BACTERIAL PATHOGENICITY

A low molecular weight outer-membrane protein of *Escherichia coli* O157:H7 associated with adherence to INT407 cells and chicken caeca

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Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 and O26:H11 have been shown to produce a low mol.wt outer-membrane protein (OMP) that is unique to a few serotypes of *E. coli*. A mutant (A10) of *E. coli* O157:H7 strain HA1 deficient in the OMP was constructed by *TnphoA* mutagenesis and assayed for its adherent ability. Adherence of A10 to intestinal epithelial cells (INT407) was significantly less than that of its parent strain (HA1). Adherence of HA1 to INT407 cells was significantly decreased by treatment with a monoclonal antibody (4E8C12) that specifically binds to the OMP. When chickens were infected experimentally with *E. coli* O157:H7 strains, the average number of cfu of strain A10 recovered from chicken caeca was significantly less than those of strain HA1 and wild-type strain 932 at 14 and 21 days after peroral inoculation. These data suggest that the OMP of EHEC is associated with adherence of *E. coli* O157:H7 to epithelial cells *in vitro* and chicken caeca *in vivo*.

Introduction

Escherichia coli serotype O157:H7, a recognised cause of haemorrhagic colitis and haemolytic uraemic syndrome, is the principal serovar within the enterohaemorrhagic *E. coli* (EHEC) [1, 2]. The organism can produce Shiga-like cytotoxins (SLT I or SLT II or both) that cause host cell death by inhibiting protein synthesis [1–3]. Also important to the pathogenicity of EHEC is its ability to attach to host cells [1, 2]. However, the mechanism of *E. coli* O157:H7 adherence to host cells is poorly understood. Previous reports have suggested that outer-membrane proteins (OMP) rather than lipopolysaccharide (LPS) or flagella of *E. coli* O157:H7 play an important role in mediating binding of the bacteria to epithelial cells *in vitro* [4–6]. The mechanism of attachment of *E. coli* O157:H7 to intestinal mucosal cells has been studied extensively by several investigators [4–13]. Genes and their products that are involved in colonisation have been studied, but their exact roles, especially in the initial attachment of *E. coli* O157:H7, remain controversial and require further elucidation [4, 7, 8, 13, 14].

Padhye and Doyle [15] produced a monoclonal antibody (MAb) 4E8C12 that reacts with a low mol. wt (originally described as 5–6 kDa, but deter-

mined to be 8 kDa in the present study) OMP of EHEC O157:H7 and O26:H11. Because of the association of the OMP with EHEC [16], it was suggested that the OMP may be associated with the virulence of EHEC, perhaps associated with the organism's adherence. *TnphoA* mutagenesis allows the creation of mutants deficient in bacterial factors that normally are secreted or exported to the bacterial cell surface [6, 17, 18]. As all bacterial proteins implicated as virulence factors are extracellular or surface associated, *TnphoA* mutants can be used to identify genes that encode adherence factors [5, 18–21]. Construction and analysis of EHEC *TnphoA* mutants can provide a foundation for the study of the molecular basis of the pathogenicity of EHEC. The objective of this study was to obtain a transposon *TnphoA* mutant deficient in the low mol. wt OMP of EHEC, and to determine its ability to adhere to human intestinal epithelial cells (INT407) and chicken caeca.

Materials and methods

Bacterial strains

E. coli O157:H7 strain 932 was used as a positive control in the chicken inoculation study. *E. coli* O157:H7 strain HA1, derived from strain 932, was used as the recipient cell in conjugation studies. Strain HA1, which produces both SLT-I and SLT-II, was used for the development of MAb 4E8C12 for EHEC [15].

E. coli strain SM10 λ *pir* (*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km*) was the donor cell in conjugation studies with *E. coli* O157:H7 strain HA1, and was kindly provided by S. Craven (Russell Research Center, USDA/ARS, Athens, GA, USA). SM10 λ *pir* carries a suicide plasmid pRT733 (*oriR6K tra- mob- Ap^r Km^r*) that bears transposon Tn*phoA*.

Media and chemical reagents

Luria broth (LB) and Luria plates were used for routine culturing of the bacteria, and MacConkey agar was used for enumeration. All media were obtained from Difco. Ampicillin (Ap, 100 μ g/ml), kanamycin (Km, 45 μ g/ml), nalidixic acid (NA, 50 μ g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (XP, 40 μ g/ml) were added when appropriate. All chemical reagents were purchased from Sigma.

Construction of Tn*phoA* mutants

A NA (50 μ g/ml)-resistant strain of HA1 was obtained by plating on to MacConkey agar plates containing different concentrations (1–50 μ g/ml) of NA. Transposon Tn*phoA* from suicide plasmid vector pRT733 was introduced into the NA-resistant HA1 by plate matings with the donor cell SM10 λ *pir*. In-frame transposon insertion into signal-sequence containing genes resulted in phenotypes of Tn*phoA* mutants that were Km^r, NA^r and formed blue colonies on LB plates containing Km, NA and XP (an indicator dye for alkaline phosphatase activity).

Screening Tn*phoA* mutants by immunodot-blot assay

Approximately 800 Km^r, NA^r blue colonies were selected from separate conjugation experiments for immunodot-blot screening. The immunodot-blot assay was performed by the following procedure. Briefly, an Immobilon-PVDF membrane (Millipore Corp., Bedford, MA, USA) was cut (8 $\times$ 12 cm), soaked in 10 ml of methanol 100% for 2 s, and placed in coating buffer (0.05 M carbonate buffer, pH 9.6) for 10 min before use. The Tn*phoA* mutants were grown in 5 ml of LB containing Km and NA for 18 h at 37°C. Fifty μ l (*c.* 10⁸ cfu) of each broth culture were placed on to the membrane assembled in a dot-blot manifold (BRL, Gaithersburg, MD, USA). The membrane was air dried for 5 min after blotting the cultures, and then submerged in bovine serum albumin (BSA) 5% w/v in 50 mM Tris buffer saline, pH 7.5, containing 150 mM NaCl with Tween-20 0.05% v/v (TBS-T) plus sodium azide 0.05% w/v at 37°C for 1 h with agitation (100 rpm). The membrane was soaked in 20 ml of MAb 4E8C12 (ascites fluid diluted 1 in 400 in TBS-T) for 1 h at room temperature, washed three times with TBS-T for 10 min each, and then incubated with alkaline phosphatase-labelled goat anti-mouse IgG (diluted at 1 in 800 in TBS) for 30 min at 37°C. The

membrane was washed four times, and placed in a substrate of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Kirkegard and Perry Laboratory, Inc., Gaithersburg, MD, USA) for 1 min at room temperature. The reaction was stopped by rinsing the membrane with distilled water for 1 min. A dark purple spot and a light purple spot indicated positive and negative reactions with MAb 4E8C12, respectively. *E. coli* O157:H7 HA1 and SM10 λ *pir* served as positive and negative controls, respectively.

Tricine-SDS-PAGE and Western blot analysis

The Tn*phoA* mutant, designated as A10 that gave a negative result in immunodot-blot assay, was selected for Tricine-SDS-PAGE and Western blot analysis to confirm that it did not express the low mol. wt OMP. The OMP of strains A10, HA1 and SM10 λ *pir* were extracted by the method of Hjelmeland and Chrambach [22]. Tricine-SDS-PAGE was performed following the procedures described by Schagger and van Jagow [23]; 20 μ g of OMP of HA1, A10 and SM10 λ *pir* were each loaded on gels consisting of a separating 20% gel, a spacer 10% gel and a stacking 4% gel in a double-slab electrophoresis unit (Protean; BioRad Laboratories, Richmond, CA, USA) and run at constant 90 mA for 7 h. Low range mol. wt markers (Sigma) were included on the gels. Protein bands were visualised by staining with Coomassie Blue 2.5% w/v for 2 h and destaining in methanol 40% v/v and acetic acid 20% v/v. Western blotting was performed according to the procedure described by Padhye and Doyle [15]. After electrophoresis, proteins were transferred to a PVDF membrane in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, methanol 20% v/v and SDS 0.05% w/v in a transblot apparatus (Multiphor II; Pharmacia, Piscataway, NJ, USA) at 400 mA for 40 min. The procedures for immunostaining were the same as the dot-blot immunoassay described above, except that MAb 4E8C12 was diluted 1 in 1600 and goat anti-mouse antibody was diluted 1 in 2000 in TBS-T.

Southern blot hybridisation

The genomic and plasmid DNA of strain A10 were extracted by a mini-preparation procedure [24, 25]. For each DNA preparation, 2 μ g were digested at 37°C for 3 h with restriction endonuclease *EcoRV* (BRL) that does not cleave within Tn*phoA*. The DNA fragments were separated by electrophoresis in agarose 0.8% w/v gel and transferred on to a Nylon membrane (Sigma). The genomic DNA of strain HA1 was also isolated and subjected to Southern hybridisation to serve as a control. The plasmid DNA of pRT733 was extracted by mini-preparation, digested with restriction endonuclease *Bgl*II, and separated in agarose 0.8% w/v gel. A 2.8-kbp *Bgl*II fragment that contains the Km resistance gene found in Tn*phoA* was isolated from the gel, purified by GeneClean II (Bio 101, La Jolla, CA, USA), and labelled with α -³²P dATP by the

random-primer method [26], to serve as the probe for Southern hybridisation. Lambda DNA digested with *Hind*III was also labelled with α -³²P dATP and included in hybridisation experiments as a molecular marker. The hybridisation was performed in a solution containing formamide 50%, 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), SDS 0.5% w/v, 5 × Denhardt's solution (Ficoll 0.1%, polyvinylpyrrolidone 0.1%, BSA 0.1%) and single strand DNA 100 µg/ml, for 16 h at 42°C.

Adherence assay

INT407 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Basal Medium Eagle (BME) plus fetal bovine serum (FBS; BRL) 15%. The adherence assay was performed by the method of Junkins and Doyle with minor modification [27]. Briefly, INT407 cells were inoculated into each well (35 mm in diameter) of a six-well tissue culture plate with lid (Corning Glass Works, Corning, NY, USA) at a concentration of 10⁵–10⁶ viable cells, and grown in BME plus FBS 15% at 37°C until confluent (2–3 days). Overnight bacterial cultures were sedimented by centrifugation (16 000 g, 1 min) and resuspended to an OD_{640nm} of 0.5 with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) plus 10 µM ZnCl₂. The bacterial suspension (2 ml) was applied to an INT407 monolayer and incubated at 37°C for 2 h without agitation. After incubation, the suspension was removed from the monolayer, and non-adherent bacterial cells were removed by rinsing five times with 3 ml of TBS plus 10 µM ZnCl₂ plus Tween 20 0.1% v/v. The monolayer was then treated with 2 ml of 2 mM Na₂EDTA in TBS for 1 min. The bacteria were separated from INT407 cells by vortex mixing. The number of viable bacteria in the suspension was determined by plating serial (1 in 10) dilutions of the suspension on MacConkey agar plates, followed by overnight incubation at 37°C. The number of INT407 cells in the suspension was determined by haemocytometry. The number of adherent bacteria/INT407 cells was then calculated. To minimise experimental error,

positive (HA1) and negative (SM10_λ *pir*) controls were always included in each tissue culture plate.

Adherence inhibition assay

Six adherence inhibition assays also were performed. The procedures of the adherence inhibition assay were the same as those of the adherence assay except that *E. coli* O157:H7 strain HA1 was treated with MAb 4E8C12 (ascites fluid) before being added to the INT407 monolayer. The MAb (diluted 1 in 200 and 1 in 400) was added to 2 ml of bacterial suspension and incubated at 37°C for 1.5 h. Strains A10 and HA1 that were not treated with MAb served as controls in each test. Data of the adherence and adherence-inhibition assays were analysed statistically by the *t*-test [28].

Chicken colonisation study

The methods used for the chicken colonisation study were described previously [29]. Briefly, *E. coli* O157:H7 strains 932, HA1 and mutant A10 were cultured at 37°C in Trypticase Soy Broth (TBS; BBL Microbiology System, Cockeysville, MD, USA) with appropriate chemicals for 24 h. Cells were washed three times and resuspended in 0.01 M phosphate-buffered saline (PBS) pH 7.5. Two trials of the chicken inoculation study were conducted (Table 1). In trial I, 105 1-day-old chicks (Peterson/Arbor-Acre) were divided into four groups, of which 15 chicks served as controls and received 0.25 ml of PBS perorally into the crop *via* a 1-ml syringe, and the remaining 90 chicks received *c.* 10⁹ cfu of *E. coli* (30 chicks/strain) by the same procedure. Five chicks from the control and 10 chicks from each test group were killed at 7, 14 and 21 days post-inoculation for bacterial enumeration. In trial II, two more sampling times (3 and 5 days) were added (Table 1).

Immediately after they were killed, the internal organs of chicks were dissected and caeca were removed aseptically and placed in plastic sterile tubes. The tissue was weighed, diluted (1 in 10) in cold PBS and

Table 1. Recovery of *E. coli* O157:H7 strains from chicken caeca after peroral inoculation

Strain no.	<i>E. coli</i> log ₁₀ cfu/g of caecal tissue and contents (SD) at post-inoculation times				
	3 days	5 days	7 days	14 days	21 days
Trial I					
932	---	---	3.68 (0.82)	4.81 (1.3)	3.75 (0.86)
HA1	---	---	2.36 (1.85)	3.50 (0.68)	4.04 (0.33)
A10	---	---	1.6 (1.79)*	<1.0†	<1.0†
Trial II					
932	4.65 (0.78)	5.37 (0.93)	5.38 (1.54)	6.11 (1.24)	3.99 (1.30)
HA1	4.05 (0.88)	3.30 (0.89)	3.17 (0.61)	3.54 (1.99)	4.02 (1.60)
A10	5.89 (0.90)	5.40 (0.94)	4.06 (0.91)	2.15 (1.98)‡	2.34 (2.51)*

*Strain A10 (<10 cfu/g of caeca; minimum level of sensitivity) detected in five of 10 chicks.

†Strain A10 (<10 cfu/g) detected in 10 chicks.

‡Strain A10 (<10 cfu/g) detected in four of 10 chicks.

homogenised aseptically for 1 min with a Polytron tissue homogeniser (Brinkman Industries, Inc., Westbury, NJ, USA). Samples were serially diluted (1 in 10) in PBS, and each dilution was plated in duplicate on sorbitol-MacConkey agar containing nalidixic acid 30 µg/ml. For enumeration of the mutant A10, kanamycin 40 µg/ml also was added to the medium. The plates were incubated at 37°C for 48 h, and colonies typical of *E. coli* were counted. At least one typical *E. coli* colony from a plate at the highest dilution of tissue was confirmed serologically as *E. coli* O157 by latex agglutination with O157-specific antiserum (Unipath, Basingstoke). Data of the chicken study were analysed statistically by the *t*-test [28].

Results

Construction of low mol. wt OMP-deficient mutants of *E. coli* O157:H7

Approximately 800 Km^r, NA^r blue colonies were selected from hundreds of separate conjugation experiments for immunodot-blot screening. A mutant, designated A10, did not react with MAb 4E8C12 in the dot blot assay. Strain A10 retained many of the characteristics of its parent strain, HA1, such as production of SLT I and SLT II, agglutination with O157 polyclonal antiserum, genomic DNA profile by pulsed-field gel electrophoresis and inability to hydrolyse 4-methylumbelliferyl β-D-glucuronide (MUG), except that A10 was Km^r, obtained from *TnphoA*.

OMP analysis

Tricine-SDS-PAGE and Western blotting were performed to analyse the OMP of mutant A10, the parent strain HA1 and donor strain SM10. The tricine-SDS-PAGE profiles indicated that one band representing the *c.* 8-kDa OMP that was present in strain HA1 was missing from strain A10. The OMP profile of SM10_λ *pir* was very different from those of strain HA1 and A10 (Fig. 1A). Western immunoblot analysis of the OMP revealed that the HA1 OMP reacted with MAb 4E8C12, whereas those of strains A10 and SM10_λ *pir* did not (Fig. 1B), further confirming that strain A10 was deficient in the low mol. wt OMP.

Genetic analysis

Hybridisation of genomic and plasmid DNA of strain A10 with the 2.8-kbp Km^r gene probe showed that A10 contained a single *TnphoA* insertion located on the chromosomal DNA, and that the plasmid DNA of strain A10 had no *TnphoA* insertion (Fig. 2). The hybridised *EcoRV* fragment of A10 genomic DNA, which contained *TnphoA* flanking the target gene, was *c.* 9.4 kb (Fig. 2). The genomic DNA of strain HA1 did not hybridise with the probe (data not shown).

Fig. 1. Tricine-SDS-PAGE Coomassie Blue staining pattern (A) and Western blot (B) of OMPs of *E. coli* O157:H7 strain HA1 (lane 2), *E. coli* O157:H7 mutant strain A10 (3), *E. coli* strain SM10_λ *pir* (4); 20 µg of OMP was loaded in each lane and the low mol. wt markers (Da) were applied in lane 1. The arrow indicates the position of the low mol. wt OMP recognised specifically by MAb 4E8C12.

Fig. 2. Southern blot hybridisation of genomic (lane 1) and plasmid (2) DNA of *E. coli* O157:H7 mutant strain A10, digested with *EcoRV* and hybridised with a mixture of α-³²P dATP-labelled 2.8-kb *Bgl*II fragment of *TnphoA* and α-³²P dATP-labelled *Hind*III-digested lambda DNA.

Adherence assay

Fifteen independent adherence assays were performed. The mutant strain (A10) was shown to be significantly less ($p < 0.005$) adherent to INT407 cells than the parent strain (HA1). The means and standard deviations (SD) of adhering bacteria/INT407 cell for strains A10, HA1 and SM10 λ *pir* were 1.62 (0.97), 133.1 (6.17) and 2.75 (2.69), respectively.

Six adherence inhibition assays also were performed. After treatment of strain HA1 with MAb 4E8C12 at 1 in 200 and 1 in 400 dilutions at 37°C for 1.5 h, HA1 was capable of adhering to INT407 cells at a mean (SD) of 3.53 (2.51) and 2.52 (2.17) bacteria/cell, respectively. As the controls, the means (SD) of adhering bacteria/INT407 cells for strains A10 and HA1 were 0.93 (0.49) and 9.49 (3.91), respectively. Results with and without treatments with MAb 4E8C12 indicate that MAb 4E8C12 significantly ($p < 0.05$) inhibited adherence of strain HA1 to INT407 cells. If strain HA1 was treated with the MAb at the 1 in 1600 dilution and held at 37°C for 1.5 h, the ability of HA1 to adhere to INT407 cells was not significantly ($p > 0.05$) different from that of bacteria that were not treated with the MAb.

Chicken colonisation study

Results of caecal colonisation with *E. coli* O157:H7 strains 932, HA1 and OMP-mutant A10 in chickens after peroral inoculation are shown in Table 1. In trial I, strain A10 was not isolated at 14 and 21 days after peroral inoculation, whereas strains 932 and HA1 were still present in the caeca at levels $>10^3$ cfu/g of caeca. Similar results were observed in trial II, in which strain A10 was not recovered after 14 and 21 days post-inoculation in at least four of 10 chicks. The average number of cfu of strain A10 recovered from caeca was significantly less ($p < 0.05$) than those of strains 932 and HA1.

Discussion

Factors involving *E. coli* O157:H7 adhesion and attaching and effacing (A-E) lesion formation have been studied by several investigators [4–12, 30]. Donnenberg *et al.* [4] reported that the *eae* gene is necessary for intimate attachment of EHEC *in vivo*, but the precise role of intimin, the product of the *eae* gene of EHEC, in the attaching and effacing phenomena is unknown [4]. Sherman *et al.* [9] suggested that a 94-kDa OMP was intimin because antiserum raised to this OMP blocked adhesion and F-actin lesion formation of *E. coli* O157:H7 strain CL56 on tissue culture cells. In later studies, Louie *et al.* [8] determined by peptide sequencing, immunoassay and expression of the carboxyl-terminal 266 amino acid of

intimin of *E. coli* O157:H7 strain CL8, that intimin was a 97-kDa OMP and was different from the 94-kDa OMP.

Dytoc *et al.* [5] determined that *E. coli* HB101 transformed with the cloned EHEC *eae* gene did not form attaching and effacing lesions on cultured epithelial cells *in vitro* or rabbit intestinal tissue *in vivo*. Louie *et al.* [8] determined that an *eae* insertional inactivation mutant of strain CL-8 (CL8-K01) gave negative results in the fluorescent actin staining (FAS) test, but adherence of CL8-K01 to HEp-2 cells was not significantly different from that of CL8. Furthermore, the *eae* gene expressed alone in *E. coli* HB101 did not significantly increase adherence to HEp-2 cells compared with the parent HB101, despite the fact that *E. coli* O157:H7 intimin was localised in the outer membrane and was surface exposed. They concluded that intimin contributes minimally if at all to the total adherence of strain CL8 to HEp-2 cells. Dytoc *et al.* [5] used *TnphoA* mutagenesis to identify factors that were necessary for O157:H7 A-E lesion formation and were not likely to be an *eae* gene product in two *TnphoA* mutants. Recently, Leroy *et al.* [31] reported the presence of the *eae* gene in non-pathogenic *E. coli* strains isolated from rabbits. Hence, it appears that production of A-E lesions by EHEC depends on the expression of several genes and these are affected by environmental conditions. Adherence of EHEC to epithelial cells still occurs without the *eae* gene. Furthermore, introduction of the *eae* gene into other non-adherent *E. coli* strains does not necessarily result in their ability to adhere to epithelial cells because other essential factors are missing.

Recent studies have revealed that a large (35 kb) region in the enteropathogenic *E. coli* (EPEC) chromosome, locus of enterocyte effacement (LEE), containing *eaeA*, *eaeB*, *sepA*, *sepB*, *sepC* and *sepD* (*sep*, for secretion of *E. coli* proteins), encodes the virulence determinants for A-E lesion formation of EPEC that have been identified to date [32, 33]. This region is conserved among other pathogens that produce the A-E lesion, including *E. coli* O157:H7, and is inserted into the *E. coli* chromosome at the identical site where a block of virulence genes or 'pathogenicity island' of uropathogenic *E. coli* is located [32]. Therefore, more studies are needed to determine the precise role of intimin, other adherence factors, the host cell receptor and their interactions in the process of colonisation.

Although *E. coli* O157:H7 has not been isolated from chickens, this organism is able to colonise well in chickens, caeca being the primary site of colonisation [29]. In the present study, chickens were used to evaluate the effect of the low mol. wt OMP on the colonisation of *E. coli* O157:H7 *in vivo*. The mutant deficient in the OMP had significantly reduced ability

to colonise chicken caeca compared to the parent strain of *E. coli* O157:H7.

Recent studies in this laboratory indicate that the OMP has a close association with the lipid A portion of LPS on the cell membrane of *E. coli* O157:H7, similar to the association of LPS and outer-membrane proteins in other *E. coli* [34, 35]. *E. coli* O157:H7 strain HA1 was obtained while attempting to isolate a variant of *E. coli* O157:H7 strain 932 that was highly adherent to Henle 407 cell monolayers [15]. Strain 932 was passaged several times on Henle 407 cell monolayers. Each time only those bacteria that remained attached to the monolayers after washing were cultured. Strain HA1 was isolated from passage 11 and was highly adherent to Henle 407 cells. This strain lacks a portion of the LPS layer of the cell outer membrane, allowing greater exposure of the unique low mol. wt OMP than its parent strain and other wild-type strains of *E. coli* O157:H7. The OMP also is expressed in greater amounts when bile salts and acriflavine are present in the growth medium [16]. Interestingly, Bilge *et al.* [36] reported that *E. coli* O157:H7 LPS-mutants were hyperadherent. Their studies suggested that the increased ability of the O157-mutants to adhere to HeLa cells could result from increased contact between the bacterial adhesin and the epithelial cell surface in the absence of O side chains. It appears that the environment of the intestinal tract of animals and man could influence *eae* genes and other factors, including expression of the OMP, in their involvement in the process of colonisation. In conclusion, the low mol. wt OMP is associated with the adherence of *E. coli* O157:H7 to cultured human epithelial cells and chicken caeca, and the mechanism of this involvement remains to be determined.

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References

1. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* 1991; **13**: 60–98.
2. Padhye NV, Doyle MP. *Escherichia coli* O157:H7: epidemiology, pathogenesis, and methods for detection in food. *J Food Prot* 1992; **55**: 555–565.
3. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. *Microbiol Rev* 1987; **51**: 206–220.
4. Donnenberg MS, Tzipori S, McKee ML, O'Brien AD, Alroy J, Kaper JB. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. *J Clin Invest* 1993; **92**: 1418–1224.
5. Dytoc M, Soni R, Cockerill F *et al.* Multiple determinants of verotoxin-producing *Escherichia coli* O157:H7 attachment-effacement. *Infect Immun* 1993; **61**: 3382–3391.
6. Sherman P, Soni R. Adherence of verocytotoxin-producing *Escherichia coli* of serotype O157:H7 to human epithelial cells in tissue culture: role of outer membranes as bacterial adhesins. *J Med Microbiol* 1988; **26**: 11–17.

7. Karch H, Heesemann J, Laufs R, O'Brien AD, Tackett CO, Levine MM. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. *Infect Immun* 1987; **55**: 455–461.
8. Louie M, De Azavedo JCS, Handelsman MYC *et al.* Expression and characterization of the *eaeA* gene product of *Escherichia coli* serotype O157:H7. *Infect Immun* 1993; **61**: 4085–4092.
9. Sherman P, Cockerill F, Soni R, Brunton J. Outer membranes are competitive inhibitors of *Escherichia coli* O157:H7 adherence to epithelial cells. *Infect Immun* 1991; **59**: 890–899.
10. Toth I, Cohen ML, Rumschlag HS *et al.* Influence of the 60-megadalton plasmid on adherence of *Escherichia coli* O157:H7 and genetic derivatives. *Infect Immun* 1990; **58**: 1223–1231.
11. Tzipori S, Gibson R, Montanaro J. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. *Infect Immun* 1989; **57**: 1142–1150.
12. Tzipori S, Karch H, Wachsmuth KI *et al.* Role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. *Infect Immun* 1987; **55**: 3117–3125.
13. Beebakhee G, Louie M, De Azavedo J, Brunton J. Cloning and nucleotide sequence of the *eae* gene homologue from enterohemorrhagic *Escherichia coli* serotype O157:H7. *FEMS Microbiol Lett* 1992; **91**: 63–68.
14. Yu J, Kaper JB. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* 1992; **6**: 411–417.
15. Padhye NV, Doyle MP. Production and characterization of a monoclonal antibody specific for enterohemorrhagic *Escherichia coli* of serotypes O157:H7 and O26:H11. *J Clin Microbiol* 1991; **29**: 99–103.
16. Clark CG, Johnson S, Johnson RP. Further characterisation of a monoclonal antibody reactive with *Escherichia coli* O157:H7. *J Med Microbiol* 1995; **43**: 262–269.
17. Hoffman CS, Wright A. Fusion of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. *Proc Natl Acad Sci USA* 1985; **82**: 5107–5111.
18. Manoil C, Beckwith J. *TnphoA*: A transposon probe for protein export signals. *Proc Natl Acad Sci USA* 1985; **82**: 8129–8133.
19. Donnenberg MS, Calderwood SB, Donohue-Rolfe A, Keusch GT, Kaper JB. Construction and analysis of *TnphoA* mutants of enteropathogenic *Escherichia coli* unable to invade HEP-2 cells. *Infect Immun* 1990; **58**: 1565–1571.
20. Jerse AE, Kaper JB. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by EAF plasmid. *Infect Immun* 1991; **59**: 4302–4309.
21. Taylor RK, Manoil C, Mekalanos JJ. Broad-host-range vectors for delivery of *TnphoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *J Bacteriol* 1989; **171**: 1870–1878.
22. Hjelm LM, Chrambach A. Solubilization of functional membrane-bound receptors. In: Venter JC, Harrison LC (eds) *Membrane, detergents and receptors solubilization*. New York, Alan R. Liss. 1984: 35–46.
23. Schagger H, van Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100 kDa. *Anal Biochem* 1987; **166**: 368–379.
24. Lech K, Brent R. Minipreps of plasmid DNA. In: Ausubel FM, Brent R, Kingston RE *et al.* (eds) *Short protocols in molecular biology—a compendium of methods from Current Protocols in Molecular Biology*. New York, John Wiley and Sons. 1989: 19–20.
25. Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE *et al.* (eds) *Short protocols in molecular biology—a compendium of methods from Current Protocols in Molecular Biology*. New York, John Wiley and Sons. 1989: 61–63.
26. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983; **132**: 6–13.
27. Junkins AD, Doyle MP. Comparison of adherence properties of

- Escherichia coli* O157:H7 and a 60-megadalton plasmid-cured derivative. *Curr Microbiol* 1989; **19**: 21–27.
28. Daniel WW. Hypothesis testing. In: Daniel WW (ed) *Biostatistics: a foundation for analysis in the health sciences*. New York, John Wiley and Sons. 1987: 188–272.
 29. Berry JT, Doyle MP, Schoeni JL. Colonization of chicken cecae by *Escherichia coli* associated with hemorrhagic colitis. *Appl Environ Microbiol* 1985; **49**: 310–315.
 30. Ashkenazi S, May L, LaRocco M, Lopez EL, Cleary TG. The effect of postnatal age on the adherence of enterohemorrhagic *Escherichia coli* to rabbit intestinal cells. *Pediatr Res* 1991; **29**: 14–19.
 31. Leroy SM, Lesage M-C, Chaslus-Dancla E, Lafont JP. Presence of *eaeA* sequences in pathogenic and non-pathogenic *Escherichia coli* strains isolated from weaned rabbits. *J Med Microbiol* 1994; **40**: 90–94.
 32. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci USA* 1995; **92**: 1664–1668.
 33. Jarvis KG, Girón JA, Jerse AE, McDaniel TK, Donnenberg MS, Kaper JB. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci USA* 1995; **92**: 7996–8000.
 34. Rocque WJ, Coughlin RT, McGroarty EJ. Lipopolysaccharide tightly bound to porin monomers and trimers from *Escherichia coli* K-12. *J Bacteriol* 1987; **169**: 4003–4010.
 35. Wu M-C, Heath EC. Isolation and characterization of lipopolysaccharide protein from *Escherichia coli*. *Proc Natl Acad Sci USA* 1973; **70**: 2572–2576.
 36. Bilge SS, Varu JC, Potter K, Dowell SF, Tarr PI. *Escherichia coli* O157:H7 lipopolysaccharide mutants are hyperadherent, abstr. B-2. Abstracts of the 95th Annual Meeting of the American Society for Microbiology, Washington, DC, USA. 1995: 166.