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Generation of an inactivated vaccine for avian pathogenic *Escherichia coli* using microarrays: A more rational approach to inactivated vaccine design

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Abstract

Background: *Escherichia coli* remains a major pathogen of poultry. Most vaccines are inactivated and produced empirically. Although inactivated *Salmonella* vaccines have been produced by culture under conditions of Fe deprivation, no vaccines have been produced which are likely to express all the proteins expressed during infection of antigen-presenting cells.

Aim: The aim was to produce a more protective inactivated vaccine by culturing the avian *E. coli* in a synthetic medium that resembled the environment of the phagolysosome.

Methods: Global gene expression in a pathogenic avian O78:K80 strain of *E. coli*, harvested from infected avian macrophage-like HD11 cells, was compared by microarray with bacteria cultured in a tissue culture medium. A liquid synthetic medium was produced based on the environmental conditions identified to which the bacteria were exposed intracellularly. A bacterin was produced from this strain and its protective ability was assessed in chickens.

Results: The changes in *E. coli* gene expression observed included the use of different electron acceptors and carbon sources such as ethanolamine, β-glucosides, galactonate, dicarboxylic acids, and amino acids, up-regulation of genes associated with Fe and Mn uptake, and up-regulation of type-1 and curli fimbriae, other adhesion genes and down-regulation of sialic acid synthesis genes. The bacterin produced in the synthetic medium was statistically more protective than a bacterin prepared from bacteria cultured in the nutrient broth when tested in vaccinated chickens challenged with a different virulent *E. coli* O78:K80 strain.

Conclusion: The approach of using gene expression to produce synthetic media for the generation of more effective bacterins could be used for a number of intracellular bacteria pathogens including Enteroinvasive *E. coli*, *Salmonella*, and the *Pasteurella/Riemerella/Mannheimia* group of organisms.

Keywords: Microarray, Vaccine, Chicken, Gene expression, Synthetic medium.

Introduction

Avian pathogenic *Escherichia coli* (APEC) is a major poultry pathogen causing septicemia and polyserositis in the immediate post-hatching period (Nolan *et al.*, 2013) or following infectious bronchitis (Dho-Moulin and Fairbrother, 1999) or turkey rhinotracheitis metapneumovirus infection (Picault *et al.*, 1987).

As a taxon, APEC comprises related clusters of strains expressing a range of virulence determinants associated with individual syndromes (Gyles and Fairbrother, 2010). No single combination of virulence determinants characterizes virulence. Identified determinants include individual fimbriae and other adhesins and invasins, serum resistance factors, and toxins (Nolan *et al.*, 2013). The availability of several annotated genome sequences (Johnson *et al*., 2007; Dziva and Stevens, 2008; Dziva *et al.*, 2013; Mangiamele *et al.*, 2013), has facilitated the identification of new candidate virulence genes. Some serotypes are associated with both avian and human infection (Johnson *et al*., 2007).

Although septicemia is characteristic of many poultry infections, the highest numbers of bacteria are isolated from the spleen, in addition to other organs, most probably located within macrophages (Smith *et al*., 1985; Barrow *et al*., 1998). The microbial behavior in

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this site and within antigen-presenting cells (APCs) is also important in initiating the immune response.

Control of infection by chemotherapy inevitably selects for antibiotic resistance, especially in countries where regulation is less stringent (Wang *et al.*, 2010). Inactivated vaccines (bacterins) are generally regarded as being less effective than live, attenuated vaccines because the latter stimulates both humoral and cellular immunity. However, issues including the use of genetically manipulated vaccines suggest that inactivated vaccines will be used for the foreseeable future. Attempts have been made to develop more rational inactivated bacterial vaccines (Woodward *et al.*, 2002) by culturing the bacteria under conditions of iron restriction. However, studies with *Salmonella typhimurium* infecting mouse macrophages (Eriksson *et al.*, 2003) and with avian serovars in chicken macrophages (Imre *et al*., 2013) indicate that Fe restriction is not the only feature characterizing the intracellular environment.

We hypothesized that an analysis of the conditions inside macrophages using data generated by bacterial transcriptional analysis could lead to the development of a medium reproducing those conditions more accurately. A bacterin produced in this way should more closely resemble bacteria within macrophages and other APCs antigenically than bacteria cultured in nutrient broth (NB) and should be more protective. The Objective was therefore to infect the avian macrophage-like cell line HD11 with an APEC strain, carry out transcriptional analysis on the harvested bacteria by microarray and identify from the pattern of gene expression the environmental conditions to which the bacteria are subjected intracellularly. These conditions would be reproduced in a synthetic medium to culture the bacteria which would then be evaluated as a bacterin in chickens challenged with a different APEC strain.

Material and Methods

Bacterial strains and culture

Escherichia coli O78:K80 strains F31 and F135 were isolated from cases of avian colibacillosis. Both are virulent for chickens when inoculated parenterally (Barrow, unpublished results; Smith *et al*., 1985). NB (Oxoid, CM67) cultures were incubated at 37°C for 24 hours. These contained between 1 and 3×10^9 cfu/ml. Bacterial enumeration was made using the method of Miles *et al*. (1938) culturing on MacConkey agar plates (Oxoid CM0007).

Cell culture and in vitro infection model

Avian macrophage-like HD11 cells (Beug *et al.*, 1979) were grown in RPMI-1640 medium supplemented with 20 mM L-glutamine (Gibco), 2.5% fetal calf serum (Gibco), 2.5% chicken serum (Sigma) and 10% tryptose phosphate broth (Sigma). For each bacterial infection, a total of 3×10^7 HD11 cells were seeded in each of three tissue culture flasks (175 cm^2, Nunc).

Overnight NB cultures of bacterial strain F31 were diluted 20-fold in the cell culture medium described above and grown statically for 2 hours (5% CO_2), then added onto the HD11 cells at a Multiplicity of Infection of ≈ 100. After co-incubation for 1 hour under the above conditions, the medium was replaced with a medium supplemented with 100 µg/ml gentamicin (Gm, Gibco). After the first 1 hour incubation, the medium was replaced with one containing 15 µg/ml Gm. Sampling points were at 0, 4, 8, 12, 24 and 48 hours post-infection. Cells were lysed (Barrow and Lovell, 1989) for bacterial enumeration.

RNA extraction and processing

The RNA extraction protocol for F31 bacteria extracted from the infected cells and broth culture was that of Eriksson *et al*. (2003) and used by Imre *et al*. (2013). At 4 and 8 hours after infection, cells were lysed with 0.1% SDS, 1% phenol, and 19% ethanol in water for 30 minutes on ice. Bacteria were collected by centrifugation (5,000 g, 10 minutes, 4°C), treated with protective lysozyme and Proteinase K, and total RNA was prepared using the RNeasy Mini Kit (Qiagen). Bacterial RNA was also harvested from bacteria cultured in RPMI for 2 hours, which had been inoculated with a 20-fold dilution from the overnight NB culture, to produce bacterial numbers similar to those applied to the cell monolayers. RNA from bacteria grown in a cell culture medium was isolated using the same RNA purification kit. The quality of bacterial RNA and host RNA contamination was checked by a 2100 Bioanalyzer (Agilent). The RNA was amplified using the MessageAmp™ II-Bacteria Kit (Ambion), resulting in aminoallyl-UTP labeled amplified RNA (aRNA). For labeling, 6 µg aliquots of the aRNA samples were coupled with the fluorescent dyes Cy3 and Cy5 (Amersham). Cy3 was coupled to *in vitro* control RNA, while Cy5 was used to label bacterial RNA extracted from macrophages.

Microarray design and data analysis

The sequence used for the array design was the APEC O1:K1:H7 reference strain (NC_008563) (Johnson *et al.*, 2007). All predicted ORFs were designated for probe design. The web-based Agilent eArray system (Agilent Technologies, https://earray.chem.agilent.com/earray/) was used with the following settings during the microarray probe design: Tm (70°C) matching methodology, 60-mer probe length, 3 probes/target. The protocol, experimental setup, RNA extraction, amplification, labelling, and hybridization are described in detail at http://www.ebi.ac.uk/arrayexpress/. Data analysis was done using GeneSpring GX 10.0 (Agilent).

The ultimate purpose of this analysis was to develop a synthetic medium reproducing these conditions as near as possible. For this reason, a full analysis of gene expression was not carried out by COGs gene classes. They were grouped according to genes likely to affect intracellular survival, response to stress, and virulence gene expression as indicated below.

Synthetic Macrophage Medium (SMM-1)

The composition of the SMM-1 was: 100 mM Tris-Cl, pH 5.0, 2 mM D-glucose, 2 mM D(+)-galactose, 20 mM glycerol, 20 mM glycerol-3-phosphate, 20 mg/; L-valine, 50 mg/l L-leucine, 50 mg/l L-isoleucine, 65 mg/l L-cysteine 2HCl, 5 mg/l L-tryptophan, 1 mM $MgCl_2$, 200 uM 2,2-dipyridyl, 500 uM $Cacl_2$, 3 mM $ZnSO_4$, 50 mM $K2SO_4$, 100 mM NaCl, 7.5 mM $(NH_4)_2SO_4$ in 100 ml ultrapure water. The pH was adjusted to 5 with hydrochloric acid and the final medium filter sterilized.

Bacterin production

A 5 ml aliquot of an overnight NB culture of *E. coli* F31 was added to 95 ml RPMI1640 medium (Invitrogen Ltd., Paisley, UK) pre-warmed to 37°C. This was incubated statically for 2 hours at 37°C in 5% CO_2. Bacterial cells were recovered and washed twice in fresh SMM-1 medium or NB before being resuspended in fresh SMM-1 or NB respectively. These cultures were incubated statically at 37°C under aerobic conditions for 4 hours and the viable counts were estimated on nutrient agar (NA). A 0.2 ml aliquot of 40% formalin was added to 10 ml of the SMM-1 and NB cultures and allowed to stand for approximately 12 hours at ambient temperature followed by a 24 hours period at 4°C.

Both formalinised preparations were centrifuged at 1,500 g for 30 minutes and both pellets were resuspended separately in 10 ml PBS. 100 µl aliquots were removed and cultured in NB (37°C, overnight) to determine sterility. These formalin-treated cell suspensions were adjusted to a density equivalent to ~1 × 10^9 CFU/ml before use as vaccine.

Vaccination studies

Three groups (A, B, and C) of 25 1-day-old Hy-Line layers were housed in separate rooms on solid floors with shavings. At 4 and 18 days of age groups, A and B were inoculated with the bacterins without adjuvant. Birds were vaccinated simultaneously orally (100 µl with a blunt-ended needle) and intra-muscularly (100, 50 µl into each breast muscle), in the case of group A, with the SMM-1 bacterin, group B with the NB bacterin and group C with PBS. At 29 days of age, all birds were challenged intravenously with 1 × 10^5 cfu of a 24 hours NB culture of an O78:K80 *E. coli* strain F135. At 30, 31, 33 days of age, 5 birds, and at 36 days, 10 birds were killed from each group and the numbers of the inoculated challenge strain in homogenized samples of liver, spleen, and blood were counted on MacConkey agar. The protective effect was measured as reductions in the severity of frequency of morbidity if these occurred or by reductions in bacterial numbers in the blood, liver, and spleen.

Ethical approval

Animal experiments were carried out under a UK government Home Office Project and Personal Licence held by Prof. Barrow and reviewed internally by the University of Nottingham Animal Welfare and Ethical Review Body prior to initiation of the work.

Results

The viable numbers of the F31 strain in the cultured HD11 cells fell from Log_{10} 3.8 at 4 hours to 3.7 at 12 hours. RNA was therefore recovered 8 hours post-infection.

Transcriptional profile of bacteria harvested from HD11 cells

The patterns of gene expression of strain F31 in the intra-macrophage environment 8 hours after infection and grouped by activity, are shown in Tables 1 and 2. Genes showing statistically significant increases in expression of more than 10-fold are shown in Table 1. A more limited selection of genes of microbiological interest that were similarly significantly down-regulated is shown in Table 2. The ultimate purpose of this analysis was to develop a synthetic medium reproducing these conditions as near as possible. For this reason, a full analysis of gene expression was not carried out by COGs gene classes. They were grouped according to genes likely to affect intracellular survival, response to stress and virulence gene expression as indicated below (i – vi).

(i) Electron transport. Upregulation of putative oxido-reductases and dehydrogenases coincided with downregulation of nitrate and nitrite reductases including *nirBD* (nitrite reductase), *narGHI* (nitrate reductase), and *napAC* (nitrate reductase with additional functions). Pyruvate kinase I (*pykF*) and formate dehydrogenase sub-unit I (*fdnG*) were also down-regulated. This suggested that the macrophage phagolysosome environment had a relatively high redox value as indicated by Turner *et al*. (2003).

(ii) Carbon sources. A number of different loci associated with carbon sources utilized by the bacteria showed changes in expression although there was no clear picture. There was evidence that dicarboxylates were utilised as carbon sources suggested by up-regulation of the *dcuB* transporter gene and a diacid regulator *cdaR*. The *gntR* gene which regulates the *gnt1* operon associated with gluconate utilization was up-regulated. The involvement of gluconate was emphasised by the up-regulation of *idnK*, a gluconate kinase. Expression of *glcC* was up-regulated. This is an activator of the *glc* operon which is associated with the transformation of glycolate to glyoxalate, and therefore may also be associated with osmotic stress rather than a prime carbon source (Núñez *et al.*, 2001). Its regulation requires Integration Host Factor which affects virulence and responses to stressful conditions. Glyoxalate is also an important intermediate in the glyoxalate bypass involved in acetate or fatty acids as major energy sources (Pellicer *et al.*, 1999). Other carbohydrates such as ethanolamine, mannose, and xylan may also have been important although the evidence through up-regulation of single genes *eutA*, *gmd,* and *yieL*, respectively was not strong as indicated by low changes in gene expression.

Table 1. Changes in gene expression in *E. coli* F31 cultured in HD11 cells measured by microarray which were up-regulated by greater than 10-fold after 8 hours culture in HD11 cells.

Gene class	Gene	Function or product	Mean change in expression 8 hours
Electron transport	*nadA*	Quinolinate synthetase A	13.65
	norV	Flavorubredoxin oxidoreductase	35.2
	sdhA	Succinate dehydrogenase flavoprotein subunit	11.17
	ycdW	Putative dehydrogenase	21.26
	ydiR	Predicted electron transfer flavoprotein, FAD-binding	12.13
	ydjG	Putative oxidoreductase	13.57
	yehL	Putative ATPase	14.96
	ygcU	Putative FAD containing dehydrogenase	11.41
	-	Putative dihydrolipoamide dehydrogenase	10.68
Carbon sources	*astA*	Arginine N-succinyltransferase	11.69
	atoA	Acetyl-CoA: acetoacetyl-CoA transferase, beta subunit	10.79
	bglH	Carbohydrate-specific outer membrane porin, cryptic	19
	cdaR	Carbohydrate diacid regulator	14.79
	dadX	Alanine racemase 2, catabolic	15.39
	dcuB	Anaerobic C4-dicarboxylate transporter DcuB	19.76
	dgoD	2-oxo-3-deoxygalactonate 6-phosphate aldolase/galactonate dehydratase	13.05
	eutA	Ethanolamine utilization protein EutA	15.23
	gclR	Putative regulatory protein GclR	13.01
	glcC	Glc operon transcriptional activator	14
	gltA	Citrate synthase	13.18
	gmd	GDP-mannose 4,6-dehydratase	13.11
	idnK	Thermosensitive gluconokinase	10.84
	metF	5,10-methylenetetrahydrofolate reductase	11.66
	rbsA	Putative ribose transport ATP-binding protein RbsA	13.98
	-	Putative hexuronate transporter	15.61
	wzb	Tyrosine phosphatase	13.49
	xapR	Santhosine operon regulatory protein	12.77
	ybbA	Putative ABC-type transport protein YbbA	14.75
	ydjH	Putative sugar kinase	10.84
	ygfT	Putative NADPH-dependent glutamate synthase beta chain	18.55
	ygjK	Putative glycosyl hydrolase	13.39
	yieL	Putative xylanase	13.72
	yphF	ABC-type sugar transport system, periplasmic component YphF	15.39
	-	Putative regulatory protein, GntR family	20.87
	-	Putative lactate dehydrogenase	10.93

Continued

Gene class	Gene	Function or product	Mean change in expression 8 hours
Ion and other transport	*cniT*	Putative carnitine transporter CniT	11.34
	cysA	Sulfate transport ATP-binding protein CysA	11.56
	cysW	Sulfate/thiosulfate transporter subunit CysW	10.66
	entS	EntS/YbdA MFS transporter	10.33
	fepC	Ferric enterobactin transport ATP-binding protein FepC	13.5
	fes	Enterochelin esterase	12.13
	livJ	Leu/Ile/Val-binding protein precursor	12.22
	mglC	Galactoside transport system permease protein MglC	23.74
	mntH	Manganese transport protein MntH	18.8
	nhaA	Na+/H antiporter, pH dependent	10.86
	sbp	Periplasmic sulfate-binding protein	10.39
	tauC	Taurine transport system permease protein TauC	19.85
	ybtU	Yersiniabactin biosynthetic protein	17.66
	ydjE	Putative metabolite transport protein	13.9
	yejB	Predicted oligopeptide transporter subunit	10.29
Key regulatory functions	*bglJ*	BglJ 2-component transcriptional regulator	17.08
	rseA	Sigma-E factor, negative regulatory protein	12.6
	ycdT	Predicted diguanylate cyclase	22.83
	ygaA	Putative sigma-54-dependent transcriptional regulator YgaA	23.69
Surface and virulence functions	*bfr*	Bacterioferritin, iron storage and detoxification protein Bfr	17.69
	csgE	Assembly /transport component in curli production	11.24
	eaeH	Attaching and effacing protein, pathogenesis factor EaeH	12.16
	fimD	Outer membrane usher protein FimD precursor	13.79
	sinH	Putative intimin/invasin (SinH-like protein)	17.4
	yfcV	Putative Yfc fimbriae subunit YfcV precursor	19.6
	-	FimF protein precursor	11.67
	wcaF	Putative colanic acid biosynthesis acetyltransferase	11.11
	yohK	Putative seritonin transporter	10.25
	betB	Betaine aldehyde dehydrogenase, NAD-dependent	10.58
Stress responses	*betI*	Regulatory protein BetI	13.57
	cpxP	Periplasmic repressor of cpx regulon by interaction with CpxA	28.27
	htrA	Periplasmic serine protease Do, heat shock protein HtrA	19.53
	osmC	Osmotically inducible protein OsmC	13.14
	proP	Proline/betaine transporter	15.89
	pspA	Phage shock protein A	28.09
	pspE	Phage shock protein E precursor	20.53

Continued

Gene class	Gene	Function or product	Mean change in expression 8 hours
Stress responses	*recA*	DNA strand exchange and recombination protein with protease and nuclease activity	11.57
	recN	DNA recombination and repair protein RecN	14.4
	recX	Regulatory protein for RecA	12.03
	rseA	Sigma-E factor, negative regulatory protein	12.6
	rspA	Starvation sensing protein RspA	10.89
	umuC	Mutagenesis and repair protein UmuC	33.2
	umuD	Mutagenesis and repair protein UmuD	17.42
	-	Putative phage lysin	54.63
	ygaA	Putative sigma-54-dependent transcriptional regulator YgaA	23.68
Miscellaneous	*marA*	Multiple antibiotic resistance protein MarA	12.75
	psiA	PsiA—plasmid SOS inhibition	10.72
	traN	TraN—mating pair stabilisation protein	12.8
	traR	TraR—quorum sensing and plasmids	14.39
	traW	TraW—transfer pilus assembly F plasmid	15.02

(iii) Transport. Many of the transport genes up-regulated in macrophages were associated with the uptake of carbohydrates and other potential carbon sources. These included galactoside transport permease (*mglC*), a C4-dicarboxylate binding protein (*dcuB*, mentioned above), a putative hexuronate transporter, and transport proteins for oligopeptides (*yejB*), and long-chain fatty acids (*fadL*). Up-regulation of genes encoding ion transport proteins for sulfate (*cysA, cysW, sbp, tauC*), manganese (*mntH*), and ferric iron (*fepC, entS, fes, ybtU*), and iron storage (*bfr*) indicated restriction for these ions.

(iv) Stress. In comparison with growth in NB, the bacteria were subjected to higher osmotic stress as indicated by a number of genes related to the biosynthesis of betaine (*betAB*), its regulator (*betL*), *proP* which transports both proline and betaine and *osmC*. The heat stress-related proteins *htrA* and *cpxP* were up-regulated. Stresses to DNA are indicated by up-regulation of *recA* and its regulator *recX*, *recN,* and *umuC,* and *umuD*. This is probably also linked to increased prophage activity with a number of genes up-regulated including *pspA* and *pspE*, a prophage integrase, *intD* and a putative phage lysin.

(v) Surface and virulence proteins. The major virulence genes up-regulated were associated with fimbriae elaboration (*csgE, fimD*, *fimF*, and *yfcV*) and adhesins/invasins (*eaeH* and *sinH*). One of the colanic acid biosynthesis genes *wcaF* was up-regulated. These changes were accompanied by down-regulation of genes associated with sialic acid capsule formation. Genes encoding surface porins OmpF, NmpC, and the OmpN precursor were also down-regulated.

Confirmation of levels of gene expression by qRT-PCR was not carried out. Although there are some limitations in the range of signal intensity obtainable with microarrays, most publications show very good correlation between levels of expression measured by microarray and by qRT-PCR or other quantitative methods, including *E. coli in vitro* (Richmond *et al.*, 1999), Enterohaemorrhagic *E. coli* in contact with rabbit cells (Dahan *et al.*, 2004), *S. typhimurium* in murine macrophages (Eriksson *et al.*, 2003), in human and canine epithelial cells (Hautefort *et al.*, 2008), the chicken intestine (Dhawi *et al.*, 2011; Harvey *et al.*, 2011) and with four different *Salmonella enterica* serovars in HD11 cells (Imre *et al.*, 2013).

Synthetic medium and vaccine development

The microarray results showed changes in gene expression indicative of availability of gluconate, succinate, and cysteine with Fe and Mn restriction and osmotic and heat stress, although the preferred carbon source was unclear. This information combined with published literature, indicating low phagosome pH, (Rathman *et al.*, 1996), was used to develop a synthetic medium (SMM-1), the full composition which is described in Materials and Methods. This was used to generate a bacterin of *E. coli* F31 for vaccination studies.

Vaccination experiment

The protective effects of the F31 bacterin produced by culture in medium SMM-1 against intravenous challenge of an O78:K80 strain F135 compared to those induced by a bacterin prepared using the same strain cultured in NB are shown in Table 3.

Table 2. Changes in gene expression in *E. coli* F31 cultured in HD11 cells measured by microarray which were down-regulated by greater than 10-fold after 8 hours culture in HD11 cells.

Gene class	Gene	Function or product	Mean change in expression 8 hours
Electron transport and metabolism	*fdnG*	FdnG, alpha subunit of formate dehydrogenase-N	13.92
	napA	Periplasmic nitrate reductase precursor	20.72
	napC	Nitrate reductase, cytochrome c-type, periplasmic	15.05
	narG	Nitrate reductase 1, alpha subunit	16.09
	narH	Nitrate reductase 1, beta (Fe-S) subunit	10.38
	narI	Respiratory nitrate reductase 1 gamma chain	11.15
	nirB	Nitrite reductase, large subunit, NAD(P)H-binding	17.43
	nirD	Nitrite reductase (NAD(P)H) subunit	20.94
	pykF	Pyruvate kinase 1	15.72
Surface and virulence functions	*kpsC*	Capsule polysaccharide export protein KpsC	13.53
	kpsM	Polysialic acid transport protein KpsM	14.68
	kpsS	Polysialic acid capsule synthesis protein KpsS	12.56
	kpsT	Polysialic acid transport ATP-binding protein KpsT	16.01
	neuA	Acylneuraminate cytidylyltransferase	50.32
	neuB	Sialic acid synthase NeuB	24.04
	neuC	Polysialic acid biosynthesis protein	45.91
	neuD	Sialic acid synthase NeuD	32.51
	neuS	Poly-alpha-2,8 sialosyl sialyltransferase NeuS	42.5
	nmpC	NmpC precursor	41.75
	ompF	OmpF	38.29
	ompN	Outer membrane protein N precursor	34.26

None of the birds showed any signs of illness. Tissue samples from a proportion of the birds from both the unvaccinated birds and those which had been vaccinated with the bacterin prepared from NB remained infected while the birds vaccinated with the bacterin cultured in the synthetic SMM-1 medium had cleared the challenge bacteria from their tissues almost by day 4 post-challenge.

A comparison of the number of samples from which the challenge strain was isolated revealed a statistically significant difference between control unvaccinated and the NB vaccine group ($\chi^2 = 3.75$, $p = 0.05$), and between the control and synthetic medium group ($\chi^2 = 8.45$, $p = <0.01$) but no statistically significant difference between the nutrient both and synthetic medium groups ($\chi^2 = 1.01$, $p = 0.4$). The differences were more marked for the blood and spleen samples than for the bacterial counts in the liver. Compared with the control group the level of significance for the synthetic medium group was higher than for the NB bacterin group.

Discussion

Vaccination is more desirable than chemotherapy to control APEC. Early protection mediated by high titre circulating specific antibodies may be important as indicated by the protection against parenteral avian *Salmonella* infections using the commercial Salenvac vaccine which stimulates high titer antibodies (Woodward *et al.*, 2002). That high titer antibody correlates to some extent with protection is indicated by the fact that passively transferred antibody may protect against challenge with the homologous strain (Arp, 1980; Bolin and Jensen, 1987; Kariyawasam *et al.*, 2004).

The ultimate purpose of this analysis was to develop a synthetic medium reproducing these conditions as near as possible. For this reason, a full analysis of gene expression was not carried out by COGs gene classes. They were grouped according to genes likely to affect intracellular survival, response to stress, and virulence gene expression.

Our microarray studies here were designed to identify genes likely to affect intra-cellular survival, response to stress, and virulence gene expression. Although detailed analyses of gene expression were not carried out it was clear that the experiments showed that *E. coli* O78:K80 when established within avian macrophages, show patterns of physiology that are different from growth in

Table 3. The effect of vaccination with bacterins produced by culture in NB or SMM-1 on the numbers of *E. coli* F135 in the tissues of chickens challenged intravenously. Log_{10} (± SD) of *E. coli* F135 in the blood, liver or spleen together with the number of chickens in each group from which the challenge strain was isolated.

Day	Medium	Synthetic			NB			Control		
		Blood	Spleen	Liver	Blood	Spleen	Liver	Blood	Spleen	Liver
Day 1	Mean	0.93 ± 1.92	0.84 ± 1.70	3.29 ± 0.31	0.94 ± 1.93	1.49 ± 1.08	2.97 ± 0.99	1.27 ± 1.66	1.93 ± 1.10	3.53 ± 0.55
	No. +ves	1 of 5	1 of 5	5 of 5	1 of 5	3 of 5	5 of 5	2 of 5	4 of 5	5 of 5
Day 2	Mean	<1±0.00	<1 ± 0.00	1.9 ± 0.69	1.08 ± 2.23	1.16 ± 1.84	2.15 ± 1.70	1.01 ± 1.88	0.9 ± 1.16	2.56 ± 1.04
	No. +ves	0 of 5	0 of 5	5 of 5	1 of 5	2 of 5	4 of 5	2 of 5	2 of 5	5 of 5
Day 4	Mean	<1 ± 0.00	<1 ± 0.00	<1 ± 0.00	<1 ± 0.00	<1 ± 0.00	0.58 ± 0.72	0.88 ± 1.79	0.69 ± 1.36	0.78 ± 1.56
	No. +ves	0 of 5	0 of 5	0 of 5	0 of 5	0 of 5	2 of 5	1 of 5	1 of 5	1 of 5
Day 7	Mean	<1 ± 0.00	<1 ± 0.00	<1 ± 0.00	<1 ± 0.00	0.32 ± 0.70	0.27 ± 0.54	0.22 ± 0.40	1.08 ± 1.50	0.37 ± 0.85
	No. +ves	0 of 10	0 of 10	0 of 10	0 of 10	1 of 10	1 of 10	1 of 10	4 of 10	1 of 10
Total +ve/organ		1 0f 25	1 of 25	10 of 25	2 of 25	6 of 25	10 of 25	6 of 25	11 of 25	12 of 25
TOTAL +ve			12 of 75			18 of 75			29 of 75	

liquid RPMI medium. These changes include a trend toward respiration with oxygen as a terminal electron acceptor indicating that oxygen was available as the preferred electron acceptor. There were indications from individual genes (*gntR*, *gmd*, *glcC*, *yieL*, and *dcuB*) that different carbohydrates (gluconate, mannose, glycolate, xylan, and dicarboxylate acids, respectively) may have been used but the picture was not clear from the small number of genes involved in each case.

Osmotic stress was indicated by the expression of *osmY* and *osmC* (Yim and Villarejo, 1992) and the *proU* operon encoding a transport system for glycine, betaine, and proline, two osmo-protectant compounds (Haardt *et al.*, 1995). In the absence of solid information on the importance of amino acids to *E. coli* within the macrophage, information was taken from the literature and from Imre *et al*. (2013) to develop SMM-1. Manganese was omitted and iron availability was restricted by the addition of 2, -2-dipyridyl. Osmolarity was adjusted with K^+ and Na^+ to a water activity less than that in RPMI.

The SMM-1 medium clearly did not reflect all of the conditions within the macrophage. This was an approximation since the major carbon sources were unclear and we modeled this to include a reduction in glucose availability. Further studies could evaluate this in more detail and include incubation at a temperature closer to that of birds (41.5°C) which could also lead to even better immunogenicity and protection.

The bacterin produced by culture in the synthetic medium produced a level of protection above the control which was more statistically significant than the bacterin produced by culture in NB. We propose that such an approach may be used for several bacterial pathogens for which inactivated vaccines are still produced regularly and this approach merits further investigation.

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Conflict of interest

The authors have no conflict of interest associated with this work.

Authors contribution

All authors consented to participation in the work and its publication. Barrow and Windhorst conceived the study and Barrow and Elazomi wrote and edited the manuscript. The work was carried out by Zhou, Imre, Bukovinski and Ruggeri (microarray work), Richards (vaccine development) and Barrow (animal work).

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