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Determination of the Proteins of *Salmonella Enteritidis* Involved in Colonization of the Chicken Ceca Using Protein Analysis

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Abstract: *The results of this investigation revealed that the whole-cell protein profile of S. Enteritidis harvested from the chicken intestine was very different to that of bacteria cultured in vitro in nutrient broth. As visualised on SDS-PAGE, the in vivo protein extract had a lower protein content and a reduced number of visible protein bands; after protein extraction by sonication, and despite the fact that the amount of protein loaded was the same for both in vivo and in vitro extracts, very few protein bands from the in vivo bacteria were detectable on SDS-PAGE. The two ceca are the main site of the colonization of Salmonella in chickens, and the bacteria can be easily harvested from the ceca for analysis; therefore this study aimed to identify protein(s) associated with colonization using protein technology.*

In this study newly-hatched chickens (within 10 hours of their hatching) were infected with S. Enteritidis, the bacteria were harvested from the ceca 18 hours post infection and bacterial proteins were extracted by sonication and analysed utilizing SDS-PAGE electrophoresis, and compared with that obtained from broth culture. This comparison showed differences between the two profiles and indicated that it is difficult to make a reasonable comparison as out of about 40 protein bands of in vitro preparation only a few 3-4 bands could be visualized from in vivo preparation, the reason behind that was thought to be the degradation of in vivo protein which was investigated in another study and the result revealed that some avian proteases might be responsible. Subsequently, some individual proteins from both preparations were identified by Mass spectrometry.

Key words: *Salmonella, Enteritidis, Chickens, colonization, protein analysis, SDS-PAGE, Mass spectrometry.*

Introduction

The bacterium *Salmonella* was discovered by Theobald Smith in 1885 and named as Salmonella in 1934 after the American pathologist Daniel Elmer Salmon who was the head of the United States Department of Agriculture (USDA).The genus was recognized as the causative agent for many significant infections in man, animals and birds, (Marthedal, 1960). The majority of *Salmonella* infection reports in different hosts including man and animals were as a result of clinical outbreaks (Jepsen, 1960, Poppe, 1999). *S. Typhimurium* was in general the most common serotype in humans until the 1980s decade of the 20th century (Doyle and Cliver, 1990, Le Bacq, 1993) when *S. Enteritidis* became dominant, with Phage Type 4 (PT4) in the European Union (EU) while PT8 was the dominant serovar in the United States of America (Rodrigue et al., 1990, Wray, 1995).

Serovars are members of the Enterobacteriaceae family and are thus Gram-negative, motile bacteria that have numerous peritrichate flagella. They are facultative anaerobic, non sporulated bacilli measuring 2-4μm in length. The majority of serovars are fimbriate, and rarely capsulated. The vast majority (>90%) of serovars are non-lactose fermenters; this can be detected using Xylose Lysine Deoxycholate Agar (XLD) (Washington et al., 2005).

All serovars are not very fastidious organisms in their nutritional requirements and growth temperature. They grow at temperatures of between 7 and 46^{o}C on a wide range of cooked or unprocessed foods (Emond et al., 1995, Golden et al., 1993, Ingham et al., 1990); storage at non -refrigeration temperatures has been shown to be important in many outbreaks (Roberts, 1986, Palmer et al., 2000)

In the UK this strain PT4 is the most involved strain in human food-poisoning cases. It's virulence in young birds was empirically proved (Barrow, 1991, Barrow and Lovell, 1991).Moreover, and beside that it is highly virulent it causes systemic disease and pericarditis in young broiler birds. It is checked periodically by slide agglutination with O-9 antisera. (Lister, 1988, O'Brien, 1988) .

Carcass contamination is the main route of entry into the human food chain. During *Salmonella* colonization of the chicken intestine the highest number of bacteria can be detected in the two ceca which are the major site of colonization in addition to some other internal organs such as gizzard and crop.

There are many factors involved in the colonization of Salmonella in chickens including the health of birds, age, stress, bird genetic conditions and the amount of preservatives added to the food (Bailey, 1988).

There is a great demand to control food-poisoning salmonellosis at both breeder and layer levels at the national and global level in order to produce *Salmonella*-free poultry products, due to the current correlation between S. Enteritidis PT4 and poultry products. Human salmonellosis continued to be a major problem in terms of both morbidity and economic cost (Barrnass et al, 1989)Certainly, there is a rising evidence that the process of the colonization is not exclusively a metabolic function since some other form of physical association with host cells or organ in the gut is implicated.

Accordingly, some fimbrial and invasion genes involved in chickens colonization were documented (Clayton et al., 2008, Morgan et al., 2004), signifying that physical attachment was a requisite. Blocking or preventing the initial colonization of *Salmonella* within farm animal hosts - particularly chickens - together with an intervention at several points in the food production chain are of great strategic importance towards reduction or elimination of the infections. The bacteria can be easily harvested from the ceca for analysis; therefore this project aimed to identify protein(s) associated with colonization using protein technology. Among the latter, Mass Spectrometry was used since it can detect substitutions of single amino acids in proteins and may be used to quantify individual protein isoforms in mixtures.

During the past two decades, mass spectrometry has become established as the primary method for protein identification from complex mixtures of biological origin. This is largely attributable to the fortunate coincidence of instrumental advances that allow routine analysis of minute amounts (typically femtomoles) of involatile, polar compounds such as peptides in complex mixtures, with the rapid growth in genomic databases that are amenable to searching with mass spectrometry (MS) data.(John E Hole, 2013)

Specific Objectives of the research

To define the proteins of S *Enteritidis* involved in colonization of chicken ceca.

Experimental Plan

Several lots of newly hatched chickens were inoculated orally within 18 hours of hatching with *S. Enteritidis* PT4 and their cecal contents were collected 16 -18 hours post infection and used for protein extraction by sonication . For a comparison to be made with proteins produced *in vitro* the same strain was grown *in vitro* in nutrient broth (NB) and this broth culture was also used for protein extraction by sonication. Both in *vivo* and *in vitro* proteins preparations were analysed by SDS-PAGE. Mass spectrometry (MS) was used to identify proteins in both preparations as highly expressed proteins which may be implicated in colonization.

Materials and Methods

Materials

Bacterial strains:

An antibiotic sensitive *S. Enteritidis* PT4 strain P125109, used in this project was obtained from a human food poisoning case resulting from consumption of poultry meat. This strain was used because its virulence attributes were known (Barrow,1991,Barrow and Lovell, 1991) and the full genome sequence had been determined: www.sanger.ac.uk/projects/salmonella;EMBL accession number: AM933172.

The strain was stored at -80°C as a culture in Luria broth (LB) containing glycerol till used. It was checked for purity before use by streaking on MacConkey agar and also checked periodically by slide agglutination with O-9 antisera. In this study PT4 was employed either as spectinomycin resistant (Spc^R) or a nalidixic acid resistant (NalR)

Chickens

Fertile one day-old commercial broiler eggs purchased from a commercial source (P D Hook), were incubated in animal house fumigated room incubators (Sutton Bonington, University of Nottingham). These birds were only provided with sterile water until they had been inoculated and were not given food at all for the very short experiments duration (18 hours).{ Work permit project license No. PPL 40-3412} . Birds were killed by cervical dislocation.

Methods

Bacterial Viable Count:

A modification of the method of Miles et al (1938) was used for bacterial enumeration, a serial decimal dilution in phosphate buffered saline (PBS) were prepared. An aliquot of 20 µL was removed aseptically

from the broth culture to be counted and transferred to the first well of a series of 8 wells in a 96-well agglutination tray, each well containing 180 µL PBS. The contents of this first well was mixed gently by sucking and aspirating using a hand-operated "Gilson" pipette with a plugged tip. 20 µL was then transferred to the next well and with a fresh tip further mixing and transfer to the next well taking place. This was continued through the complete series resulting in a decimal dilution of the original culture. This was then followed by plating 100 µL of each dilution (triplicate) to the surface of a single quadrant of a nutrient agar (NA) plate. The viable count was then made and recorded as colony forming units per ml (cfu/ml), after overnight incubation at 37^{o}C. The quadrant chosen for counting was that which contained between 30 and 300 colonies.

Determination of optimal harvesting time for analysis of *in vitro* culture

Colony margin of *S. Enteritidis* PT4 sensitive strain was transferred into 10 ml tube of NB and incubated for overnight (O/N) at 37^{o}C, then 100 µL of this O/N culture were transferred into pre-warmed 100 ml NB in a 300 ml conical flask which was incubated at 37^{o}C with shaking for different time points (after 1, 2, 3, 4, 5 and 24 hours of incubation), the viable count was made for different time point utilizing serial dilution method. The experiment was also performed in triplicate using 300 ml conical flasks instead of 10 ml universal bottles

Growth culture – mid logarithmic phase

100 µL of an overnight (O/N) statically incubated broth of *S. Enteritidis* were transferred into pre-warmed 10 ml tube of NB and incubated at 37^{o}C shaking incubator for 2.5 hrs. One ml of this culture was transferred into pre-warmed 100 ml NB in a 300 ml conical flask, which was incubated at 37^{o}C with shaking (200 rpm) for 2.5 hrs.

Protein extraction of *Enteritidis* PT4

Several universal tubes containing nutrient broth (NB) were inoculated with this strain, then incubated for overnight (O/N) at 37°C. After that all tubes were centrifuged 4000 for 20 minutes at 4°C, then the pellet was re-suspended 3 times in phosphate buffer saline (PBS). The suspension in each tube was sonicated in ice using different programs of sonication followed by centrifugation 20000 at 4°C; the protein concentrations were measured by BCA assay and Nanodrop, then the supernatant from each tube was transferred to a new tube (aliquots) and stored at -80°C.

Proteins extraction from both *in vivo* and *in vitro* grown bacteria

Same protocol was used for bacterial protein extraction from both *in vivo* and *in vitro* condition as in the next two sections.

Protein Extraction Procedure for *in vitro* Grown Bacteria

Stationary phase broth culture of S. *Enteritidis* PT4 sensitive strain was used. This culture was centrifuged 20000 for 5 minutes at 4°C, and then the pellet was washed twice in PBS by centrifugation 20000 for 5 minutes at 4°C followed by sonication of the pellet in 5 ml of PBS and addition of bacterial protease inhibitor cocktail (Sigma) immediately prior the sonication for 5 minutes, then centrifugation at 15000 for 10 minutes at 4°C. Finally the pellets were discarded and supernatants were stored at -80°C.

Preparation of *in vivo* Culture

Chicken Inoculation and Sample Collection for Protein Extraction

Four lots of eggs (a total of 100 fertile eggs in each lot) were received from PD Hook Hatcheries Ltd (Cote Bampton, Oxfordshire, UK). Chickens were hatched in pre-fumigated incubators located in the Breeding and Rearing Unit (BRU), Sutton Bonington Campus, Nottingham University. All hatched chickens were orally inoculated within 10 hours of their hatching to avoid the development of gut flora. For each lot chicks were hatched over an approximately three day period so that three batches of chicks were used on three different days respectively. Chickens were housed in fumigated cages and were handled with sterile gloves to avoid contamination. Chicks were infected orally with 0.1 ml of a culture of the *S. Enteritidis PT4* sensitive strain, grown for 16 hours in NB at 37ºC and diluted in sterile NB to contain 10^{7} cfu/ml. Sterile water only was provided during the infection period since the yolk sac is not fully absorbed for up to 3-4 days providing sufficient nutrients. After 16–18 hrs chicks were killed individually and cecal contents were harvested into universal bottles held on dry ice and later stored at -80^{o}C. The cecal contents of three randomly chosen chicks were transferred to 3 separate sterile universal bottles which were placed in ice for checking purity and viable number estimation on MacConkey agar.

Protein Extraction Procedure for *in vivo* grown Bacteria

One day-old chicks were inoculated orally with *S. Enteritidis PT4* sensitive strain. On the following day the cecal contents which contained the highest number of bacteria harvested in dry ice using 50 ml falcon tubes, were diluted in PBS and then the same sonication procedure used for *in vitro* preparation mentioned above was applied.

Viable Count by Measurement of Bacterial Optical Density (OD):

A thermo- scientific spectrophotometer was used for this purpose.

The viable count was also verified using OD_{600} spectrophotometer as the spectrometric reading were taken using OD_{600} spectrophotometer for each time point of every dilution.

SDS-PAGE of Proteins

The electrophoresis running buffer 10X was prepared as following: Tris base, 30.3 g; glycine 144.4 g; SDS powder10 g and RO water was added to complete one litre volume. For each electrophoresis run 100 ml of this 10X stock solution were mixed with one litre of RO-water to get 1X running buffer. The electrophoresis loading (sample) buffer - Tris-glycine SDS- PAGE loading buffer (2X) was prepared as following:1M Tris-HCl (phH6.8) 1.6 ml;10% SDS 4 ml; glycerol (100%) 2 ml; B-Mercabtoethanol 1 ml; bromophenol blue 4 mg and DdH2O 1.4 ml for a total volume of 10 ml. This sample buffer was stored at 4°C until needed for sample loading into gel lanes.

Gel Electrophoresis Apparatus P9DS and P10DS (Thermo Scientific) were used to run SDS-PAGE gel and the bacterial proteins were detected using Imperial protein stain. 12% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to a well-established procedure (Laemmli 1970).

After the gel apparatus was set up, the separation gel monomer was prepared according to Laemmli (1970) method as follows:

1. 50mls per gel, volume in mls was made up for 10% gels (per gel)

Water (RO-water)	19.8 ml
30% acrylamide mix	16.7 ml
1.5mM Tris (pH 8.8)	12.5 ml
10% SDS	0.5 ml
Ammonium persulphate (AMPS)	50mg

2. The mixture was well mixed to ensure that AMPS is dissolved, then 20µl of TEMED were added, mixed and poured into gel plates, (a room at the top was left for stacking gel).

3. Approximately 0.5 ml water saturated butanol were used to cover the top of each gel.

4. The water saturated butanol layer was removed and the top of the gel was washed with water to remove the excess butanol.

After the gel has polymerized, the overlay was decanted and the stacking monomer was prepared as follows:

To prepare 20ml stacking gel

Water (BDH)	12.2ml
30% acrylamide mix	2.6 ml
0.5mM Tris (pH6.8)	5.0 ml
10% SDS	200 µl
Ammonium persulphate (AMPS)	20mg

1.The mixture was well mixed to ensure that the AMPS is dissolved, after that 20µl TEMED were added just prior to pouring gel, mixed and pipetted into gel plate; the comb was rinsed with methanol and placed in between the gel plates, then the gel was allowed to polymerize completely before running.

2. While the gel was allowed to set, the samples were prepared, boiled for 5 minutes in heating block previously switched on to the maximum, the samples were spun down for 1 minute in the microfuge.

Staining and Destining of the gel

Immediately after running the gels, they were stained by Imperial colloidal Coomassie blue (Sigma): gels were placed in Coomassie blue stain solution for two hours, and then destained by placing the gels in distilled water until protein bands became visualized, followed by scanning using a Bio-Rad densitometer scanner GS800.

Identification of *Enteritidis* Proteins Harvested from the *in vivo* and *in vitro* Environments by Mass Spectrometry

All gels were scanned and individual bands were cut out with a sharp blade and transferred to separate wells of a 96 well microtitre plate with a protective cover, 50 – 100 µl of HPLC grade water were added to each well of the plate (100 mM ammonium bicarbonate solution which is composed of 100 ml HPLC grade water and 0.79 g ammonium bicarbonate). Samples excised from the gel were processed using the Proteome Works Mass PREP robotic liquid handling station (Waters Ltd, Hertfordshire, UK). In this, samples were de-stained by incubating in 100 µL of 50 mM NH_4HCO_3, 50% acetonitrile 3 times (i.e. solution removed and replenished with fresh solution

between each incubation) for 10 min at 40°C and then after removal of final aliquot the gel pieces were dehydrated by incubation at 40°C in 50 µL of acetonitrile for 5 min. Then the samples were incubated at 40°C for a further 10 min after removal of the acetonitrile allowing evaporation to take place. Samples were then incubated for 30 min at 40°C in 50 µL of reducing solution (10 mM dithiothreitol, 100 mM NH_4HCO_3) followed by further incubation at 40°C for 20 minutes in 50 µL of alkylation solution (55 mM iodoacetamide, 100mM ammonium bicarbonate). After that the gel pieces were washed for 10 minutes in 50 µL of 100mM ammonium bicarbonate at 40°C, 50 µL of acetonitrile for 5 minutes followed by dehydration, then by two further washes in 50 µL in acetonitrile for 5 minutes at 40°C and 5 minutes evaporation. After cooling of the gel slices in the micro plate at 6°C for 10 min, 30 µL of trypsin gold (Promega) at 12 ng μL^{-1} in 50 mM ammonium bicarbonate; were added to each well. The plate was incubated at 6°C for 30 minutes to allow the trypsin to enter into the gel plugs with minimal autocatalysis, and then incubated at 40°C for 5 hours. Samples were stored at 4°C until used for MS analysis.

Mass Spectrometry of Digested Proteins

A Q-TOFII mass spectrometer was used fitted with a nanoflow ESI (electrospray ionization) source (Waters Ltd Hertfordshire, UK). Tandem MS data were obtained by automated data-dependent switching between MS and MS/MS scanning modes, based upon ion intensity, mass and charge state (data directed analysis, DDA™). In this automated acquisition type of experiment, a method was created in the MassLynx 4.0 software where charge state recognition was used to select doubly, triply and quadruply charged precursor peptide ions for fragmentation. Four precursor masses at a time were selected for tandem MS acquisition. The collision energy was automatically selected based on the charge and mass of each precursor and varied from 15 to 55 electron volts (eV).

MS Data Processing and Mascot Search

Protein Lynx Global server (version 2.0) (Waters Ltd, Hertfordshire, UK) was used to process MS DDA data into peak list (pkl) files. These data files were searched against all entries in SwissProt (a high quality, manually annotated and reviewed protein database) using the MASCOT MS/MS ions search tool (http://www.matrixscience.com/). The oxidation of methionine and carbamidomethylation of cysteine were set as variable modifications. One missed cleavage by trypsin was accepted. All other search values were preset defaults, with the exception of instrument type (ESI-QUAD-TOF) and file type (Micromass pkl).

Results

Bacterial Viable Count

In vitro Bacterial Counts

The viable numbers of *S. Enteritidis* P125109 in 10 ml NB tube cultures incubated for 18 hours indicated that the use of a 150 minute time point represents a mid-Log phase culture and is appropriate for inoculation of the 100 ml pre-warmed NB flasks. The viable Log numbers of *S. Enteritidis* P125109 in 100 ml NB flask cultures incubated for 24 hours again indicated that the use of a 150 min time-point represents a mid-log phase culture and is appropriate for harvesting. In addition, the viable count at each hour time-point here was combined with optical-density (OD_{600}) spectrophotometer readings which indicated that the curve of Log viable numbers and OD_{600} are very similar and the latter may be used in place of the former for estimating sampling time.

In vivo Bacterial Counts

The average of viable bacterial Log numbers obtained from the cecal contents collections of the 3 randomly selected chicks were determined. Counts were made on MacConkey agar ($\leq 2X10^2$ cfu/ml); no other bacterial cells were detected indicating purity and absence of contamination. The counts were very high as expected. No anaerobic bacteria are expected in birds at this age.

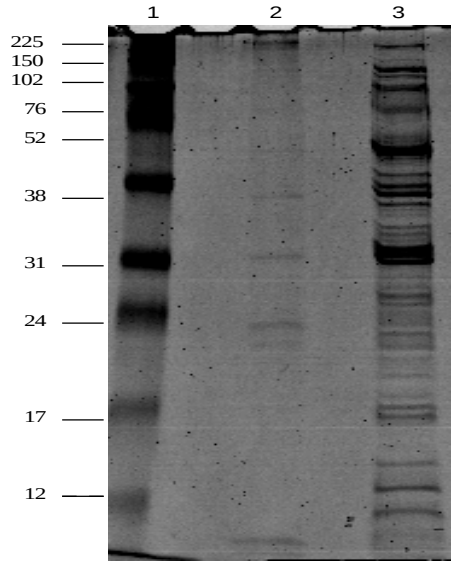
Table 1. The Viable Bacterial Numbers Obtained from the 3 Randomly Selected Birds During the Days of Chicken Cecal Contents Collections of all Experiments

Experiment	Total number of hatched eggs	Mean of bacterial count cfu/ml in 3 birds randomly selected	Mean of bacterial count cfu/ml of all experiments
1	72	3.1x1010	5.28x109
11	77	8.56x109	
111	62	5.78x109	
1v	76	5.28x109	

Comparison of *in vivo* and *in vitro* Protein Profile of *S. Enteritidis*

There was a great difference between the protein profiles of *S. Enteritidis* harvested from chicken ceca and that of *in-vitro* grown bacteria (Fig 1). Around 40 protein bands ranging from high to low molecular weights from the *in vitro Salmonella* protein extract could be clearly seen in SDS-PAGE stained with Colloidal Coomassie blue stain, whilst fewer than

five bands were visualized for the proteins obtained from *S. Enteritidis* harvested from chicken ceca. This was repeated three times and all results were similar suggesting that *S. Enteritidis* protein extracts harvested from chickens might have been exposed to some kind of degradation.

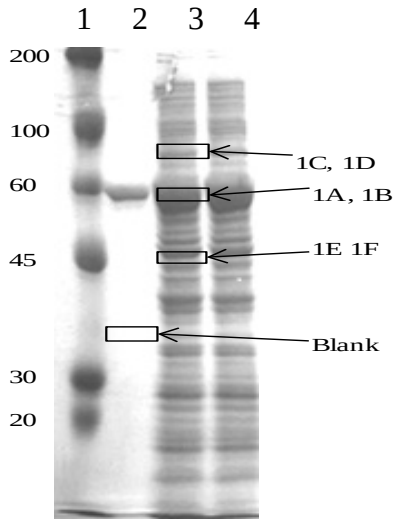


"Figure 1. SDS-PAGE Protein Profiles of *Enteritidis* Whole Cell Sonicated from *in vivo* and *in vitro* Cultures Stained with Colloidal Coomassie blue.1, Molecular Weight Marker. 2, *Salmonella* Harvested from Chicken Cecal Contents. 3,*Salmonella* Harvested from Nutrient Broth Culture".

Identification of *in vivo* and *in vitro Salmonella* Proteins

Use of SDS- PAGE bands for separation of protein mixtures is a successful tool in assessing protein size and purity. Several experiments to identify proteins present were performed and are as following:

In the initial experiment a few bands were cut from SDS-PAGE of *in-vitro* preparation of S. *Enteritidis* (Fig 1). Stationary phase NB cultures were used in contrast to mid log phase cultures to ensure that sufficient protein was available. In table 2. Bands 1A and 1B were thought likely to be flagella judging from their abundance and apparent molecular weight on SDS-PAGE, the results were as expected as these bands (1A, 1B), contained *S. Enteritidis* flagellin with observed molecular weight of ~52 kDa with mascot score 204 and 5 matched peptides, in addition to *S. Typhimurium* flagellin of molecular weight ~51 kDa with a lower score of 77, coverage of 7% and only 2 matched .peptides. Band1C contained a ~60 kDa chaperonin from the sequenced strain *S. Enteritidis* PT4 P125109 with a relatively low coverage (12% of the protein length). Some other bacterial proteins matching the same set of peptides were present. B and 1E contained glyceraldehyde-3-phosphate dehydrogenase of *S. Typhimurium* (~35.5kDa), plus other proteins of other bacteria including some other strains of *Salmonella* matching the same set of peptides. The blank was clear as expected, as it has been cut from clear gel



"Figure 2. Pierce Colloidal Coomassie Blue Stained Gel of *S .Enteritidis in vitro* Proteins Showing the Three Proteins Bands Used for the Evaluation of Use of Mass Spectrometry for Protein Analysis and Identification, Plus the Blank. Lane 1= MW Marker, 2=Blank, 3&4= in *vitro* Protein.

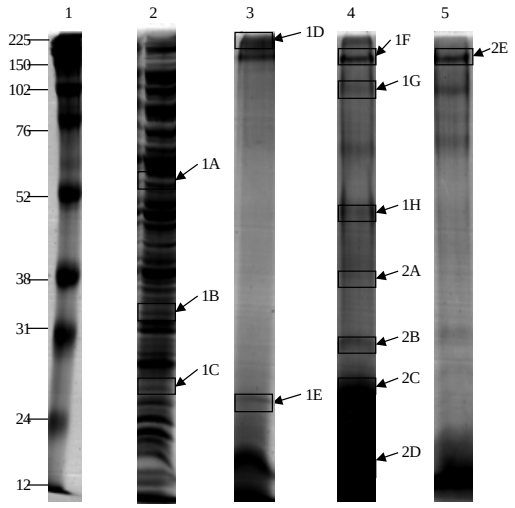
"Table 2. Proteins Detected in Bands 1A, 1C and 1E with their MASCOT Score and Predicted Mass Using the Web Version of the MASCOT MS/MS Ions Search Tool".

sample	Swiss Port Entry	Protein/ Gene name	Protein mass kDa	Sequence coverage	Mascot score	No, of peptides
1A	FLIC SAEN	Flagellin OS=Ss. Enteritidis/*fliC*	52950	16%	204	5
	FLIC SALTY	Flagellin OS=Typhimurium/*fliC*	51581	7%	77	2
1C	CH 60	60 kDa chaperonin OS=S Enteritidis PT4(P125109) /groL Glyceraldehyde-3-phosphate dehydrogenase of S	57250	12%	314	4 4
1E	G3P1	Typhimurium/*gapA*	35564	20%	353	

Notes:

♦ Generally, a minimum of two peptides per protein was a requirement for a positive identification.
♦ Predicted MW = Predicted MW (Da) of the protein sequence from the database entry.
♦ Observed MW = Molecular weight estimated from migration on SDS-PAGE.
♦ MASCOT score =MASCOT score associated with protein identification.
♦ Number of peptides = Number of peptides associated with protein identification by MASCOT

In another experiment (expt11) 3 protein bands were cut from SDS-PAGE and analysed by MS including the blank (plain or clear gel), each band was designated as illustrated in Figure 2. The proteins identified in sample 1A were enolase of *S. Enteritidis* PT4 (P125109) observed molecular weight of ~45.5 kDa and mascot score of 89, plus citrate synthase ~48 kDa of *S. Typhimurium* with a relatively lower score and coverage. Three *S. Enteritidis* PT4 (P125109) proteins were found in band 1B, (ProP effector (proQ) of (~25.5 kDa), transcriptional regulator (kdgR) of (~30.5 kDa), with two matched peptides for each and similar coverage and Mascot score in addition to D-alanyl-D-alanine dipeptidase of *Salmonella* (pdgL).In contrast, no significant hits were reported in sample B2. Sample 1C contained flavoproin (wrbA) of *Enteritidis* PT4 (125109) with a molecular weight of ~ 21.0 kDa with a relatively high score (119) and two matched peptides, additionally, gllutatthionine S-transferase of *Typhimurium* (gst) was found to be present in band C1. F2 was cut from a clear gel and considered as blank therefore it is clear.



"Figure 3. SDS-PAGE of *S. Enteritidis in vitro* and *in vivo* Protein Preparations Showing Bands Which were Selected and Identified by Mass Spectrometry (MS Experiment II), lane 1, Molecular Weight Marker, Lane 2 in *vitro* Preparations, Lane 3, 4 and 5 are *in vivo* Preparations

Discussion

The principle of performing a bacterial growth curve is to determine the mid-logarithmic point, at which period the bacterial cells are active, viable, and show their typical characteristics. There were no major problems associated with the growth curve. The mid-log phase point was established by viable counting and verified by optical density measurement (OD_{600}). It was decided to carry out *in vivo* infection experiments using one -day old chicks primarily to avoid the development of intestinal microflora, which would be likely to have a significant effect on interference in interpreting the patterns of protein expression in *S. Enteritidis* as well as to enable the bacterium of interest (*S. E*nteritidis) to multiply extremely well in the absence of competitive colonizers (Barrow et al., 1987; Barrow et al., 1988a).. A protein analysis of *S. Enteritidis* in the ceca of one -day old chicks (*in- vivo*) together with a comparison with nutrient broth medium (*in- vitro*) was used to detect changes in the pattern of protein expression during infection and in particular to identify proteins that enable this strain to colonize the ceca. It was decided to use *S. Enteritidis* proteins extracted from stationary phase as the protein concentration of mid log phase was very low. Proteomic analysis has proved to be a reliable and reproducible approach to analysis of microbial behavior in different environments in comparison with more established methods (Cordwell, 2006, Hecker and Volker, 2004, Volker and Hecker, 2005, Cash, 2003, Brotz-Oesterhelt et al., 2005)

We are therefore reasonably confident that with the method that we were using sufficient changes of interest would be detected. Unfortunately, because of absence of the majority of *in- vivo* bands (just very few bands can be visualized in Coomassie blue stained SDS-PAGE (Figure 1), compared with more than 40 bands of *in- vitro* preparations, it was difficult to perform a reasonable comparison between the two protein profiles. Mass spectrometry (MS) was the method used for our protein identification as it is the method of choice for that (Blackstock and Weir, 1999). The MS data were used to search publicity available sequence data base using the MS/MS Ion search tool. In this, identification is based on the fragment peptide masses of the unprocessed MS/MS data from one or more peptides matching the equivalent

data predicted from database entries (http://www.matrixscience.com/search_form_select.html). The preliminary exploratory study of individual bands identified major proteins (flagellin of *S. Enteritidis* and *Typhimurium fliC*) and mixtures of proteins including 60 kDa chaperonin *groEL* and glyceraldehyde-3-phosphate dehydrogenase *gapA*. We were therefore confident that the method chosen would be sufficient to characterize the potentially complex mixtures present in SDS-PAGE separated proteins from the *in vivo* and *in vitro* cultures.

Some proteins of significance may be expressed equally both *in- vivo* and *in -vitro* (e.g. fimbrial – flagellar, outer membrane protein, metabolic –, regulatory – and LPS-synthesis encoded genes). These proteins were predicted to play a major role in colonization. And indeed, changes in proteins (and other molecules) can occur during sample extraction and preparation e.g. protease mediated degradation.

In the current study *S. Enteritidis* fimbrial 14 (sefA), was identified from the *in- vivo* preparation suggesting a role in cecal colonization. Moreover, fimbrial proteins SEF14, SEF 17 and SEF 21 have been shown to mediate *Salmonella* (Muller et al., 1991, Collinson et al., 1993). In addition, Turner et al., (1998) and Morgan et al., (2004) indicated that a number of metabolic changes are involved and also response to stresses including high temperature. Therefore, proteins of *S. Enteritidis* which we might expect to be up-regulated in the ceca for colonization include those which encode T3SS of SPI-1 & SPI-2, LPS biosynthesis, flagella (motility & chemotaxis), fimbria and outer membrane-proteins in addition to those responsible for metabolic and physiological changes in the intestine. (Berndt et al., 2007) Any differences seen in *in vivo* may relate to differences in environmental conditions between *in-vitro* culture and the chicken intestine. These conditions include a higher temperature (chicken body temperature is 41.5^{o}C), low oxygen tension and different nutrient supply. This latter point may relate specifically to the high concentration of yolk proteins and different levels of phosphate, sulphates, nitrogen, calcium, and iron in the intestine as a result of the incomplete re-absorption of the yolk in the newly hatched chick.

In our results at least one fimbrial protein sefA (*S. Enteritidis* fimbrial 14 - sef14) was identified from the *in vivo* preparations, suggesting a role in chicken colonization; however, previous microarray work by Elazomi together with colleagues (Dhawi et al 2011), showed that most of fimbrial associated genes were down regulated in chicken gut and only few fimbrial genes were up-regulated including (SEF21) and other fimbrial subunit genes encoded by *pegA*, *stdA*, *lpfA* (SEF14), so that *fimA*, *pegA*, *stdA* and *lpfA* genes might be involved in cecal colonization, sefA was not expressed or was equally expressed *in vitro* and in the gut (Dhawi et al., 2011). This participation is thought to be via physical attachment of *Salmonella* to host mucosal surface and epithelial cells (Gophna et al., 2001, Edelman et al. 2003, Morgan et al., 2004, Snyder et al., 2004). However, previous research conducted by Allen-Vercoe and Woodward had supported the array work and indicated that fimbriae were not important in cecal colonization (Allen-Vercoe and Woodward, 1999). Other previous research has also suggested some involvement of SEF21, SEF17 and flagella, and reported that SEF14 is not implicated in the early stages of chicken colonization by *S. Enteritidis* (Dibb-Fuller and Woodward, 2000). Cogan and others have also reported that SEF21, SEF17 are involved in the colonization of chicken reproductive tract and egg contamination (Cogan et al., 2004). It has been reported that most fimbrial subunit of *Enteritidis* (phage type 4) are not implicated in chicken colonization (Clayton et al., 2008).wo proteins involved in lipid modification were identified, major outer membrane lipoprotein (lpp), and major outer membrane lipoprotein 1 (lpp1) which might be involved in gut colonization. In contrast with array work lpp is also upregulated at. genome level in chicken, while IPPI is neither up regulated nor down regulated or might be equally expressed in chicken and broth culture or not expressed in both (Dhawi et al., 2011) Lpp is essential to maintain the integrity of the bacterial envelope, (Cohen and Glauser, 1991, Braun and Hantke, 1974), and has a role in the virulence of *Salmonella* . Moreover, Lpp is essential to maintain the integrity of the bacterial envelope, and considered as most abundant component of the outer membrane (Cohen and Glauser, 1991, Braun and Hantke, 1974) and has a role in the virulence of *Salmonella* and induction of systemic infections (Sha et al., 2004). Other work suggested that lipoprotein may play a role in the induction of cytokine production and pathologic changes associated with gram negative bacterial infection.(Zhang et al., 1998).

Enolase (eno), which plays a role in gluconeogenesis and citrate synthase (*gltA*) which is involved in cellular carbohydrate metabolism TAC were identified from *in- vitro* preparation of *S. Enteritidis* .Some proteins involved in transcription and signal transduction mechanisms were also detected in *S. Enteritidis in-vitro* protein preparations including transcriptional regulator (*kdgR*), DNA-directed RNA polymerase subunit beta of *S. Enteritidis* (*rpoB*), transcription elongation protein (*nusA*), Flavoprotein of *S. Enteritidis* (*wrbA*), and transcriptional regulatory proteins, regulator of virulence determinants (*phoP*), with exception of *phoP* which was clearly down regulated in gut, all others might not be expressed or equally

expressed in chicken ceca compared with broth culture (Dhawi et al., 2011).

Conclusions

Due to the importance of *S Enteritidis as* the main cause of human food poisoning through contamination of the food chain, it was found worthwhile to investigate its colonization to the chicken ceca.

The main purpose of this study was to identify proteins associated with colonization of chicken ceca by *Salmonella* using protein technology.

Proteomic analysis has proved to be a reliable and reproducible approach to analysis of *Salmonella* protein.

Mass spectrometry is the method of choice for protein identification; it characterizes the potentially complex mixtures present in SDS-PAGE separated proteins from *in vivo* and *in vitro* cultures.

The result of this study revealed that the whole-cell profile of *S Enteritidis* harvested from the chicken ceca was very different to that of bacteria cultured in nutrient broth. In SDS- PAGE, despite the fact that the amount of protein loaded was the same for both *in vitro* and *in vivo* extracts more than 30 bands were observed for *in vitro* protein extract compared to only 3-4 bands for the *in vivo* one suggesting that most are degraded and disappeared off the gel.

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