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Identification of protein expression changes in milk in experimental bovine mastitis using difference gel electrophoresis

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ABSTRACT

In order to identify the extent of protein changes in milk during mastitis as a guide to detecting markers for prompt management of the disease, milk from cows in which clinical mastitis was experimentally induced were subjected to difference gel electrophoresis (DiGE) analysis. Pooled samples from 6 udders (from 6 cows) were analysed at selected time points: 0, 81 and 312 hours post-challenge with *Streptococcus uberis* mastitis. These corresponded to samples from the pre-infection, peak and resolution phases of the mastitis challenge. After the preliminary sample preparation, concentration and pooling steps, samples were labelled with CyDyes (Cydye 2, 3 and 5), after which isoelectric focusing and gel electrophoresis were carried out respectively. DiGE gels were subsequently scanned and ImageQuant, ImageJ and DeCyder™ 2D (version 7.0) softwares were used to crop, obtain jpeg images and carry out 2-D differential analysis and processing of the images, respectively. Biological variation analysis (BVA) software (GE Healthcare Life Sciences, Buckinghamshire, UK) was also used to analyse the gels and create gel to gel matching of spots (qualitatively and quantitatively) within the three gels produced. Overall, a total of 521 protein spots were identified as significantly differentially expressed (qualitatively or quantitatively) in mastitis milk during the course of the intramammary infection. This demonstrates the large repertoire of protein biomarker candidates available, revealed through this technique. Further studies are required to elucidate the merits and demerits of these changing proteins in order to identify the one most suitable for clinical application in mastitis diagnosis.

Key words: mastitis; milk; proteins; *Streptococcus uberis;* difference gel electrophoresis

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Introduction

Protein analysis has been carried out in samples for decades using immuno and biochemical assays, but these assays are usually limited to detection of a particular protein or quantitative analysis, such as total protein (CECILIANI et al. 2014b), and often require the use of antibodies. Current approaches to proteomic studies include gel based and non-gel based methods and have additional advantages over the immuno and biochemical assay techniques in identifying a wider panel of proteins within a single experiment, without the need for specific antibodies (BOEHMER et al. 2010). Two fundamental principles underlie modern proteomics studies. These are the separation of individual proteins from a complex mixture of proteins (fractionation) and the identification or characterization of these proteins (CECILIANI et al. 2014a).

Gel-based proteomics employs the use of an electric field (electrophoresis) through a gel matrix, such as a polyacrylamide gel, for fractionation or separation of different proteins within a complex mixture of proteins, as found in biological samples. Examples of gel-based methods of proteomics include: one dimensional SDS-polyacrylamide gel electrophoresis (1DE SDS-PAGE), 2 dimensional (2DE) SDS-PAGE and difference gel electrophoresis (DiGE) (LILLEY et al. 2002).

1 DE and 2 DE are the most frequently used gel-based proteomics techniques with outstanding results in bovine milk proteomics (HOGARTH et al. 2004; BOEHMER et al. 2008; THOMAS et al. 2016). However, a number of shortcomings have been associated with these, for example in 1 DE, limited variations can be observed for the discrepant samples the large bands contain, and thus mask numerous small protein changes. In the case of 2 DE, the method suffers from a lack of reproducibility due to marked between-gel variabilities (VAN DEN BERGH et al. 2003).

Difference gel electrophoresis was first described by UNLU et al. (1997) and was developed in order to overcome the problem of the lack of reproducibility of 2DE. It utilizes different fluorescent dyes to label proteins in different samples, and an internal standard (pool of samples) is also labelled with another dye, and these samples are then electrophoresed in combination within a single 2D gel.

Mastitis, udder inflammation, has continued to pose a major challenge to the dairy sector worldwide. As part of the efforts towards control and management of this disease, reliable biomarkers are urgently needed that would facilitate prompt diagnosis on-line on farms. Hence, the potentials of emerging proteomic techniques in uncovering these likely analytes need to be evaluated. Most gel-based proteomic analyses of bovine mastitis milk have been based on either 1 DE or 2 DE, with very few reports on DiGE research in bovine mastitis milk, and none specifically dealing with *S uberis* mastitis (ABDELMEGID et al. 2020). We believe that given the added advantage of DiGE analysis, more insights on milk proteome variability can be gleaned by exploiting the advantageous features of the DiGE technique over 1 DE and 2 DE electrophoresis. In particular, it is projected that the DiGE may reveal more changes in the proteome that could potentially provide a wider panel of compounds from which suitable biomarkers of mastitis can be selected.

The objective of this study was to utilize DiGE in pre-fractionation and preliminary identification of the qualitative and quantitative protein changes in the proteome of milk during the course of an experimental mastitis challenge.

Materials and methods

Experimental Samples. A putative host adapted strain of *S. uberis*, strain FSL Z1-048, was infused into one hind quarter each of six healthy mid-lactation Holstein cows, as described earlier (TASSI et al., 2013). All infected quarters were confirmed to be clinically mastitic by the presence of clinical signs, including redness of the udder, pain on palpation and swelling, as well as an increase in somatic cell counts (SCC) by 48 hours post-infusion. All animal experiments were conducted at the Moredun Research Institute (Penicuik, UK) with the approval of the Institute's Experiments and Ethical Review Committee, in accordance with the Animals (Scientific Procedures) Act 1986.

Skimmed milk was prepared by two repeated centrifugations of approximately 50 ml of milk at 2800 x g at 4°C for 20 minutes (min). The fat layer was discarded and the supernatant was transferred into new Falcon tubes. Pooled samples from each of three selected time points (0, 81 and 312 hours (h) post challenge) following intramammary inoculation were analysed using DiGE. These corresponded to samples from the pre-infection, peak and resolution phases of the experimental mastitis, respectively.

Sample Preparation. All chemicals used (except where stated otherwise) were purchased from Sigma-Aldrich, (USA) and were of analytical grade. Whole milk samples (~50 ml) were centrifuged at 2800 x g at 4°C for 20 minutes (min), followed by the removal of the fat layer, transfer of the supernatant into new tubes, and a repeat of the centrifugation step. Bradford assay with bovine serum albumin as standard (BSA) was used for quantification of the total protein in each sample, and 1 mg/ml of protein from each of the six skimmed milk samples from time points 0 h, 81 h and 312 h was pooled to give a volume with a total protein content of 6 mg for each of the 3 time points during infection. Acetone precipitation was then carried out on each of the pools by adding 4 times the sample volume of ice cold 100% acetone to the samples. This was mixed thoroughly and kept at -20°C overnight. The precipitate was separated from the supernatant by centrifugation at 1400 xg for 30 min, at 4°C. The pellets formed were then washed by mixing thoroughly in ice cold 80% (v/v) acetone, and then centrifuged at 1400 xg for 30 min once again. This step was repeated twice more. Finally, all the supernatant was separated from the pellet and the pellet was allowed to air dry. Subsequently the pellet was re-suspended in 250 µl DiGE lysis buffer (7 M urea, 2 M Thiourea, 4% CHAPS, 25 mM Tris HCl). The total protein concentration of the re-suspended pellet was then determined by the Bradford method, and adjusted to 5 mg/ml by addition of more DiGE lysis buffer as required. One microliter of CyDye was added to 10 µl of sample and incubated in the dark at 4°C for 30 min, to achieve conjugation. To stop the reaction, 1 µl of 10 mM lysine was added, and it was further incubated in the dark for 10 min.

Difference Gel Electrophoresis Procedure. The DiGE samples (labelled Cy3, Cy5 and pool standard labelled Cy2; ~12 µl of each) were mixed together and 424 µl of rehydration buffer (RHB) was added into the sample mixture and mixed thoroughly. Fifty µl of the unlabelled pooled standard was added to 410 µl of the RHB. Four hundred and fifty µl of each DiGE pool and preparative gel pool was then carefully pipetted along the length of each strip holder to avoid bubble formation. The IPG strip (24 cm pH 4-7, GE Healthcare, UK) was put onto the strip holder, 1 ml of mineral oil was applied over the top of the strip, and it was then covered and left for ~30 min for the gels to rehydrate passively. The lid was replaced and the strip holder placed on the IPGphor system (GE Healthcare, UK). Isoelectric focussing was carried out on the IPGphor using the manufacturer's protocol.

After isoelectric focusing, the strips were taken out of the holders, washed briefly in milli Q water to remove excess mineral oil, and put into plastic tubes. Ten ml of SDS equilibration buffer (EB) I (1.5M Tris HCl, 216.21 g urea, 180 ml glycerol, 12 g SDS and 100 mg DTT, pH 8.8) were placed onto the strips and the plastic tubes were put on a large flat rocker desk for 15 min. The buffer was poured off and SDS EB II (pH8.8, 1.5 M Tris HCl, 216.21 g urea, 180 ml glycerol, 12 g SDS 280 and 250 mg iodoacetamide) was added and it was rocked gently for another 15 min. The buffer was poured off and the strip(s) were inserted horizontally between the DiGE gel (precast Ettan DALT® gels; 26 x 20 cm) cassettes and allowed to make contact with the gel. 1 ml of 0.5% (w/v) agarose was added on top of the DiGE gel (before putting the gel in the electrophoresis running tank). Running buffer was added into the tank assembly and electrophoresis was carried overnight at 1W/gel.

Scanning and Staining of Gels. Upon completion of electrophoresis, the DiGE gels were scanned on a 3 Laser Typhoon 9400 scanner (GE Healthcare life sciences, Buckinghamshire, UK). A preliminary scan was performed of each gel at a low resolution (1000 microns). Spot saturation was checked on each of the resulting images, and the photomultiplier tube voltage adjusted so that the most intense protein spots were not

saturated (according to DiGE instruction manual, GE Healthcare). A high resolution (100 microns) scan of each DiGE gel was then performed. Once satisfactory images were obtained, the gels were stored in a wet tray and kept at 4°C for a maximum of one week. The preparative gel was fixed in 7% (v/v) acetic acid + 10% (v/v) methanol for 1-2 h, and then rinsed in water and stained with Sypro-orange (1/10, 000 dilutions in 7% (v/v) acetic acid for 2 h). The prep gel was scanned using the green filter of the Typhoon scanner at high resolution (100 microns). The prep gel was wrapped and stored at 4°C until spot picking.

DiGE images were cropped using ImageQuant software (GE Healthcare Life Sciences, Buckinghamshire, UK) on the Typhoon scanner. The cropping removed all the edges of the gel image (gel spacers, IPG strip and dye front). ImageJ software (National Institute of Health, Maryland, USA) was also used to obtain jpeg format images of each DiGE gel. The images were then loaded into DeCyder™ (GE Healthcare life sciences, Buckinghamshire, UK) 2-D differential analysis software for processing.

Statistical Analysis. DeCyder Biological Variation Analysis. DeCyder 2D (version 7.0) differential In-gel analysis (DIA) and biological variation analysis software (BVA) (GE Healthcare life sciences, Buckinghamshire, UK) were used to analyse the gels and create gel to gel matching of spots (qualitatively and quantitatively) within the three gels produced. Normality of data was assessed using the Shapiro-Wilk test. The software produced statistical comparisons (one-way ANOVA) after normalization of the ratio of spot volume between the spots of different dyes on a single gel using the internal standard (pool of all samples labelled with Cy2), between the 3 different gels across the 3 time points (designated: before, peak and after for 0, 81 and 312 h post-infection times, respectively). It was therefore possible to identify spots which were quantitatively and qualitatively different across gels.

A few of the significantly varying spots, once identified, were then excised from the preparative gel (gel 4) using an Ettan spot picker (GE Healthcare life sciences, Buckinghamshire, UK) and processed by trypsin digestion for protein identification using LC-MS/MS, as described in (MUDALIAR et al., 2016a).

A one-way ANOVA test was carried out by the BVA module of the DeCyder software following normalization and ratio matching of spots, to determine the spots that changed across the 3 time points in each of the gels. The Student's t-test was also used to determine differences in spot volume at pre-infection (0 h) and peak infection time (81 h). A P value of < 0.005 was considered significant.

Results

The ImageQuant software images of the 3 different DiGE gels are shown in Fig. 1 to 3. Numerous new protein spots were seen at time 81 h which were not present at 0 h. Also, some major differences in the intensity of spots present at 0 h and 312 h were observed at 81 h. There were no appreciable differences between spots at 0 h and at 312 h (Fig. 2). The preparative (prep) gel image is shown in Fig. 4. This gel comprised a pool of the 3 time points considered for the DiGE experiment, from which spots of interest were excised (spots indicated as numbers on the image).

A total of 2154 spots were identified on the first DiGE gel (0 h + 81 h) by the DeCyder software, and 1077 of these were matched on the prep gel, while 2577 and 2358 spots were seen in the second and third gels, respectively (0 h + 312 h and 81 h + 312 h). A total of 2577 spots were matched in the preparatory gel in the second DiGE gel while 1077 matched to the prep gel on the third DiGE gel. Therefore, because the second DiGE gel had the highest number of matches in the preparatory gel, it was selected for matched-spot picking to detect proteins of interest (spots varying significantly across the 3 time points and in the three gels). After the BVA test, 728 showed differences across the pre-infection, peak-infection and resolution phase of the challenge study. A total of 521 of these spots had a statistically significant variation (ANOVA, $P<0.05$) with time, while the variations in 207 spots were not statistically significant ($P=0.05$-0.97). Out of the significantly varying spots, 29 were selected on the basis of the level of change, for protein identification. Table 1 shows the identities and type of regulation of 16 of these spots picked from the preparatory gel for LC-MS/MS analysis (others were not determined (ND) due to the poor-quality spot picked).

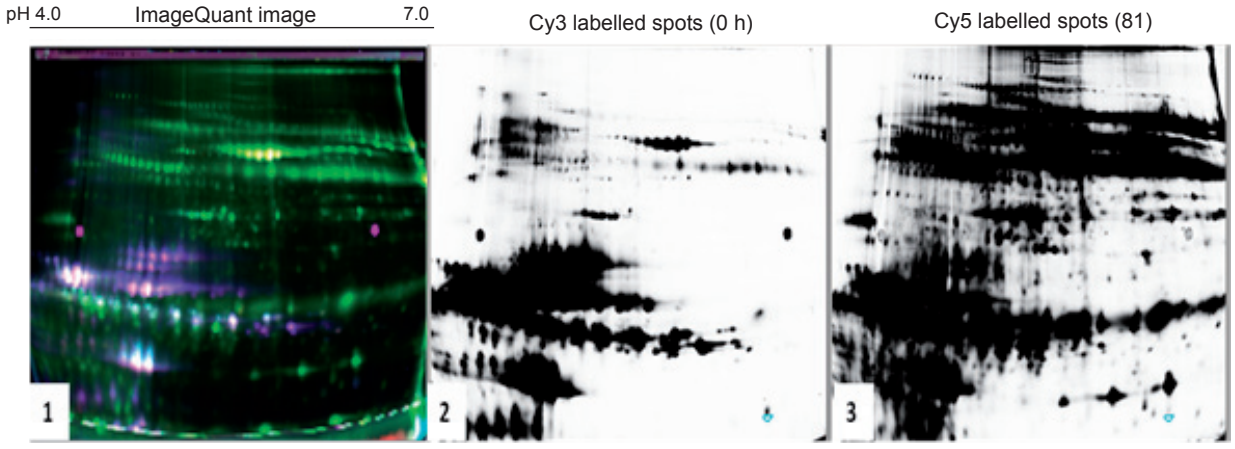

Fig. 1. Difference gel electrophoresis (DiGE gel 1) on bovine whey from experimental *S. uberis* mastitis showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots

Pool of time point 0 labelled with Cy3/red fluorescent dye (appearing purple) and pool of time point 81 labelled with Cy5/blue (appearing green) on ImageQuant

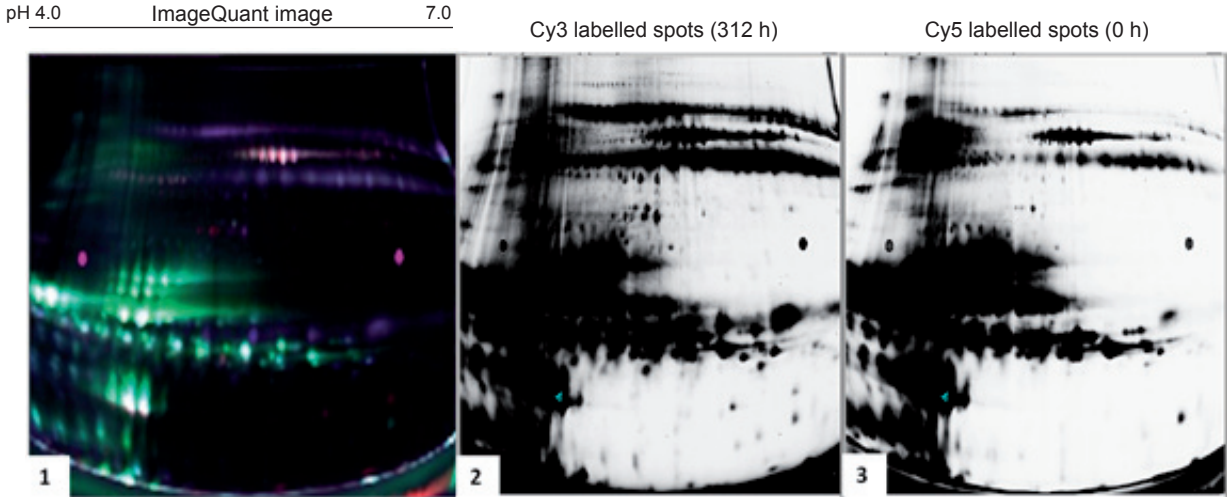

Fig. 2. Difference gel electrophoresis (DiGE gel 2) on bovine whey from experimental *S. uberis* mastitis, showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots

Pool of time point 0 labelled with Cy5/blue (appearing green) fluorescent dye and pool of time point 312 labelled with Cy3/red (appearing purple) on ImageQuant

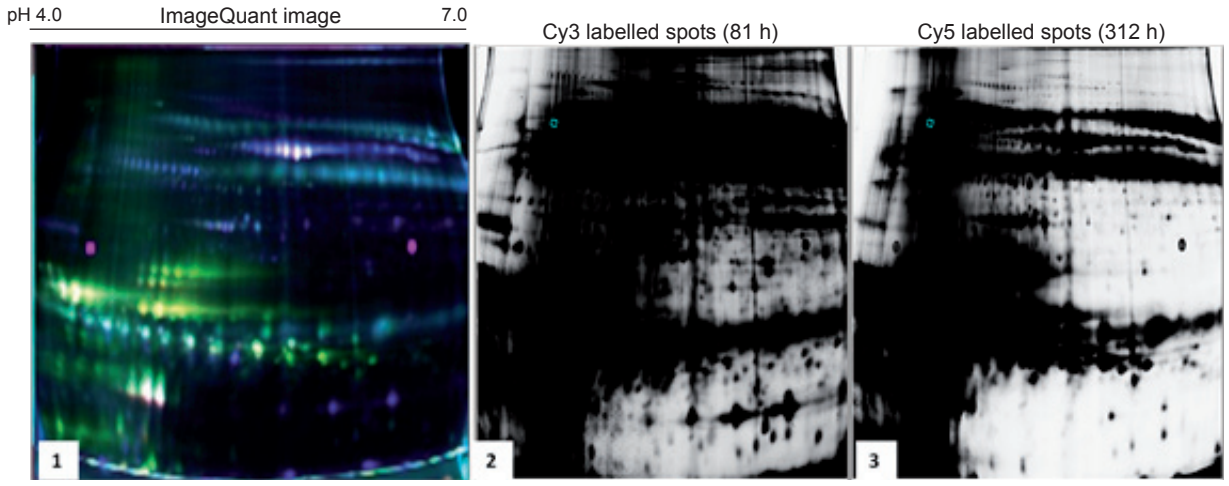

Fig. 3. Difference gel electrophoresis (DiGE gel 3) on bovine whey from experimental *S. uberis* mastitis, showing juxtaposed ImageQuant image (1), DeCyder differential scans of Cy3 (2) and Cy5 (3) spots

Pool of time point 81 labelled with Cy3/red (appearing bluish/purple) fluorescent dye and pool of time point 312 labelled with Cy5/blue (appearing green) on ImageQuant

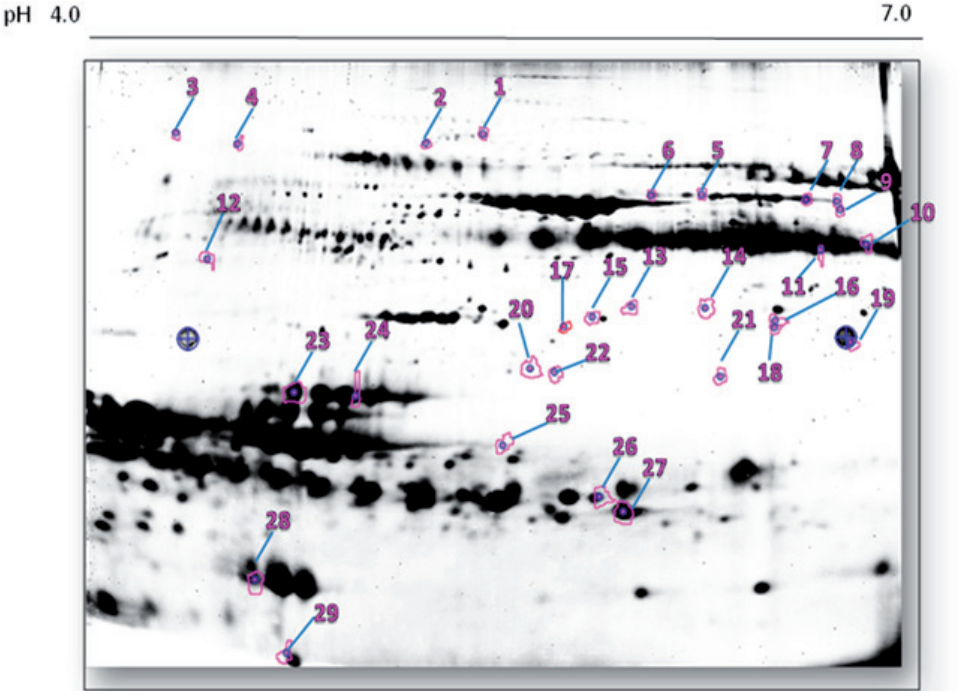

Fig. 4. DiGE preparative gel comprising the pool of 3 time points (0, 81 and 312 h)

Spots of interest (selected on the basis of changing profile during the course of the 3 time points) (n=29) excised for LC-MS/MS analysis, are shown numbered and identified as listed in Table 1

Table 1. Protein identification using LC-MS/MS of a few spots excised from the DiGE preparative gel (Fig. 4), following spot matching with 3 DiGE gels

S/N	Spot ID	Protein	Ac. Number	Calculated pI	Mass (Da)	MOWSE Score	Characteristics at peak infection
1	141	Ankyrin repeat and sterile alpha motif domain-containing protein 1B	XP_005198370	5.41	90486	18	down regulated
2	495	Albumin	754920A	5.76	68083	51	up regulated
3	562	Albumin	754920A	5.76	68083	51	up regulated
4	607	Serotransferrin	Q29443	6.75	79870	81	up regulated
5	636	IQ motif containing E-like	DAA15162	8.43	88179	21	up regulated
6	960	Albumin	754920A	5.76	68083	74	up regulated
7	962	Albumin	754920A	5.76	68083	48	up regulated
8	1042	Refeldin A-inhibited guanine nucleotide-exchange protein 3	XP_871474	5.54	245336	16	down regulated
9	1117	Factor XIIa inhibitor precursor	NP_777246	6.19	51919	16	up regulated
10	1137	Centromere protein A-like	DAA25906	11.19	52963	16	up regulated
11	1155	RAC-beta serine/threonine protein kinase isoform x4		5.98	55905	20	up regulated
12	1236	SH2 domain-containing adapter protein B	NP_001179863 XP_618475	9.07	55667	21	up regulated
13	1336	RAC-beta serine/threonine-protein kinase	NP_001193075 XP_870006	5.98	56004	23	up regulated
14	1375	Albumin	754920A	5.76	68083	76	up regulated
15	1701	Immunoglobulin heavy chain variable region	CAA10182	8.62	12561	19	down regulated
16	2236	Beta-lactoglobulin	CAA32835	4.85	20307	25	down regulated

Discussion

In this study, three time points representing pre-infection, peak infection and resolution phase of an experimental clinical mastitis model were analysed using DiGE. This procedure provides a platform for comparison of similar samples, and in this study it was possible to identify small changes in protein spots from different samples run on a single gel.

Using the Decyder™ 2-D image analysis (DIA) software, it was possible to match and recognize differing spots across the three time points considered within the same gel using the internal pooled standard. In particular, quantitative changes in protein across the pre-infection, peak infection and resolution of infection stages were recognizable. The BVA module of the Decyder software also made it possible to compare the spots and their variation across the 3 different gels reproducibly, thus improving the robustness of the DiGE proteomic methodology.

From this study, however, there were a number of spots observed in the DiGE gels which were not detected in the preparative gel, possibly due to the relatively lower protein concentration of each time point sample in the prep gel. This may pose a problem for identification of key proteins which are relevant to the diagnosis of IMI, as they cannot be picked for further MS analysis.

The technique enabled the detection of up to 728 protein spots which changed across the pre-infection, peak infection and resolution phases of the challenge course. Two hundred and thirty-eight protein spots were up-regulated during the course of infection, while 283 protein spots were down-regulated as the infection progressed. Among the 29 spots selected for LC-MS/MS identification, 12 spots were of proteins up-regulated during infection and were identified as serum related proteins, such as albumin and serotransferrin, which are known to increase in milk during mastitis. One other up-regulated protein, benzodiazepine receptor (peripheral) associated protein 1, represents a rare finding in mastitic milk. It has been reported to have roles in steroidogenesis, apoptosis and oxidative processes, and is localized in the mitochondria (CASELLAS et al., 2002). This suggests the reason for its detection and up-regulation in mastitic milk as probably being the part it plays in the phagocytic process, and also as a consequence of seepage from serum into the milk. Factor XIIa inhibitor precursor, a serine–type endopeptidase inhibitor with molecular roles in regulating important physiological pathways, including complement activation, blood coagulation, fibrinolysis and the generation of kinins (www.uniprot.org/uniprot/P50448), was also identified as up-regulated, probably as a result of seepage from serum into the milk. Acyl-protein thioesterase 1 isoform X1, leucine-rich repeat flightless-interacting protein 2, RAC-beta serine/threonine-protein kinase, and glial fibrillary acidic protein were all up-regulated, and are also probably related to the compromise of the blood milk barrier (HOGARTH et al., 2004).

Some of the down-regulated proteins identified, such as nuclear receptor ROR-alpha, thyroid receptor-interacting protein 6 and zinc finger protein, have no clear significance in milk during mastitis.

Generally, DiGE is has been proven to be a useful and robust quantitative method for analysing protein differences between samples. However, this method is limited by the number of samples that can be compared per experiment, due to the number of dyes available for labelling samples. Newer approaches for quantitative proteomics studies are being developed to overcome these limitations, for example, label-free quantification proteomics, which does not require labelling of proteins prior to MS analysis, but is based instead on precursor signal intensity or on spectral counting. A high abundance proteins proteomic study, as well as the label-free method were applied to study the proteomic changes in the milk samples following this particular experimental *S. uberis* mastitis challenge (MUDALIAR et al. 2016; THOMAS et al. 2016), a total of 77 and 570 proteins were quantified in these studies respectively. Compared to the 521 significantly varying proteins seen in the present study, it may be concluded that the DiGE technique shows high potential in being able to expose a large repertoire of protein changes during mastitis.

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Conflict of Interest

The authors declare that no conflicts of interest exist.

Code or data availability – data is available on request.

Authors' contribution

Thomas F. C. – writing – original draft, wrote the code and the manuscript; Ajibola E. S. – reviewed the manuscript; Alan S. – methodology; Tassi R. – methodology; Andre M. Santana – methodology, data curation; McNeilly, T. N. - provided the research ideas and theoretical analysis; Zadoks R. – provided the research ideas and theoretical analysis; Hayley Haining – wrote and edited the manuscript; Burchmore R. – provided the research ideas and theoretical analysis, formal analysis; Eckersall, P. D – provided the research ideas and theoretical analysis.

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Ethics approval statement

All animal experiments were conducted at the Moredun Research Institute (Penicuik, UK) with the approval of the Institute's Experiments and Ethical Review Committee, in accordance with the Animals (Scientific Procedures) Act 1986 as described by TASSI et al., 2013.

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SAŽETAK

Cilj je bio ustanoviti opseg promjena u sastavu proteina mlijeka za vrijeme mastitisa i na taj način dati doprinos smjernicama za otkrivanje markera kojima bi se ubrzalo liječenje ove bolesti. Mlijeko krava u kojih je eksperimentalno izazvan klinički mastitis podvrgnuto je razlikovnoj elektroforezi u gelu (DiGE). Analizirani su objedinjeni uzorci iz 6 vimena (od 6 krava) u odabranim vremenskim točkama 0, 81 i 312 sati nakon infekcije bakterijom *Streptococcus uberis*. Navedene vremenske točke odgovaraju trima fazama mastitisa u kojima su uzeti uzorci: u predinfektivnoj fazi, u fazi u kojoj je infekcija dosegnula vrhunac i u fazi povlačenja mastitisa. Nakon preliminarne pripreme odnosno više koraka koncentracije i objedinjavanja, uzorci su obojeni pomoću CyDyes (Cydye 2, 3 i 5) te je provedeno izoelektrično fokusiranje i elektroforeza u gelu. DiGE gelovi zatim su skenirani te su primjenom softvera ImageQuant, ImageJ i DeCyder™ 2D (verzija 7.0) snimke obrezane, dobivene su jpeg slike i provedena je 2D diferencijalna analiza i obrada snimki. Upotrijebljen je i softver za analizu bioloških varijacija (BVA) (GE Healthcare life sciences, Buckinghamshire, UK) kako bi se gelovi analizirali i formiralo podudaranje točaka (kvalitativno i kvantitativno) unutar tri proizvedena gela. Utvrđeno je da je ukupno 521 mjesto proteina znakovito različito izraženo (kvalitativno i kvantitativno) u mlijeku krava s mastitisom za vrijeme intramamarne infekcije. Navedeno upućuje da je primjenjena tehnika ponudila veliki broj kandidatnih proteina koji mogu poslužiti kao biomarkeri mastitisa. Potrebna su daljnja istraživanja kako bi se razjasnile prednosti i nedostaci metode otkrivanja promjena u proteinima sa svrhom pronalaska najprikladnije kliničke primjene u dijagnostici mastitisa.

Ključne riječi: mastitis, mlijeko; protein; *Streptococcus uberis*; razlikovna elektroforeza u gelu