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1 **SHORT COMMUNICATION**

2

3 **Vaccination of calves with EspA, a key colonisation factor of *Escherichia coli* O157:H7,**
4 **induces antigen-specific humoral responses but does not confer protection against intestinal**
5 **colonisation.**

6

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Abstract

1 Enterohaemorrhagic *Escherichia coli* (EHEC) infections in humans are frequently associated
2 with direct or indirect contact with ruminant faeces and may result in haemorrhagic colitis and
3 severe renal and neurological sequelae. Broadly cross-protective vaccines for control of EHEC
4 do not yet exist and the molecular mechanisms that influence bacterial persistence in the
5 intestines of ruminants are incompletely understood. We sought to determine the role in
6 colonisation and protective efficacy of EspA, which forms a filamentous extension of the locus
7 of enterocyte effacement-encoded type III secretion system that injects EHEC proteins into
8 enterocytes. A non-polar deletion of *espA* severely impaired the ability of *E. coli* O157:H7 to
9 colonise the intestines of calves. Vaccination of calves with highly purified recombinant EspA
10 induced high-titre antigen-specific IgG1 (also reactive to native EspA) and salivary IgA
11 responses, however these responses did not protect calves against intestinal colonisation by *E.*
12 *coli* O157:H7 upon experimental infection.
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15 Key words: *Escherichia coli*, Cattle, Colonisation, Vaccine, EspA.

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1 **1. Introduction**

2 Enterohaemorrhagic *Escherichia coli* (EHEC) comprise a group of zoonotic enteric
3 pathogens of worldwide importance. The predominant serotype affecting humans in Europe and
4 North America is O157:H7 and infections may involve bloody diarrhoea (reviewed in Paton &
5 Paton, 1998). The leading antecedent to human EHEC infection is contact with ruminant faeces
6 and strategies to reduce the carriage of EHEC in ruminants are predicted to lower the incidence
7 of human infection (reviewed in Stevens et al., 2002a). By random mutagenesis and allelic
8 replacement, genes located within the locus of enterocyte effacement (LEE) have been found to
9 play key roles in colonisation of the bovine intestines by EHEC (Dean-Nystrom et al., 1999;
10 Cornick et al., 2002; Dziva et al., 2004; Naylor et al., 2005; van Diemen et al., 2005). The LEE
11 encodes a type III secretion system (T3SS) that injects a set of bacterial proteins into enterocytes,
12 some of which modulate cellular pathways (reviewed in Caron et al., 2006). This process
13 requires type III secreted proteins EspA, -B and -D, encoded within the LEE4 operon.

14 EspA forms a filamentous extension of the T3SS needle complex and creates a physical
15 bridge between the bacteria and host cells (reviewed in Stevens et al., 2005). Resolution of the
16 3D structure of EspA filaments of enteropathogenic *E. coli* (EPEC) revealed the subunits to be
17 arranged in a helical tube enclosing a central channel (Daniell et al., 2003). A type III secreted
18 protein (Tir, translocated intimin receptor) is secreted from the tips of EspA filaments (Crepin et
19 al., 2005) and serves both as a receptor for the bacterial outer membrane protein intimin and as a
20 nucleator of cellular and bacterial proteins involved in actin assembly. These events aid the
21 formation of ‘attaching and effacing’ (AE) lesions on intestinal epithelia which are characterised
22 by intimate bacterial adherence to enterocytes on actin-rich pedestals and localised destruction of
23 microvilli.

1 170 nal^R carrying the temperature-sensitive λRed helper plasmid pKD46 following induction of
2 λRed recombinase with 10 mM L-arabinose at 30°C. A recombinant (85-170nal^R Δ*espA*::kan^R)
3 was selected on Luria Bertani medium containing nalidixic acid (nal, 25 μg/ml) and kanamycin
4 (kan, 50 μg/ml) and cured of pKD46 by growth at 42°C. The kan^R cassette was excised from
5 strain 85-170nal^RΔ*espA*::kan^R following transient expression of Flippase (FLP) recombinase
6 from the temperature-sensitive plasmid pFT-A in the presence of 20μg/ml heat-inactivated
7 chlortetracycline at 30°C to leave an 84-nucleotide scar (Posfai et al., 1997). The resulting
8 mutant (85-170nal^R Δ*espA*) was verified by PCR using the *espA* flanking primers *espA1* (5'-
9 ATATATGAGCTCTCTTCCCGAGCGTGAGA-3') and *espA4* (5'-
10 ATATATGAGCTCGGCTGTTAATGCGACGC-3'). Plasmid pFT-A was cured by growth at
11 37°C in the absence of ampicillin.

12 To verify the mutant and assess polarity, the mutant and parent strains were cultivated in
13 Dulbecco's Modified Eagles Medium (DMEM) to induce type III secretion and secreted proteins
14 prepared as described (Stevens et al., 2004). Production of EspA and EspD was assessed by
15 Western blotting as previously reported (EspA, Stevens et al., 2002b; EspD, Stevens et al.,
16 2004). The growth rates of the mutant and parent strains were assessed in rich and minimal
17 media using a BioScreen C real-time spectrophotometer (Oy Growth Curves AB Ltd., Helsinki,
18 Finland).

19 20 2.2. Purification of recombinant EspA.

21 A construct for the expression of *E. coli* O157:H7 EspA with a carboxyl-terminal 6xHis-
22 affinity tag was generated by PCR amplification of *espA* from strain EDL933 (Riley et al., 1983)
23 using primers EspA-Fr (5'CATGCCATGGATACATCAAATGCAACATCC 3')

1 and EspA-Re (5'CGGAATTCAGTTTACCAAGGGATATTGCTGAAATAG 3') and ligation of
2 the *NcoI*- and *EcoRI*-digested amplicon into pET28 opened with the same enzymes (Novagen[®],
3 Merck Biosciences Ltd., Nottingham, UK). The resulting plasmid was transformed into *E. coli*
4 K-12 strain BL21 (DE3) Star cells which lack RNaseE to stabilise mRNA (Novagen[®]) and the
5 protein expressed under isopropyl- β -D-thiogalactopyranoside (IPTG) induction and affinity
6 purified with nickel resin using standard protocols. The yield, size, stability and purity of the
7 expressed protein was assessed by SDS-PAGE. Protein concentration was determined by the
8 BCA method (Pierce Biotechnology, Inc, Rockford, IL, USA).

9

10 2.3. Oral inoculation of calves with *E. coli* O157:H7.

11 Procedures for oral inoculation of calves with *E. coli* O157:H7 have been described
12 previously (Stevens *et al.*, 2004) and were conducted in compliance with the Animals (Scientific
13 Procedures) Act 1986 (license no. 30/2009) with the approval of the Local Ethical Review
14 Committee. Three 14 day-old Friesian bull calves for each strain were separately inoculated with
15 ca. 1×10^{10} colony forming units (CFU) 85-170 na^R or 85-170 na^R $\Delta espA$ in 20 ml of antacid.
16 The magnitude and duration of faecal excretion of the bacteria was followed twice daily for two
17 weeks. Wild-type and mutant bacteria were enumerated by plating of triplicate serial dilutions of
18 fresh faeces collected by rectal palpation to sorbitol MacConkey agar (T-SMC) supplemented
19 with potassium tellurite (2.5 μ g/ml) and nalidixic acid (25 μ g/ml). Recovery of wild-type and
20 the mutant bacteria was confirmed by PCR from selected colonies using *espA*-flanking primers.
21 The sensitivity of detection was 10^2 CFU/g. Faecal excretion data were statistically analysed for
22 the effect of mutation by means of an F-test, with the data taken as repeated measurements and
23 the animal as a covariant (Proc Mixed, Statistical Analysis System 1995, SAS Institute, Cary,
24 NC, USA). *P* values of <0.05 were considered significant.

1

2 *2.4. Vaccination studies.*

3 Calves for vaccination were confirmed to be free of EHEC by PCR of presumptive *E.*
4 *coli* recovered from faeces on T-SMC using universal primers for *stx1*, *stx2*, *ehxA* and *eae* as
5 described (Stevens et al., 2004). Animals had received comparable quantities of colostrum in the
6 first few days of life as evidenced by the zinc sulphate turbidity assay (McEwan et al., 1970). For
7 intramuscular (i.m.) vaccinations, rEspA or mock antigen (sterile water) were formulated in
8 Freund's Incomplete adjuvant (Sigma, St. Louis, Mo, USA) at 100 µg protein/dose. The trial
9 commenced when the calves were 14 days old (day 0). The vaccines (2ml per dose) were each
10 separately given to 4 conventionally reared Friesian bull calves 3 times at day 0, day 28 and day
11 49 in the neck muscle. For intranasal (i.n.) boosting of the same animals, rEspA (300 µg/dose) or
12 water were mixed with cholera toxin B subunit (CT-B 100 µg/dose, Sigma, St. Louis, Mo., USA)
13 as a mucosal adjuvant and delivered on day 49 of the trial. A syringe fitted with a device to
14 create a fine aerosol was inserted as far as possible into the nostril and 2.5 ml of the solution was
15 delivered into each nostril during inhalation. Vaccinated animals were challenged 2 weeks after
16 the final boost with ca. 1×10^{10} CFU *E. coli* O157:H7 strain EDL933nal^R (Dziva et al., 2004) and
17 the course of faecal excretion monitored twice daily for one week and once daily for the second
18 week essentially as described above. Statistical analysis of the data from control and rEspA-
19 vaccinated groups was performed as above.

20

21 *2.4. Serology.*

22 Venous blood and saliva samples were collected at weekly intervals to monitor sero-
23 conversion following vaccination. An enzyme-linked immunosorbent assay (ELISA) was
24 developed to detect serum IgG1 and salivary IgA (sIgA) antibodies to rEspA. Pre-determined

1 sample dilutions (serum 1:640, saliva 1:20) were added to the antigen-coated wells (1 µg of
2 rEspA per well) of a microtitre plate. After incubation for 1 h at room temperature and
3 subsequent washing, wells were incubated with horseradish peroxidase-conjugated sheep anti-
4 bovine Ig (Serotec, Oxford, UK). After further washing, tetramethylbenzidine substrate (TMB,
5 Sigma) was added. Colour formation was stopped after 10 minutes with 2.5 N sulphuric acid.
6 The optical density was measured at 450 nm (OD₄₅₀) with background correction of 620 nm. The
7 ability of calf anti-rEspA to bind native EspA antigen was examined by ELISA assays using pre-
8 absorbed calf sera. Agar-grown mutant bacteria were suspended in 1 ml of neat calf sera (week
9 5) and incubated at 37 °C for 2 h with agitation. Mutant-absorbed sera were collected following
10 centrifugation (13,000 x g for 30 min) and used in whole cell ELISAs. Whole-cell ELISAs were
11 performed essentially as above using formalin-fixed DMEM-grown bacteria. Briefly, 85-170nal^R
12 and its isogenic *espA* mutant were separately inoculated into DMEM supplemented with 25mM
13 HEPES buffer and incubated statically at 37 °C for 6 hours. Bacteria were killed and fixed by
14 addition of 0.5% (v/v) formalin, then harvested by centrifugation and standardized for ELISA as
15 described (Zhu et al., 2006). Western blotting assays using purified rEspA were performed to
16 confirm the magnitude and specificity of the responses measured by ELISA.

17

18 3. Results & Discussion

19 An *espA* deletion mutant of *E. coli* O157:H7 strain 85-170nal^R was constructed by λRed-
20 mediated linear recombination. The mutation removed the entire *espA* open reading frame and
21 did not impair bacterial growth in rich or minimal media (data not shown). Western blot analysis
22 of secreted proteins from DMEM-grown 85-170nal^R and 85-170nal^R Δ*espA* confirmed the
23 absence of EspA in the mutant strain but revealed that EspD (which is encoded immediately 3'
24 of *espA*) was secreted in comparable levels to the wild-type, indicating that the mutation is non-

1 polar (data not shown). The mutant strain was also confirmed to exhibit reduced adherence to
2 HeLa cells and to be incapable of nucleating actin at the sites of bacterial attachment as
3 previously reported (Ebel et al., 1998).

4 To assess the role of EspA in colonisation of the bovine intestines by *E. coli* O157:H7, the
5 wild-type and mutant strains were each separately administered to three 14 day-old
6 conventionally reared Friesian bull calves by the oral route. 85-170nal^R efficiently colonised the
7 intestines of calves, being excreted at in excess of 10⁵ CFU/g for the duration of the experiment
8 (Fig. 1). The isogenic $\Delta espA$ mutant was shed in significantly lower numbers than the parent
9 strain from day 1 post-inoculation (Fig. 1; *P* values <0.05) and was shed for a shorter duration,
10 being undetectable in the faeces following direct plating by day 11 post-inoculation. The extent
11 of attenuation was similar to that observed with an 85-170nal^R Δtir mutant that was tested in
12 parallel (Vlisidou et al., 2006) and this may reflect the importance of EspA-mediated delivery of
13 Tir into enterocytes. Our data support previous findings that EspA is required for the carriage
14 and virulence of rabbit EPEC in an infant rabbit model (Abe et al., 1998) and *E. coli* O157:H7 in
15 mice (Nagano et al., 2003). Further, they extend the observations of Naylor et al, who reported
16 that deletion of the entire LEE4 operon which encodes EspA, but also other type III secreted
17 proteins, impaired persistence of *E. coli* O157:H7 in 13-25 week-old cattle (Naylor et al., 2005).

18 Given the key role of EspA in intestinal colonisation and the promise shown by *E. coli*
19 O157:H7 secreted protein vaccines in experimental cattle studies (Potter et al., 2004), we sought
20 to determine if purified EspA could elicit protective responses upon vaccination of calves. A
21 6xHis-tagged derivative of *E. coli* O157:H7 strain EDL933 EspA was expressed in *E. coli* K-12
22 BL21 (DE3) Star under isopropyl- β -D-thiogalactopyranoside (IPTG) induction and purified by
23 affinity chromatography. SDS-PAGE analysis revealed the protein to be highly purified and
24 stable (Fig. 2A). Starting at 14 days of age, groups of 4 calves were vaccinated by the

1 intramuscular route with rEspA or mock antigen formulated in Freund's Incomplete adjuvant at
2 days 0 (start of trial), 28 and 49 as described in Materials and Methods. On day 49, calves were
3 also boosted with the same antigens via the intranasal route mixed with CT-B subunit. CT-B is
4 known to be an effective mucosal adjuvant in a variety of model systems and is well tolerated by
5 this route in cattle.

6 Antigen-specific sIgA was observed in calves vaccinated with rEspA 2 weeks after
7 vaccination and levels were almost always significantly higher than those in mock-vaccinated
8 calves (Fig. 2B; P values <0.05). Only day 56 (week 8) sIgA levels showed no significant
9 differences between the two groups. Although temporal increases in the level sIgA were
10 observed in the mock-vaccinated group during the experiment, these remained significantly
11 lower than those from the rEspA-vaccinated calves. The increase in sIgA titres over the
12 vaccination duration was thought to be due to the relatively low specificity of IgA, which could
13 be induced by a variety of other antigens encountered during the period. Calves were also
14 confirmed to have raised IgG1 and IgA responses to CT-B, indicating that it was effectively
15 delivered and recognised via the intranasal route (data not shown), consistent with recent
16 observations (van Diemen et al., 2007). Administering a second booster did not confer any
17 further increase in sIgA levels.

18 All vaccinated calves mounted a significant ($P < 0.001$) antigen-specific serum IgG1
19 response to rEspA after the first vaccination (day 14, Fig. 2C). Sustained high levels of anti-
20 rEspA IgG1 were detected at subsequent time intervals and immediately prior to challenge. Very
21 low levels of antibody reactive to the antigen were detected in mock-vaccinated calves by
22 ELISA. The magnitude and specificity of the serum IgG1 response against rEspA was confirmed
23 by Western blotting using sera collected at day 0, day 35 (week 5) and day 63 (week 9; Fig. 2D).

1 Vaccinated animals were challenged 14 days after the final boost with 1×10^{10} CFU *E. coli*
2 O157:H7 strain EDL933nal^R. No differences in the magnitude or duration of faecal excretion of
3 *E. coli* O157:H7 were observed between calves given rEspA and those given mock antigen (Fig.
4 3; $P < 0.05$). In order to confirm that calf antibodies against rEspA could bind to native EspA
5 filaments, we performed ELISAs using formalin-fixed *E. coli* strain 85-170nal^R and with its
6 isogenic *espA* mutant and compared the titres of calf sera pre-absorbed with fixed $\Delta espA$
7 bacterial cells. A significant difference in the titre of the same sera on the wild type strain and the
8 mutant was evident (P value < 0.05 ; Fig. 2E), confirming that antibodies against rEspA are
9 capable of binding to native filaments. The low reactivity in whole cell ELISAs likely reflects
10 the fact that a denatured antigen, possibly lacking similar conformational epitopes of surface
11 exposed EspA, was used for vaccination. It could also be due to low amounts of surface-exposed
12 EspA antigen, studies have indicated that not all *E. coli* O157:H7 bacteria in a given population
13 express EspA filaments at any one time under inducing conditions (Roe et al., 2003). Previous
14 work has shown that whilst monoclonal and polyclonal anti-EspA bind native surface-exposed
15 EspA filaments and can inhibit actin nucleation by *E. coli* O157:H7 in vitro, adherence to HEp-2
16 cells was not affected (La Ragione et al., 2005). Further, whilst egg yolk-derived IgY against
17 EspA inhibits adherence of human EPEC to porcine intestinal explants, it did not interfere with
18 adherence of the porcine EPEC strain from which the EspA antigen was derived or that of *E. coli*
19 O157:H7 (Girard et al., 2006). A vaccine based on the secreted proteins of *E. coli* O157:H7
20 induced humoral responses against type III secreted proteins, as well as potent anti-
21 lipopolysaccharide responses, and reduced the magnitude and duration of faecal excretion of *E.*
22 *coli* O157:H7 and the total number of animals shedding following experimental inoculation of
23 calves (Potter et al., 2004). However, in subsequent trials in 218 pens of feedlot cattle in 9
24 feedlots in Alberta and Saskatchewan there was no significant association ($P > 0.20$) between

1 vaccination with EHEC secreted proteins and pen prevalence of faecal *E. coli* O157:H7
2 following vaccination or prior to slaughter (van Donkersgoed et al., 2005).

3 It is known that differences in the antigenicity of EspA filaments exist among AE pathogens
4 (Neves et al., 2003) and this may be a barrier to the design of broadly cross-protective EspA-
5 based vaccines. Further, EspA filaments are only transiently expressed during AE lesion
6 formation (Knutton et al., 1998; Ebel et al., 1998). Subunit vaccines based on other well-
7 documented EHEC colonisation factors (intimin and Efa1) have also been shown to elicit
8 humoral responses in cattle that are not protective (van Diemen et al., 2007) and it is clear that
9 the rational development of effective vaccination strategies for the control of EHEC in ruminants
10 will continue to be a major challenge for the future.

11

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15

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16 **Figure legends**

17 Figure 1. Course of faecal excretion of *E. coli* O157:H7 strain 85-170 nal^R (-◇-) and an isogenic
18 non-polar *espA* deletion mutant (-▲-) following oral inoculation of calves. Bacterial recoveries
19 from faeces were measured twice each day. The mean daily faecal count ± standard error of the
20 mean is shown.

21

22 Figure 2. The purity of the rEspA antigen used in these experiments and for immunisation of
23 calves, is shown in panel A. Induction of salivary IgA and serum IgG1 responses to rEspA
24 following immunisation of calves as measured by ELISA (B & C respectively). The specificity

1 of the IgG1 response was confirmed by Western blotting using purified rEspA and serum
2 samples from a representative calf collected at three different time points (panel D). Serum
3 antibodies were confirmed to bind native EspA by ELISA using whole cells (wild type and
4 $\Delta espA$ strains) and $\Delta espA$ -pre-absorbed sera (panel E). Triplicate ELISA measurements were
5 performed for each animal and the data represent the mean of four animals. ^a not significant; *
6 $P < 0.05$; ** $P < 0.001$.

7

8 Figure 3. Course of faecal excretion of *E. coli* O157:H7 strain EDL933 nal^R following oral
9 challenge of calves primed i.m. with rEspA (-◇-) or mock-antigen (-■-), then boosted with the
10 same antigens intranasally (n = 4 per group). Vaccinated calves were challenged two weeks after
11 the last booster. Data represent the mean daily faecal count \pm standard error of the mean.

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Figure 1

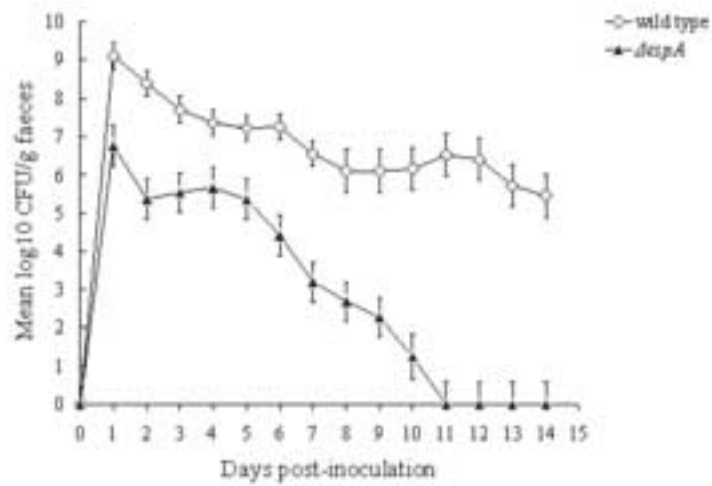
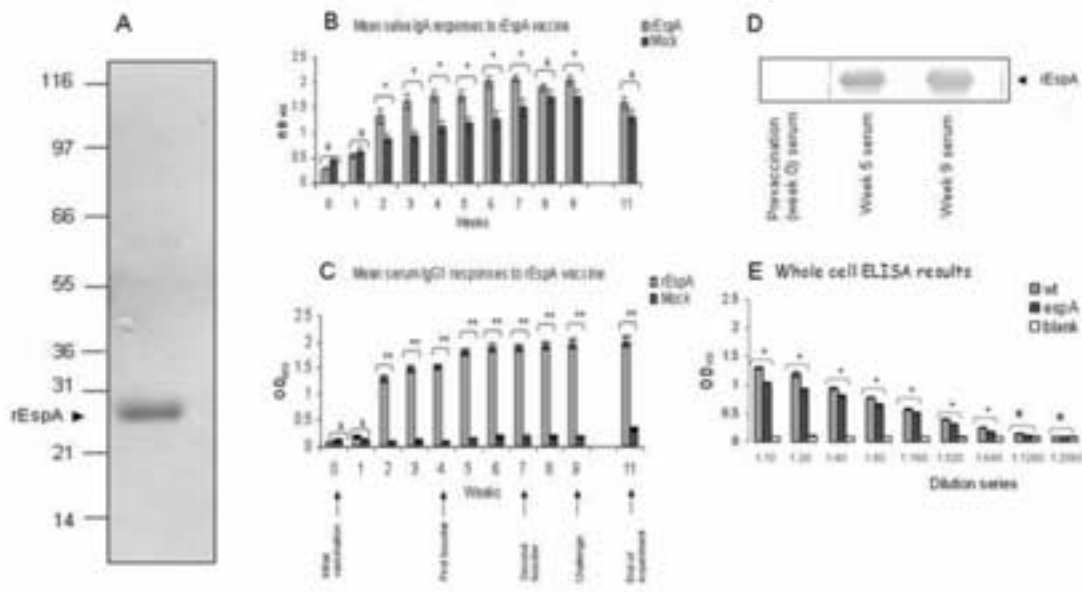


Figure 2



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Figure 3

