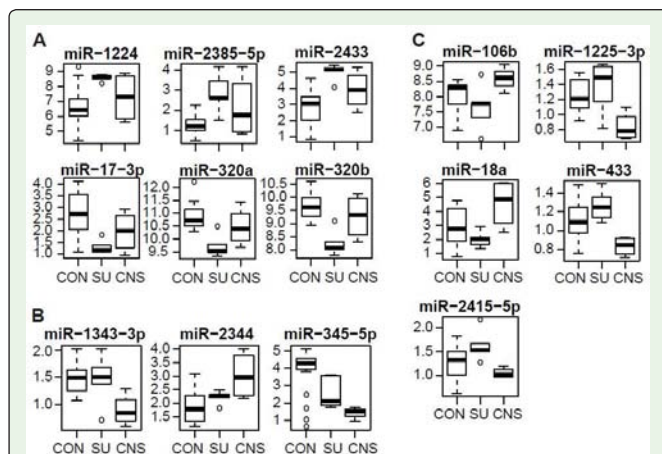

Figure 6: Levels of differentially expressed bovine miRNAs unique to each pairwise group comparison.
(With reference to Figure 5B) (A) Representative differentially expressed bovine miRNAs unique to control-versus-SU group comparison based on biggest fold change (up/down). Differentially expressed bovine miRNAs unique to control-versus-CNS (B), and CNS-versus-SU (C) group comparison. CON: control; SU: S. uberis-positive mastitis; CNS: coagulase negative staph-positive mastitis. Y-axis: log2 intensity.

We would also suggest that the measurement of readily obtainable whole milk samples is sufficient for the sensitive detection of circulating miRNA (without the need for lengthy fractionation procedures) and allows for a more systemic 'secretome' measure of mastitis. It is noteworthy that we have been able to detect miRNAs previously linked with mammary epithelial cell differentiation, such as miR-200a [11], and stem cell recruitment and tissue regeneration, such as miR-200c [26], despite epithelial cells constituting only a small proportion (2%) of the total somatic cells found in whole milk [3]. Many of differentially expressed miRNAs are also known to relate to milk composition and/or quality, including the bta-let-7, miR-29 and miR-30 families. Based on Target Scan Release 7.1 prediction [27], the 3'-UTR of alpha-lactalbumin (LALBA) and UDP-Galactose-4-Epimerase (GALE) genes (both are involved in lactose synthesis) contain a highly conserved site for members of the let-7 miRNA family, including miR-98 which is found within the let-7/miR-98 miRNA cluster [28], suggesting that these miRNAs may interact with LALBA and GALE genes. Indeed, elevated SCC is associated with decreased lactose, LALBA protein and fat levels due to reduced synthesis in the mammary tissue [2]. Other miRNAs we have identified may be biologically relevant to perturbed mammary function. Amongst these are members of the bta-let-7, miR-29, miR-30 and miR-99 families. The let-7 family and the let-7f/miRNA-98 cluster, are also well known for their roles in immunity [29,30] and cellular differentiation [28], although their precise mechanism of action remains to be elucidated. Members of the miR-29 family (as with many miRNAs) appear pleiotropic influencing broad biological effects from immunity [31,32] to cellular fibrosis [33]. Most notably however, miR-29s were recently shown to epigenetically regulate lactation by modulating DNA methylation levels or target genes in bovine primary mammary epithelial cells [7]. Furthermore, anti-miR-29b treatment was shown to induce excess fibrosis [34]. Chronic or recurrent infection can result in fibrosis of the mammary gland [35,36]. Although we have not assessed mammary tissue fibrosis in this study, our finding of decreased miR-29s is consistent with these reports of increased fibrosis of the mammary gland following mastitis. Members of the miR-99 family are meanwhile critical in the maintenance of tissue identity [37] and miR-99a and miR-99b (as well as miR-100) shown to decrease epithelial cell proliferation and migration (characteristics of wound healing) by down-regulating AKT/mTOR signalling [38]. Mir-30s are implicated in regulating lactation, given recent reports inrodents [10,39]. Indeed, miR-30b over expression *in-vivo* led to histological defects in lactating mammary gland and specifically delayed involution [39]. Disruption of alveolar cell integrity, increase in epithelial cell shedding and apoptosis, and increased appearance of poorly-differentiated epithelial cells, are believed to be directly related to loss of mammary function in mastitis [40]. Taken together, down-regulation of these various miRNAs is consistent with the notion of compromised mammary tissue integrity, resulting in perturbed mammary function.

Few studies have directly compared global changes in miRNA response in the context of mastitis, but existing findings from Jin et al. comparing changes in miRNA profiles between Gram-positive and Gram-negative bacteria support the utility of miRNA as a useful molecular tool to identify pathogen-specific mastitis [25]. Similarly, we found several miRNAs that were unique to the presence of SU and CNS pathogens and distinct from many found with S aureus infection (Sun, et al. 2015). Interestingly, differentially expressed

miR-320a and miR-320b were unique to SU-positive mastitis. Host innate and acquired immune responses to intra- mammary infections vary between pathogen species [41,42]. Very recently, miR-320a was demonstrated to down-regulate NOD2 expression (a cytosolic receptor that senses bacterial wall peptides and induces pro-inflammatory transcriptional responses to eliminate pathogens) during inflammation [43]. Interestingly, miR-320 also plays a role in inhibiting chaperone-mediated autophagy by targeting the chaperone Hsc70 [44]. Given that NOD2 signalling and autophagy both activate trained innate immunity, it is tempting to speculate that SU's ability to establish persistent intra-mammary infections may in part be attributed to its ability to influence host trained immune activity via miR-320s to favour its survival.

The current work differs from previous studies in that we have deliberately chosen to examine levels of miRNA in milk samples after 'natural levels of exposures' and in response to different causative agents 'on farm'. This more holistic context contrasts from the existing literature which reports the effects of either exogenous dosing with a singular agent or of dosed exposures in the context of single cell types (which individually contribute in only very minor ways to SCC) in an empirical ex vivo setting. Given the constraints of 'on farm' trials, a limitation of our field study is sampling of milk at a single time point (i.e., upon detection of mastitis as indicated by elevated SCC). MiRNAs display differing expression kinetics following bacterial challenge in bovine mammary epithelial cells; furthermore, the temporal response of these miRNAs may vary between different bacterial species [25]. In spite of this, we were able to identify miRNA signatures in SU-positive and CNS-positive mastitis. However, future studies to assess temporal expression patterns of miRNAs would reasonably generate a more comprehensive pathogen-specific miRNA signature. It is worth noting that longitudinal (non-invasive) milk sampling is feasible and is advantageous for monitoring the progression of an infection episode or the recovery process following treatment. More importantly, accurate detection of mastitis is key to avoiding the issue of antibiotic resistance arising from inappropriate timing or dosing with antibiotic administrations. Collectively, these differentially expressed miRNAs are potentially useful for detection of mastitis (even when asymptomatic), assessment of clinical status and identification of causative agent. If confirmed by independent testing, they should provide an invaluable tool for improving the efficacy of disease management.

Current technologies rely on analytical platforms generally provided by dedicated and specialised technical support. We envisage a technology based on the measurement of miRNAs in readily obtainable biological samples which could be assessed 'on site', 'in line' and in real time, at much less cost [45,46]. Further, this could be used for constant surveillance, in addition to diagnosis, of disease. While current detection methods are indicative of exposure but not necessarily diagnostic of disease, miRNA profiling is an accurate measure of dynamic responses [47] and allows for classification of causative agents [19,24,25] which is not true of SCC, and consequently a better guide to a more effective or efficient intervention strategy.

Acknowledgements

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