Proteomic profiling of plasma-derived small extracellular vesicles: a novel tool for understanding the systemic effects of tick burden in cattle

Natalie Turner*, Pevindu Abeysinghe*, Hassendrini Peiris*, Kanchan Vaswani*, Pawel Sadowski†, Nick Cameron‡, Nathanael McGhee‡, Jayden Logan* and Murray D. Mitchell*.

* Centre for Children’s Health Research (CCHR), Queensland University of Technology (QUT), 62 Graham Street, South Brisbane, QLD 4101, Australia.
† Central Analytical Research Facility (CARF), QUT Gardens Point, 2 George Street, Brisbane City, QLD 4000, Australia.
‡ Nindooibah, 272 Nindooibah House Road, Beaudesert, QLD 4285, Australia.

Natalie Turner: natalie.turner@hdr.qut.edu.au
Pevindu Abeysinghe: abeysinghe.abeysingh@hdr.qut.edu.au
Hassendrini Peiris: nel.peer@gmail.com
Kanchan Vaswani: k2.vaswani@qut.edu.au
Pawel Sadowski: pawel.sadowski@qut.edu.au
Nick Cameron: Nick@goannacorp.com.au
Nathanael McGhee: nat.mcghee@nindooibah.com.au

© The Author(s) 2022. Published by Oxford University Press on behalf of the American Society of Animal Science. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Cattle ticks are a significant burden to cattle industries globally. Current methods to treat cattle ticks are costly and inefficient in the long-term. It has been noted that while some cattle may exhibit a natural resistance to ticks, others carry a heavy tick burden. The study of small extracellular vesicles, or exosomes (EX), isolated from cattle blood plasma provides a non-invasive way of analysing changes at the cellular level and may be of use in understanding the systemic effects of tick burden or factors leading to natural resistance. The aim of this study was to assess HTR and LTR cattle identified using a tick burden scoring system by analysing the protein content of circulating EX via qualitative proteomics analysis. We found that a class of proteins related to defense/immunity comprised 50% of proteins unique to HTR cattle, while this protein class was not detected in proteins unique to LTR cattle. Additionally, epidermal growth factor-calcium binding protein domains were 2-fold increased in LTR cattle compared to HTR cattle, indicating a possible mechanism for widespread metabolic change. This is the first study to employ proteomic analysis of exosomal cargo as an approach to understanding the systemic effects of tick burden in cattle.

Teaser Text
Small extracellular vesicles of size ~50 – 150 nm have been isolated and enriched from the blood plasma of cattle exhibiting high and low natural resistance to tick infestation. Proteomic analysis of these small extracellular vesicles by mass-spectrometry has provided valuable information regarding the systemic effects, and particularly the immune challenges, of tick burden on animals exhibiting low tick resistance.
Abstract

Cattle ticks pose a significant threat to the health and profitability of cattle herds globally. The investigation of factors leading to natural tick resistance in cattle is directed towards targeted breeding strategies that may combat cattle tick infestation on the genetic level. Exosomes (EX), small extracellular vesicles (EV) of 50 – 150 nm diameter, are released from all cell types into biofluids such as blood plasma and milk, have been successfully used in diagnostic and prognostic studies in humans, and can provide essential information regarding the overall health state of animals. Mass-spectrometry (MS) is a highly sensitive proteomics application that can be used to identify proteins in a complex mixture and is particularly useful for biomarker development. In this proof of principle study, EX were isolated from the blood plasma of cattle (Bos taurus) with high (HTR) and low tick resistance (LTR) (n = 3/group). Cattle were classified as HTR or LTR using a tick scoring system, and EX isolated from the cattle blood plasma using an established protocol. Exosomes were subjected to MS analysis in data-dependent acquisition mode and protein search performed using Protein Pilot against the Bos taurus proteome. A total of 490 unique proteins were identified across all samples. Of these, proteins present in all replicates from each group were selected for further analysis (HTR = 121; LTR = 130). Gene ontology analysis was performed using PANTHERGO online software tool. Proteins unique to HTR and LTR cattle were divided by protein class, of which 50% were associated with immunity/defense in the HTR group, whereas this protein class was not detected in EX from LTR cattle. Similarly, unique proteins in HTR cattle were associated with B cell activation, immunoglobins, immune response, and cellular iron ion homeostasis. In LTR cattle, unique exosomal proteins were associated with actin filament binding, purine nucleotide binding, plasma membrane protein complex and carbohydrate derivative binding. This is the first study to demonstrate that MS analysis of
exosomes derived from the blood plasma of HTR and LTR cattle can be successfully applied to profile the systemic effects of tick burden.

Key words: Cattle, exosomes, extracellular vesicles, mass-spectrometry, tick resistance
| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| ACN          | Acetonitrile                                     |
| AMBIC        | Ammonium bicarbonate                            |
| BCA          | Bicinchoninc acid                               |
| BSA          | Bovine serum albumin                            |
| DPBS         | Dulbecco’s phosphate buffered saline            |
| DTT          | Dithiothreitol                                   |
| EGF          | Epidermal growth factor                         |
| EGF-CA       | Epidermal growth factor-calcium binding         |
| EV           | Extracellular vesicle/s                         |
| EX           | Exosome/s                                        |
| FASP         | Filter-aided sample preparation                 |
| FDR          | False discovery rate                            |
| GO           | Gene ontology                                    |
| HTR          | High tick resistance                            |
| IAA          | Iodoacetamide                                    |
| IG           | Immunoglobulin                                   |
| IGc1         | Immunoglobulin constant type-1                  |
| IGv          | Immunoglobulin variable type                    |
| LTR          | Low tick resistance                              |
| MS           | Mass-spectrometry                                |
| NTA          | Nanoparticle tracking analysis                  |
| SDC          | Sodium deoxycholate                              |
| SEC          | Size exclusion chromatography                    |
| TOF   | Time-of-flight |
|-------|----------------|
| UC    | Ultracentrifugation |
1. Background

Cattle ticks and tickborne diseases represent a huge burden to cattle industries globally, with 80% of the world’s cattle situated in areas of tick prevalence (De Castro 1997). In Australia, the financial losses to industry associated with cattle-ticks is estimated to exceed $160 million annually (Mahony 2021). While there is evidence to suggest that susceptibility to tick infestation is cattle species-dependent, the mechanisms underlying natural resistance to ticks remain unclear (Robbertse, Richards, and Maritz-Olivier 2017). There is some evidence to suggest that the ability of the host to acquire immunity to tick infestation over time encompasses both adaptive and innate immune response, however it is clear that this immunity fails to develop in a significant portion of cattle (RECHAV et al. 1991; Jonsson, Piper, and Constantinoiu 2014). Strategies to combat cattle tick infestation include acaricides and vaccine-based treatments, however these are not long term solutions due to sustainability and environmental concerns, and promotion of tick resistance to acaricide treatment (Jonsson 2006; De Castro 1997).

Studies investigating natural resistance in cattle to tick infestation include the sampling of blood and skin from cattle to perform genetic and immunological studies with the hope of developing biomarkers of natural resistance (Marima et al. 2020; Akbar et al. 2015; Bagnall et al. 2009). Genetic, proteomic and peptidomic studies of cattle ticks have also been employed to gain understanding of the parasite-host relationship that underlies tick infestation (Xiong et al. 2020; Garcia et al. 2020; Sajiki et al. 2021). The sampling of blood plasma is ideal as a diagnostic or prognostic tool as it is relatively simple to obtain and carries information at the systemic level. Secreted cell proteins and genetic material are released into the bloodstream, including cytokines, chemokines and immune cells and thus carry important information regarding the overall health state of the animal. Proteomic profiling of various biological fluids, including blood plasma, has been used as a method for developing...
biomarkers for a variety of conditions in cattle (Faulkner et al. 2012; Miller et al. 2019). More recently, blood plasma-derived extracellular vesicles (EV) have been the focus of many clinical diagnostic and prognostic studies in relation to their potential as biomarkers of health and disease (Raimondo et al. 2011; Madhavan et al. 2015; Rashed et al. 2017). Small extracellular vesicles (~50 – 150 nm diameter), termed exosomes (EX), are released by cells into the external milieu and carry molecular cargo such as proteins, nucleic acids and lipids specific to their cell type of origin (Zhang et al. 2019; Stoorvogel, Kleijmeer, and Geuze 2002). While they are essential in cell-cell communication and signalling, there is also an increasing body of evidence linking unique EX cargo to cancer and disease in humans (Meng et al. 2018; Han et al. 2018; Riva, Battaglia, and Venturin 2019).

The majority of studies involving EX or other extracellular vesicle subtypes have been performed in relation to human health, however they have also gained interest in the agricultural sector for their ability to predict or improve fertility in dairy cows (Wang et al. 2020; Almughlliq et al. 2018; Mitchell et al. 2016). To date, no studies have been performed in relation to using blood plasma-derived EX to identify cattle with inherent susceptibility to tick infestation. As a tool for understanding physiological perturbations leading to susceptibility to disease states, proteomic analysis of EX is particularly useful, as proteins are the effectors of physiological change within the body. As such, any significant differences in proteomic content between two or more divergent groups may shed light on the underlying mechanisms of, for example, immune dysregulation in cattle with extremely high tick burden as compared to cattle with low or negligible tick burden.

The aim of the present study is to determine whether qualitative proteomic analysis of blood plasma-derived EX can identify notable differences in cattle with high or low tick burden. A secondary aim of this study is to establish whether proteomic analysis of blood plasma-
derived EX can be used as a tool to assess the physiological effects of high versus low tick burden in a genetically similar group of beef cattle.

2. Material and Methods

2.1 Animals, management and blood collection

The animals, management, and sample collections were approved by the Animal Welfare Unit, UQ Research and Innovation, the University of Queensland (UQCCR/459/16). A total of 199 animals were selected randomly and tick scores were given as described below. The authors confirm this study was carried out in compliance with ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines).

The cattle used in this study were of species *Bos taurus*. All animals were tick exposure naïve, with no prior prophylactic tick treatments. The cattle under study were released into a paddock with known tick prevalence and natural tick infestation was allowed to occur.

Each cow underwent careful assessment for evidence of tick infestation as part of a thorough physical exam as follows; Animals were hand checked for the presence and absence of ticks on their hind regions and belly over a three-month period. A scoring system was developed (1-5, A or B), (1) no identifiable tick burden, (2) < 10 ticks, (3) 20 to 100 ticks, (4) 100 to 200 ticks, (5) > 200 ticks with (A) representing crusting and (B) no crusting. Animals with a score of <3 were left untreated. Detailed information of cattle histories and other relevant information (e.g., weight, pasture location) was recorded. Cattle from group 1A or B were considered high tick-resistant, and those from group 4 or 5A or B low tick-resistant. Three HTR (classification 1B) and three LTR (classification 4B and 5A) cattle (*n* = 6) from the same paddock were chosen at random for this study. The cattle selected were all female, 1.5 ± 0.3 years of age at the time of baseline tick scoring (no exposure), with weight 362.2 ± 40.6
kg measured at 1.1 ± 0.1 years of age. Tick scoring was performed every 2 – 3 weeks for up to six assessments including baseline score. Cattle with heavy tick burden following first tick exposure were assessed until tick score reached 4A or higher (minimum 3 separate tick scoring assessments).

Blood was collected from cattle in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. Plasma was separated by centrifugation at 3000 x rcf for 10 min at 4°C. The plasma was aspirated and stored at −80°C until thawed for EV/EX isolation. One 10 mL aliquot of plasma per biological replicate was thawed on ice on the same day as EX isolation and enrichment was initiated.

2.2 Extracellular vesicle isolation and enrichment

2.2.1 Sequential centrifugation and ultracentrifugation (UC)

Ultracentrifugation was performed as previously described (Koh et al. 2018). Briefly, EV were isolated from 8 mL thawed blood plasma using an established sequential centrifugation protocol. Plasma was centrifuged at 2,000 x rcf for 30 min at 4°C and 12,000 x rcf for 30 min at 4°C to remove cellular debris and apoptotic bodies. It was then filtered through a 0.22-μm polyether sulfone membrane filter (Corning Inc., Corning, NY) cleared and filtered blood plasma supernatant was transferred into 32.4 mL OptiSeal Polypropylene Tube (361625, Beckman Coulter), and brought to equal volumes with Dulbecco’s Phosphate Buffered Saline (DPBS, pH 7.0 – 7.2) (Vitrolife, Australia). Samples were centrifuged at 100,000 × g for 2h at 4°C (Beckman, Type 50.2 Ti, Fixed angle ultracentrifuge rotor). The supernatant was discarded and the pellet containing EVs was resuspended in 500 μL DPBS. Following UC, samples were stored at -80°C until the next day.
2.2.2 Size exclusion chromatography (SEC)

Samples were thawed on ice to perform SEC as previously described (Koh et al. 2018). Briefly, the columns and filtered DPBS were brought to room temperature prior to loading the sample onto the column bed. The 500 µL EV sample was loaded onto the column gel bed and 500 µL fractions collected as follows; 1 – 6 as void volume fraction (3 mL total), 7 – 10 as exosomal (EX) fractions, and 11 – 16 as non-exosomal (non-EX) fractions known to contain soluble plasma proteins, protein aggregates, and nucleic acids. Columns were used up to three times each. In between uses, the columns were flushed with 0.5 mL 0.5M NaOH solution, followed by 15 – 20 mL filtered DPBS. Exosomal fractions 7 – 10 and non-EX fractions 11 – 16 were pooled separately to final volume 1 mL and used for downstream analyses. The remaining fraction volumes were stored at -80°C until required.

2.3 Characterisation of small extracellular vesicles (exosomes)

2.3.1 Protein quantification

The total protein concentrations of pooled EX and non-EX fractions were determined by micro bicinchoninic acid (BCA)™ Protein Assay Kit (cat number 23235, Thermofisher Scientific, Australia) following the microplate assay protocol as per manufacturer’s instructions. Briefly, bovine serum albumin (BSA) standards and EX samples (diluted 1:10) were solubilised 1:1 (v/v) in lysis buffer (1% w/v sodium deoxycholate (SDC) and 20 mM Tris-HCl pH 8.5), sonicated in an ice bath for 2 min and incubated on ice with gentle agitation for 20 min prior to assay. Protein standards were prepared in triplicate and samples in duplicate. 140 µL of protein standard or sample/lysis buffer was transferred onto a 96 well flat-bottom microplate (N2936, CELLSTAR, Greiner, Sigma) in triplicate (standard) or duplicate (sample). Micro BCA working reagent was added at a ratio of 1:1 with standard/sample and incubated at 37°C in the dark for 2 hrs, cooled to room temperature and absorbance read at 562 nm.
2.3.2 Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis measurements were performed using a NanoSight NS500 instrument (NanoSight NTA 3.1 Build 3.1.46). Instrumentation calibration was performed using 100 nm synthetic beads at a 1:250 dilution. Measurements of samples included particle concentrations, mean and mode sizes of nanoparticles enriched from blood plasma in individual fractions (representative sample) (Supplementary file 1, Fig. S1).

2.4 Sample preparation for mass spectrometry analysis

2.4.1 Filter-aided sample preparation (FASP)

Pooled 7 – 10 fractions and were combined for each method to create a master pool per method and processed for MS analysis using a modified FASP (Wiśniewski et al. 2009). For the master pool EX samples, a volume of protein extract corresponding to ~10 – 20 µg total protein was mixed at a ratio of 1:1 with lysis buffer (1% w/v SDC, 100 mM dithiothreitol (DTT) in 100 mM Tris-HCl pH 8.5, cOmplete-mini EDTA-free protease inhibitor cocktail (Roche)). All samples were sonicated in an ice bath for 2 min and incubated on ice for 20 min. Samples were loaded onto Nanosep® Centrifugal Devices with Omega™ Membrane 30K (PALL) and centrifuged at 14,000 x g for 15 min at 21°C. As EX sample volumes exceeded device capacity, EX samples were loaded onto device in 450 µL aliquots and centrifuged as described. This was repeated until all the sample had passed through the filter with flow-through discarded. Proteins bound to the filter membrane were reduced with 200 µL of DTT-Urea buffer (8M urea, 100 mM Tris-HCl pH 8.5, 25 mM DTT) for 60 min at RT with gentle agitation. Samples were centrifuged at 14,000 x g for 15 min at 21°C. Filters were washed with 200 µL Urea-Tris buffer (8 M urea, 100 mM Tris-HCl pH 8.5) and centrifuged at 14,000 x g for 15 min at 21°C. Reduced samples were alkylated with 100 µL IAA-Urea buffer (50 mM iodoacetamide (IAA), 8 M Urea-Tris buffer) and incubated at RT for 20 min on agitator. The filters were centrifuged at 14,000 x g for 15 min at 21°C. The filters were
washed twice with 200 μL Urea-Tris buffer and centrifuged at 14,000 x g for 15 min at 21°C each. The filters were equilibrated with two washes, 200 μL 100 mM ammonium bicarbonate (AMBIC) and centrifugation at 14,000 x g for 15 min at 21°C. Samples were digested overnight (16 h) with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) at 37°C in a humidified chamber with gentle agitation, with volume of trypsin added at an enzyme to protein ratio of 1:50. The next day, filters were transferred to clean 1.5 mL Eppendorf tubes and peptides collected by centrifugation at 14,000 x g for 15 min at 21°C. One additional elution was performed by adding 60 μL 100 mM AMBIC and centrifugation at 14,000 x g for 15 min at 21°C.

2.4.2 Peptide desalting

Peptide digests were acidified by mixing 1:1 with 4% trifluoroacetic acid (TFA) solution. StageTips were produced with double SCX membrane (part no: 2251, Empore) as described in Supplementary file 2. 50 μL 100% acetonitrile (ACN) was passed through the tip using centrifugal force (2 min spin at 2,000 rpm) and positive pressure. 50 μL of 5% ammonium hydroxide/80% ACN (Buffer DE) was added to the tips and passed through the tip using centrifugal force and positive pressure. 50 μL of 0.2% TFA (Buffer DW) was added to the tip and passed through the tip using centrifugal force and positive pressure. Each sample was loaded onto a StageTip and passed through the tip using centrifugal force and positive pressure. 50 μL of DW was added to the tip and passed through the tip using centrifugal force or positive pressure, three times in total. The tip was placed in a clean 1.5 mL tube and 80 μL DE was added to the tip and passed through the tip using centrifugal force or positive pressure. The eluted peptides were dried in a vacuum centrifuge and reconstituted in 15 μL iRT buffer (Biognosys-11).
2.4.3 Peptide assay

Samples were assayed for peptide concentration with Pierce™ Quantitative Colorimetric Peptide Assay according to manufacturer’s instructions (cat number 23275, Thermofisher Scientific). Peptide concentration in all samples were equalized by an appropriate addition of iRT buffer.

2.5 Mass-spectrometry (MS)

All peptide samples were analysed by LC-MS/MS as follows. Reversed-phase chromatography was conducted on an Eksigent ekspert nanoLC 400 System (Eksigent Technologies) using trapping for 3 min at flow rate of 10 μL/min onto a Trajan ProteCol trap (120 Å, 3 μm, 10 mm × 300 μm) followed by separation on an Eksigent ChromXP C18 3 μm 120 Å (3C18-CL-120, 3 μm, 120 Å, 0.3 x 150 mm) analytical column at a flow rate of 5 μL/min maintained at 40 °C. Trapping utilized mobile phase A only whereas separation utilised a combination of mobile phase A and B. Mobile phase A consisted of 0.1% FA in water and mobile phase B was made of 0.1% FA in ACN. Peptides were separated by 68 min linear gradient of 3-25% mobile phase B followed by 5 min linear gradient of 25-35% mobile phase B. After peptide elution, the column was flushed with 80% mobile phase B for 5 min and re-equilibrated with 97% A for 8 min before next injection. Mass spectrometry was conducted on Triple time-of-flight (TOF) 6600 (SCIEX) instrument equipped with DuoSpray Ion Source configured for micro flow HPLC applications.

2.5.1 DDA-MS data acquisition

High resolution (30,000) TOF MS scan was collected over range of m/z 400 – 1250 for 0.25 s, followed by high sensitivity TOF MS/MS scans over a range of m/z 100 – 1800 on up to the 30 most abundant peptide ions (0.05 s per each scan) that had intensity greater than 150 cps and charge state of 2-5. The dynamic exclusion duration was set at 15 s. Ion fragmentation in the collision cell used rolling collision energy with the collision energy
spread set to 5 eV. The declustering potential was set to 80 V and the remaining gas and source parameters were adjusted as required.

2.5.2 Protein identification

Mass spectrometry data files were added to ProteinPilot (v. 5.0.2.0, 5346) and processed individually, using Paragon Algorithm (v. 5.0.2.0, 5174). The fragmentation spectra were searched against cattle proteome (23,847 sequences, downloaded Aug 2020, available in fasta format, Uniprot), combined with sequences of cRAP (ftp://ftp.thegpm.org/fasta/cRAP) and iRT peptides. The following search parameters were entered: Urea denaturation; alkylation with iodoacetamide; species ‘none’; amino acid substitution; thorough ID; false discovery rate (FDR) analysis 0.01. The protein list was exported to a .xls file, which was subject to an additional refinement. The final list of proteins for each method required minimum 2 peptides per protein ID (1% FDR at the protein level; 5% FDR at the peptide level). All EX fractions were compared to non-EX fractions analysed in the same way.

2.6 Gene ontology analysis

Proteins identified in ProteinPilot as described above were analysed for gene ontology (PANTHERGO, Gene Ontology Phylogenetic Annotation Project, v 16.0. Available online: http://www.pantherdb.org/), including molecular function, cellular component, biological process, pathway analysis, and protein class. Protein accession codes were entered into the search window and searched against species Bos taurus and analysed for functional classification. FunRich (Functional Enrichment analysis tool) was used to perform enrichment analysis on proteins identified from each enrichment method. The complete Vesiclepedia database (version 4.1, downloaded on 27/07/2021, available at http://microvesicles.org/Archive/VESICLEPEDIA_PROTEIN_MRNADETAILS_4.1.txt) was imported into FunRich and all data were searched against the cattle proteome.
3. Results

3.1 Mass spectrometry

A total of 490 unique proteins were detected in EX pooled fractions from high tick resistant (HTR) and low tick resistant (LTR) cattle with 1% false discovery rate (FDR) cut-off at the protein level (Supplementary data file 1, Table S1). After additional FDR cut-off of 5% at the peptide level, 121 proteins were present in all HTR replicates, and 130 proteins in all LTR replicates (Supplementary data file 1, Fig. S2). There were 91 EX proteins shared between HTR and LTR cattle.

3.2 Gene ontology analysis

To obtain a clear profile of EX proteins identified in HTR and LTR cattle in association with functions at the molecular, biological and cellular level, protein uniprot IDs were subjected to gene ontology analysis using online software tool PANTHERGO (http://www.pantherdb.org/). Shared proteins identified in EX and non-EX fractions were profiled to determine whether there were clear differences in the representation of proteins by protein class (Fig. 1A, Supplementary data file 1, Fig. S3). EX shared proteins were divided into twelve separate categories, with structural proteins (18%), scaffold/adapter proteins (14%) and protein modifying enzymes (14%) accounting for most proteins, with all others being 9% or less. In non-EX fractions, shared proteins were divided into seven different categories, with protein-binding activity modulators (44%), defense/immunity proteins (20%) and transfer/carrier proteins (16%) representing the largest number of proteins, all others were 8% or less.

Exosomal proteins unique to HTR or LTR cattle were then analysed in the same way to assess similarities and differences between groups (Fig. 1). There were 8 protein classes
detected in HTR cattle EX and 6 in LTR cattle EX. While 50% of proteins unique to HTR cattle were classified as defense/immunity proteins, this class of protein was not detected in EX proteins unique to LTR cattle. Conversely, protein binding activity modulator proteins represented 23% of EX proteins unique to LTR cattle but this protein class was absent in HTR cattle. Cytoskeletal proteins contributed to 38% of unique proteins in LTR cattle, but only 6% unique proteins in HTR cattle. Intercellular signal molecule proteins (6%) and storage proteins (13%) were unique to HTR cattle and absent in LTR cattle.

Next, gene ontology analysis focused on protein families and their subclasses that were associated with EX proteins unique to HTR and LTR cattle (Table 1). High tick resistant cattle EX proteins were associated with immunoglobulins (PC00123) and various immune system processes such as B cell/lymphocyte activation (GO:0042113) and humoral response (GO:0006955), whereas these were absent in LTR cattle. Interestingly, genes associated with vesicle-mediated transport (GO:0006900) were present in HTR cattle and absent from LTR cattle. In LTR cattle, cytoplasmic proteins and their associated processes (GO:0005737) were highly represented, in addition to actin (GO:0051015), carbohydrate derivative (GO:0097367) and genes upstream of ATP binding (GO:0017076).

Finally, we examined the pathways associated with EX proteins unique to HTR and LTR cattle to assess whether these may have association with tick burden (Supplementary data file 1, Tables S2 and S3). While there were only 8 pathways associated with proteins unique to HTR cattle, there were 45 pathways associated with proteins unique to LTR cattle. Pathways involving three or more genes were considered enriched. Of these, pathways related to inflammation (P00031), G-protein signalling (P00026), and cytoskeletal regulation (P00016) were enriched in LTR cattle, while only the integrin signalling pathway (P00034) was enriched in HTR cattle EX.
3.3 Functional enrichment analysis

Differences in exosomal protein composition between HTR and LTR cattle may translate to functional differences associated with tick burden. To explore differences in protein composition as related to function, FunRich software analysis tool (http://funrich.org/index.html) was used to perform functional enrichment analysis of exosomal proteins in HTR and LTR cattle.

Several differences were identified between HTR and LTR cattle in various categories of protein function (Supplementary data file 1, Fig. S4 and S5). Biological processes relating to iron ion transport and immune response contributed to a significantly larger percentage of proteins in HTR cattle compared to LTR cattle (4.82% vs 1.19% and 10.84% vs 2.38%, respectively). The calcium-binding epidermal growth factor (EGF-CA) protein domain was increased nearly 2-fold in proteins identified in LTR cattle (7.41% vs 3.80%), while immunoglobulin (IG) and IG variable (v)-type domains were increased 3-fold in proteins identified in HTR cattle (11.39% vs 3.70% in both cases). The IG-constant 1 (c1)-type domain was also increased in HTR cattle (11.39% vs 8.64%).

4. Discussion

Plasma-derived EX are diverse in origin and can provide essential information regarding the health status of the animal. This is the first study that has utilised MS to analyse the
proteomic cargo of EX in relation to tick burden in cattle. Regarding the first aim of the present study, we have provided evidence that MS analysis of the EX-proteome derived from HTR and LTR cattle plasma identified several differences that indeed correlates with tick burden status. The secondary aim of this study addressed the validity of EX analysis as a novel screening tool for assessing physiological effects of tick burden. Analysis of EX proteomic cargo identified protein classes, pathways and protein domains that are directly related to immune status and function. Therefore, EX proteomic cargo analysis provides a previously unused method for studying and understanding biological perturbations associated with tick infestation.

4.1 Increased EGF-CA protein domains may result in widespread alterations to signalling pathways

Protein domains are substructures of proteins that allow for a diverse array of functions within the same molecule. They may give clues to alterations in protein function resulting from evolutionary changes that occur via the incorporation of additional domains at the genetic level or represent unique post-translational modifications of existing domains. In this study, the number of proteins containing the protein domain EGF-CA was 2-fold increased in LTR cattle. The EGF-CA protein domain is a recent evolutionary adaptation (Wouters et al. 2005). It has been studied with regard to its effects on blood coagulation, and genetic mutations produce biologically inactive proteins that give rise to developmental and clotting disorders in humans (Stenflo, Stenberg, and Muranyi 2000). In cattle, expression of genes associated with Ca\(^{2+}\) signalling were found to be increased in the skin of HTR cattle (Bagnall et al. 2009). As ion channels can import Ca\(^{2+}\) directly into cells, there may be direct or indirect stimulation of Ca\(^{2+}\) signalling in HTR cattle. Additionally, as Ca\(^{2+}\) binding EGF domains represent >25% of the 600+ identified EGF modules, any small change in EGF-CA domains between groups may have a significant impact on the multitude of biological
pathways in which they participate (Stenflo, Stenberg, and Muranyi 2000). If the observed increase in EGF-CA protein domains in LTR cattle is indeed a result of genetic variance, this theory is somewhat supported by the apparent innate tick resistance difference between cattle breeds, suggestive of a link to genetic traits in determining natural resistance (Jonsson, Piper, and Constantinou 2014). While this is not a definitive finding, further investigation of the EGF-CA domain in follow-on proteomic or genomic studies would be beneficial to better understand the physiological effects of alterations in this domain prevalence and its relationship to tick resistance.

4.2 EX proteome profiling identifies a significant reduction of proteins associated with defense/immunity in LTR cattle

A predictable consequence of tick infestation in cattle is the immunosuppressive effects of heavy tick burden on the animal (Inokuma et al. 1993). EX proteins unique to LTR cattle did not fall into the defense/immunity protein class, which signifies that there is indeed a physiological shift associated with high tick burden. The reduction of IG, IGv, and IGc1 protein domains in LTR cattle is in further support of impaired immune function in LTR cattle. Of note, EX from HTR cattle were found to contain proteins related to B cell and T cell activation, whereas this was not present in the LTR group. Whether this immune dysregulation is innate or acquired through heavy tick burden is unclear, however further studies may wish to analyse plasma EX of cattle prior to tick infestation at a baseline stage, as this will provide an unbiased assessment of natural tick resistance in cattle. This study has demonstrated the ability of EX to carry vital immune proteins, in addition to validating the use of EX as a tool for physiological assessment of HTR and LTR cattle.

It is known that immune cells such as B and T cells are capable of producing EV, including EX (Zhou et al. 2020). What is currently unknown is the proportion of EX in blood plasma that are produced by immune cells versus those that are produced by other cell types and
released into systemic circulation. Interestingly, EV can mediate both innate immune activation and immunosuppression (Zhou et al. 2020). Extracellular vesicles interact with antigen presenting cells (APC) via a range of uptake mechanisms to produce an immune response (Robbins and Morelli 2014; Zhou et al. 2020). As such, they are of ongoing interest in studies related to immune function. The suppression of immune response in the LTR group correlates with heavy tick burden, and potentially a reduction of immune cell derived EVs in the blood plasma of LTR cattle. This idea is further supported by the significant loss of immune proteins identified in EX from LTR cattle. The divergent EX immune profiles of HTR and LTR cattle in this study suggests that circulating EX play an important part in mediating the immune response to tick exposure and follow-on studies may delineate their role in this regard.

5. Conclusion

For the first time we have successfully applied a MS workflow to analyse the proteomic cargo in blood plasma EX of cattle with high or low tick burden. The divergent EX proteomic profiles established using this method is proof of principle for establishing a blood plasma-derived EX screening tool for cattle with predisposition to tick infestation, which may be of great benefit to cattle farmers when implementing tick management strategies. Sampling cattle at regular timepoints from birth through to adulthood and past first tick exposure would further clarify any biological, physiological or pathological processes that are either genetically or environmentally acquired leading to increased tick resistance or susceptibility. As this is a global issue, there would be significant financial and environmental gains associated with improvements in diagnostics or prognostics for tick resistance. Future studies may focus on quantitative proteomics analyses of blood plasma-derived EX to identify differences in shared proteins between high and low tick resistant cattle. Quantitative differences in shared proteins can be developed into biomarker panels and further assist in
determining the resistance status of cattle at earlier timepoints and thus warrants further
investigation.

6. Supplementary Data

Supplementary data are available at Journal of Animal Science online.

7. Acknowledgements

This project is funded by Advance Queensland Innovative Partnership (AQIP01115-16RD1)
program. Nindooinbah Pastoral Company partnered in this project. NT and PA are supported
by a student scholarship from the Australian Research Council (Grant No: ARC
LP160101854) and QUT HDR Tuition fee sponsorship. Our laboratory experiments were
funded, in part, by funding from a partnership fund (DRCX1302) between the New Zealand
Ministry of Business, Innovation and Employment and New Zealand dairy farmers through
DairyNZ Inc.

8. Conflict of interest statement

The authors declare no conflict of interest.
9. Literature Cited

Akbar, H., T.M. Grala, M. Vailati Riboni, F.C. Cardoso, G. Verkerk, J. McGowan, K. Macdonald, et al. 2015. “Body Condition Score at Calving Affects Systemic and Hepatic Transcriptome Indicators of Inflammation and Nutrient Metabolism in Grazing Dairy Cows.” *Journal of Dairy Science* 98 (2): 1019–32. https://doi.org/10.3168/jds.2014-8584.

Almughlliq, Fatema B., Yong Q. Koh, Hassendrini N. Peiris, Kanchan Vaswani, Scott McDougall, Elizabeth M. Graham, Chris R. Burke, Buddhika J. Arachchige, Sarah Reed, and Murray D. Mitchell. 2018. “Proteomic Content of Circulating Exosomes in Dairy Cows with or without Uterine Infection.” *Theriogenology* 114: 173–79. https://doi.org/10.1016/j.theriogenology.2018.03.024.

Bagnall, N., J. Gough, L. Cadogan, B. Burns, and K. Kongsuwan. 2009. “Expression of Intracellular Calcium Signalling Genes in Cattle Skin during Tick Infestation.” *Parasite Immunology* 31 (4): 177–87. https://doi.org/10.1111/j.1365-3024.2008.01092.x.

Castro, Julio J. De. 1997. “Sustainable Tick and Tickborne Disease Control in Livestock Improvement in Developing Countries.” *Veterinary Parasitology* 71 (2–3): 77–97. https://doi.org/10.1016/S0304-4017(97)00033-2.

Faulkner, Simon, Giuliano Elia, Michael P. Mullen, Padraic O’Boyle, Michael J. Dunn, and Dermot Morris. 2012. “A Comparison of the Bovine Uterine and Plasma Proteome Using ITRAQ Proteomics.” *Proteomics* 12 (12): 2014–23. https://doi.org/10.1002/pmic.201100609.

Garcia, Gustavo R., José Marcos Chaves Ribeiro, Sandra Regina Maruyama, Luiz Gustavo Gardinassi, Kristina Nelson, Beatriz R. Ferreira, Thales Galdino Andrade, and Isabel...
K. Ferreira de Miranda Santos. 2020. “A Transcriptome and Proteome of the Tick Rhipicephalus Microplus Shaped by the Genetic Composition of Its Hosts and Developmental Stage.” *Scientific Reports* 10 (1): 1–23. https://doi.org/10.1038/s41598-020-69793-3.

Han, Yineng, Lingfei Jia, Yunfei Zheng, and Weiran Li. 2018. “Salivary Exosomes: Emerging Roles in Systemic Disease.” *International Journal of Biological Sciences* 14 (6): 633–43. https://doi.org/10.7150/ijbs.25018.

Inokuma, H., R.L. Kerlin, D.H. Kemp, and P. Willadsen. 1993. “Effects of Cattle Tick (Boophilus Microplus) Infestation on the Bovine Immune System.” *Veterinary Parasitology* 47 (January): 107–18.

Jonsson, N. N., E. K. Piper, and C. C. Constantinou. 2014. “Host Resistance in Cattle to Infestation with the Cattle Tick Rhipicephalus Microplus.” *Parasite Immunology* 36 (11): 553–59. https://doi.org/10.1111/pim.12140.

Jonsson, N. N. 2006. “The Productivity Effects of Cattle Tick (Boophilus Microplus) Infestation on Cattle, with Particular Reference to Bos Indicus Cattle and Their Crosses.” *Veterinary Parasitology* 137 (1–2): 1–10. https://doi.org/10.1016/j.vetpar.2006.01.010.

Koh, Yong Qin, Fatema B Almughliq, Kanchan Vaswani, Hassendrini N Peiris, and Murray D Mitchell. 2018. “Exosome Enrichment by Ultracentrifugation and Size Exclusion Chromatography.” *Frontiers in Bioscience (Landmark Edition)* 23: 865–74. http://www.ncbi.nlm.nih.gov/pubmed/28930577.

Madhavan, Bindhu, Shijing Yue, Uwe Galli, Sanyukta Rana, Wolfgang Gross, Miryam Müller, Nathalia A. Giese, et al. 2015. “Combined Evaluation of a Panel of Protein and
MiRNA Serum-Exosome Biomarkers for Pancreatic Cancer Diagnosis Increases Sensitivity and Specificity.” *International Journal of Cancer* 136 (11): 2616–27. https://doi.org/10.1002/ijc.29324.

Mahony, Timothy J. 2021. “Evaluation of Anti-Tick Vaccines for Tick Immunological Control in Cattle.” *Final Report*. Vol. 364. North Sydney.

Marima, J. K., C. L. Nel, M. C. Marufu, N. N. Jonsson, B. Dube, and K. Dzama. 2020. “A Genetic and Immunological Comparison of Tick-Resistance in Beef Cattle Following Artificial Infestation with Rhipicephalus Ticks.” *Experimental and Applied Acarology* 80 (4): 569–90. https://doi.org/10.1007/s10493-020-00480-8.

Meng, Xiaodan, Jinchang Pan, Shifang Sun, and Zhaohui Gong. 2018. “Circulating Exosomes and Their Cargos in Blood as Novel Biomarkers for Cancer.” *Translational Cancer Research* 7 (Suppl 2): S226–42. https://doi.org/10.21037/tcr.2017.09.17.

Miller, Blake A., Amy Brewer, Paolo Nanni, Joseph J. Lim, John J. Callanan, Jonas Grossmann, Laura Kunz, André M. de Almeida, Kieran G. Meade, and Aspinas Chapwanya. 2019. “Characterization of Circulating Plasma Proteins in Dairy Cows with Cytological Endometritis.” *Journal of Proteomics* 205 (June): 103421. https://doi.org/10.1016/j.jprot.2019.103421.

Mitchell, M.D., K. Scholz-Romero, S. Reed, H.N. Peiris, Y.Q. Koh, S. Meier, C.G. Walker, et al. 2016. “Plasma Exosome Profiles from Dairy Cows with Divergent Fertility Phenotypes.” *Journal of Dairy Science* 99 (9): 7590–7601. https://doi.org/10.3168/jds.2016-11060.

Raimondo, Francesca, Lavinia Morosi, Clizia Chinello, Fulvio Magni, and Marina Pitto. 2011. “Advances in Membranous Vesicle and Exosome Proteomics Improving
Biological Understanding and Biomarker Discovery.” *Proteomics* 11 (4): 709–20.
https://doi.org/10.1002/pmic.201000422.

Rashed, Mohammed H., Emine Bayraktar, Gouda K. Helal, Mohamed F. Abd-Ellah, Paola Amero, Arturo Chavez-Reyes, and Cristian Rodriguez-Aguayo. 2017. “Exosomes: From Garbage Bins to Promising Therapeutic Targets.” *International Journal of Molecular Sciences*. MDPI AG. https://doi.org/10.3390/ijms18030538.

RECHAV, Y., F. C. CLARKE, D. A. ELS, and J. DAUTH. 1991. “Development of Resistance in Laboratory Animals to Adults of the Tick Rhipicephalus Evertsi Evertsi.” *Medical and Veterinary Entomology* 5 (1): 29–34. https://doi.org/10.1111/j.1365-2915.1991.tb00517.x.

Riva, Paola, Cristina Battaglia, and Marco Venturin. 2019. “Emerging Role of Genetic Alterations Affecting Exosome Biology in Neurodegenerative Diseases.” *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms20174113.

Robbertse, Luïse, Sabine A. Richards, and Christine Maritz-Olivier. 2017. “Bovine Immune Factors Underlying Tick Resistance: Integration and Future Directions.” *Frontiers in Cellular and Infection Microbiology* 7 (DEC): 1–16.
https://doi.org/10.3389/fcimb.2017.00522.

Robbins, Paul D., and Adrian E. Morelli. 2014. “Regulation of Immune Responses by Extracellular Vesicles.” *Nature Reviews Immunology* 14 (3): 195–208.
https://doi.org/10.1038/nri3622.

Sajiki, Yamato, Satoru Konnai, Yoshinori Ikenaka, Kevin Christian Montecillo Gulay, Atsushi Kobayashi, Luís Fernando Parizi, Benvindo Capela João, et al. 2021. “Tick Saliva-Induced Programmed Death-1 and PD-Ligand 1 and Its Related Host
Immunosuppression.” *Scientific Reports* 11 (1): 1–11. https://doi.org/10.1038/s41598-020-80251-y.

Stenflo, Johan, Yvonne Stenberg, and Andreas Muranyi. 2000. “Calcium-Binding EGF-like Modules in Coagulation Proteinases: Function of the Calcium Ion in Module Interactions.” *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1477 (1–2): 51–63. https://doi.org/10.1016/S0167-4838(99)00262-9.

Stoorvogel, Willem, Monique J Kleijmeer, and Hans J Geuze. 2002. “The Biogenesis and Functions of Exosomes.” *Traffic*, no. 3: 321–30.

Wang, Xiangguo, Xinxin Yao, Tongtong Xie, Zhenyu Chang, Yong Guo, and Hemin Ni. 2020. “Exosome-Derived Uterine MiR-218 Isolated from Cows with Endometritis Regulates the Release of Cytokines and Chemokines.” *Microbial Biotechnology* 13 (4): 1103–17. https://doi.org/10.1111/1751-7915.13565.

Wiśniewski, Jacek R., Alexandre Zougman, Nagarjuna Nagaraj, and Matthias Mann. 2009. “Universal Sample Preparation Method for Proteome Analysis.” *Nature Methods* 6 (5): 359–62. https://doi.org/10.1038/nmeth.1322.

Wouters, Merridee A., Isidore Rigoutsos, Carmen K. Chu, Lina L. Feng, Duncan B. Sparrow, and Sally L. Dunwoodie. 2005. “Evolution of Distinct EGF Domains with Specific Functions.” *Protein Science* 14 (4): 1091–1103. https://doi.org/10.1110/ps.041207005.

Xiong, Caixing, Krzysztof Kaczmarek, Janusz Zabrocki, Ronald J. Nachman, and Patricia V. Pietrantonio. 2020. “Activity of Native Tick Kinins and Peptidomimetics on the Cognate Target G Protein-Coupled Receptor from the Cattle Fever Tick, Rhipicephalus Microplus (Acari: Ixodidae).” *Pest Management Science* 76 (10): 3423–31. https://doi.org/10.1002/ps.5704.
Zhang, Y, Y Liu, H Liu, and W H Tang. 2019. “Exosomes: Biogenesis, Biologic Function and Clinical Potential.” *Cell Biosci* 9: 19. https://doi.org/10.1186/s13578-019-0282-2.

Zhou, Xiaoxue, Feng Xie, Lin Wang, Long Zhang, Suping Zhang, Meiyu Fang, and Fangfang Zhou. 2020. “The Function and Clinical Application of Extracellular Vesicles in Innate Immune Regulation.” *Cellular and Molecular Immunology* 17 (4): 323–34. https://doi.org/10.1038/s41423-020-0391-1.
Table 1: Gene ontology (GO) analysis of proteins unique to HTR and LTR cattle.

| Category ID | Mapped IDs | Name | Parent | Child | Family and Subfamilies |
|-------------|------------|------|--------|-------|------------------------|
| PC00123     | BOVIN|Ensembl=ENSBTAG00000045659|UniProtKB=G3N033,BOVIN|Ensembl=ENSBTAG00000050088|UniProtKB=A0A3Q1MI29,BOVIN|Ensembl=ENSBTAG00000052689|UniProtKB=A0A3Q1MT50 |
|             |             | imm | unog   | defe | nse/i                  |
|             |             | lobu| mmu    | nity | protei                 |

Downloaded from https://academic.oup.com/jas/advance-article/doi/10.1093/jas/skac015/6511758 by guest on 29 January 2022
| GO:0006955 | BOVIN|Ensembl=ENSBTAG00000045659|UniProtKB=G3N033,BOVIN|Ensembl=ENSBTAG00000050136|UniProtKB=A0A3Q1MSF6,BOVIN|Ensembl=ENSBTAG00000017305|UniProtKB=F1N160,BOVIN|Ensembl=ENSBTAG00000052689|UniProtKB=A0A3Q1MT50 | immune response;type 2 immune response;cell activation involved in immune response;innate immune response;adaptive immune response | immunune immune response;type 2 immune response;cell activation involved in immune response;innate immune response;adaptive immune response | 863 |
|----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| GO:0006900 | BOVIN|Ensembl=ENSBTAG00000050088|UniProtKB=A0A3Q1MI29,BOVIN|Ensembl=ENSBTAG00000048268|UniProtKB=G3N1H5 | vesicle budding | vesicle budding | Golgi vesicle budding | 813 |
| GO:0005737 | BOVIN|Gene=FTH1|UniProtKB=O46414,BOVIN|Ensembl=ENSTAG00000013343|UniProtKB=O46415,BOVIN|Gene=LIMS1|UniProtKB=F6QGZ0. | cytoplasm | intracellular | SMN complex;endoplasmic reticulum;cell cortex;cytoplasmic vesicle;cytoplasmic microtubule;cytoplasmic region;sarcoplasm;cyt | 245 | 92 |
| osol;cytoplasmic | ribonucleoprotein;mitochondrion;SMN-Sm protein complex;microbody;endoplasmic reticulum-Golgi intermediate compartment;contractile fiber;perinu |
| GO:0042113 | BOVIN|Ensembl=ENSBTAG00000050088|UniProtKB=A0A3Q1MI29,BOVIN|Ensembl=ENSBTAG00000048268|UniProtKB=G3N1H5 | B cell activation | B cell proliferation; B cell activation involved in immune response; B cell differentiation | 150 |
| GO:0006879 | BOVIN|Gene=FTH1|UniProtKB=O46414,BOVIN|Ensembl=ENSBTAG00000013343|UniProtKB=O46415 | cellular iron homeostasis | iron import into cell | 153 |
| Low tick resistance |  |  |  |  |  |  |  |  |
| Category ID | Mapped IDs                                                                 | Name          | Parent      | Child Families and Subfamilies |
|-------------|----------------------------------------------------------------------------|---------------|-------------|--------------------------------|
| GO:0017076   | BOVIN|Ensembl=ENSBTAG00000050904|UniProtKB=P62833,BOVIN|Ensembl=ENSBTAG0000006969|UniProtKB=Q2KJD0 | purine nucleotide binding | nucleotide binding | ATP binding | 913 |
| GO:0098797 | BOVIN|Ensembl=ENSBTAG00000020645|UniProtKB=A7MBH9|BOVIN|Gene=GNB1|UniProtKB=P62871 | plas | plas | plasma membrane respiratory chain complex; voltage-gated calcium channel complex | 594 |
| GO:0005737 | BOVIN|Ensembl=ENSBTAG00000017970|UniProtKB=Q08DQ6,BOVIN|Gene=GNB1|UniProtKB=P62871,BOVIN|Ensembl=ENSBTAG00000006969|UniProtKB=Q2KJD0 |
| cytoplasm | intercellular | SMN complex; endoplasmic reticulum; cell cortex; cytoplasmic vesicle; cytoplasmic microtubule; cytoplasmic region; sarcoplasm; cytoplasmic ribonucleoprotein granule; mitochondrial | 245 | 92 |
| ochondrion ;SMN-Sm protein complex;microbody;endoplasmic reticulum-Golgi intermediate compartment;contractile fiber;perinuclear region of cytoplasm;vacuole;membrane |
| GO:0097367 | BOVIN|Ensembl=ENSBTAG00000050904|UniProtKB=P62833,BOVIN|Ensembl=ENSBTAG00000006969|UniProtKB=Q2KJD0 | coat;Golgi apparatus;philagophore assembly site;SNARE complex;plastid;eukaryotic translation initiation factor 3 complex | carbohydrate derivative binding | lipopolysaccharide binding;ATP binding;glycosaminogl | 110 9 |
| GO:0051015 | BOVIN|Ensembl=ENSBTAG00000008631|UniProtKB=Q92176,BOVIN|Ensembl=ENSBTAG00000021455|UniProtKB=Q5E9F7 | binding | ycan binding | actin filament binding | actin binding | 473 |
Figure Legend

**Figure 1:** Exosomal proteins by protein class that are shared and unique to high (HTR) and low (LTR) tick resistant cattle. PC = Protein class.