

BACTERIAL PATHOGENICITY

An in-vitro model for studying the interaction of *Escherichia coli* O157:H7 and other enteropathogens with bovine primary cell cultures

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Sections of kidney, trachea, ileum, colon, rectum and rumen were removed at post mortem from a neonatal calf and, with the exception of the rumen, primary cell lines were established for each of the cell types. The adherence of enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, enteropathogenic *E. coli* (EPEC) serotype O111, *E. coli* K12 (a laboratory adapted non-pathogenic strain) and *Salmonella enterica* serotype Typhimurium was assayed on each cell type. For all adherence assays on all cell lines, EHEC O157:H7 adhered to a significantly greater extent than the other bacteria. *S. Typhimurium* and EPEC O111 adhered to a similar extent to one another, whereas *E. coli* K12 was significantly less adherent by 100-fold. In all cell types, >10% of adherent *S. Typhimurium* bacteria invaded, whereas *c.* 0.01–0.1% of adherent EHEC O157:H7 and EPEC O111 bacteria invaded, although they are regarded as non-invasive. EHEC O157 generated actin re-arrangements in all cell types as demonstrated by fluorescent actin staining (FAS) under densely packed bacterial micro-colonies. EPEC O111 readily generated the localised adherent phenotype on bovine cells but generated only densely packed micro-colonies on HEp-2 cells. The intensity of actin re-arrangements induced in bovine cells by EPEC O111 was less than that induced by EHEC O157:H7. The intimate attachment on all cell types by both EHEC O157:H7 and EPEC O111 was clearly demonstrated by scanning electron microscopy.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 infection of man is a food-borne zoonosis of considerable current public concern. Infection is associated with a distinctive gastrointestinal illness of severe bloody diarrhoea without fever, termed haemorrhagic colitis (HC), first described by Riley *et al.* [1]. Sequelae may include acute renal failure, thrombocytopenia and micro-angiopathic haemolytic anaemia, designated haemorrhagic uraemic syndrome (HUS), first recognised by Karmali *et al.* [2]. Infection is potentially fatal in the elderly and the very young and major incidents in the USA, Japan and the UK have been documented [3–5].

Molecular pathogenesis studies of EHEC O157:H7 indicate that virulence determinants and their regulatory factors are encoded by the chromosome, a 60-Mda plasmid and by toxin-converting bacteriophage [6]. The role of fimbriae in adherence remains unclear [7–9]. Transfer of the pO157 60-Mda plasmid to *E. coli* K12 resulted in elaboration of a novel fimbria by *E. coli* K12 and enhanced adherence to intestinal cells [7]. Other studies have demonstrated that O157 isolates without plasmids adhere to Henle-407 cells and that most O157 isolates are non-fimbriate [10, 11]. The principal factors for *E. coli* O157 colonisation and adherence to the intestinal epithelium are intimin and the signal transduction apparatus. Intimin is an outer-membrane protein of 94–97 kDa which is encoded by the *eaeA* gene located within a chromosomally encoded pathogenicity island [12]. The signal transduction apparatus comprises a number of secreted proteins (EspA) that form a filament and a pore in the enterocyte (EspB and D) to deliver the Tir effector protein in to the enterocyte [13–15]. The result is intimate attachment of the bacterium to the enterocyte

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surface mediated by bacterial docking proteins with actin re-arrangements causing pedestal formation and consequent effacement of villi.

EHEC O157:H7 are present in the faecal flora of a number of animals including cattle, sheep, goats, pigs, dogs, cats, chickens and gulls, with the primary source of infection for man considered to be cattle and bovine-derived meat products [16]. In conventional and gnotobiotic pigs, EHEC O157:H7 produces extensive 'attaching and effacing' lesions, whereas *eaeA* mutants defective for the elaboration of intimin failed to colonise [12, 17, 18]. Calves and older cattle may be readily infected with *E. coli* O157:H7, both naturally and experimentally, with mild transient diarrhoea in calves and an asymptomatic transient carrier status in older animals [19]. The bacterium may persist in cattle for many months, but generally persists for about 30 days or less [20, 21]. In experimental infections, Dean-Nystrom *et al.* [22, 23] showed that with a dose of 10^{10} cfu of a five-strain mixture of EHEC O157:H7, normal neonatal calves of 12–36 h of age gave attaching and effacing lesions in both small and large intestines and lesions were detectable in the rectum of 3-month-old weaned calves. These data indicate that the mechanisms for intimate attachment encoded by the locus of enterocyte effacement of EHEC O157:H7 do mediate mucosal colonisation in bovines. However, there is still little evidence for mucosal colonisation of naturally infected bovines.

Studies are underway in this laboratory to investigate the mechanisms by which EHEC O157:H7 persists in the bovine gastrointestinal tract. This paper describes the derivation of bovine primary cell cultures for adherence assays.

Materials and methods

Bacterial strains and inocula

EHEC O157:H7 strain VT⁻ (NCTC 12900) was confirmed by gene probe analysis to possess *eaeA*, *sepABCD*, *espA*, *etpD*, *katP* and *hlyA* and to lack genes encoding VT1 and VT2. Southern hybridisation of the strain NCTC 12900 plasmid profile confirmed the presence of *hlyA* on a plasmid *c.* 60 MDa in size, assumed to be the highly conserved pO157 plasmid, and no other plasmids were observed. Enteropathogenic *E. coli* serotype O111 strain NM:B171 was isolated from an infant with diarrhoea [24] and was kindly provided by Professor Gordon Dougan (Imperial College, University of London). *Salmonella enterica* serotype Typhimurium DT104 strain 3530 was a bovine isolate obtained from the reference collection at the Veterinary Laboratory Agency (Weybridge) (Addlestone, Surrey). *E. coli* K12 strain DH5 α was from a commercial source (Gibco-BRL). All bacteria were maintained on Dorset's egg medium at room temperature. To prepare inocula, each strain was streaked to

single colonies on Blood Agar (Oxoid) and a sweep of up to 20 colonies was taken into nutrient broth and grown overnight at 37°C. Numbers of viable bacteria were determined by serial dilution and plating on to nutrient agar. Confirmation of the serotype of bacteria recovered from these experiments was by slide agglutination with appropriate sera (VLA, Weybridge).

Development of primary bovine cell lines

Sections of the alimentary tract including the rumen, ileum, colon and rectum, and also trachea and kidney were removed aseptically from a necropsied calf that had been taken at birth and deprived of colostrum. Tissues were treated separately. Sections of ileum, colon and rectum were tied at one end with sterile catgut and the luminal surface was washed three times with pre-warmed Ringer's solution before adding 5 ml of Accutase (Tissue Culture Services, Botolph Claydon, Bucks) to enzymically detach the outer layer of cells. After incubation for 60 min at 37°C, the Accutase was removed and centrifuged at 100 *g* for 5 min. The resulting pellets were washed twice in Ringer's solution and resuspended in complete tissue-culture medium containing L-glutamine (Sigma) 1% non-essential amino acids (NEAA; Sigma) 1% fetal calf serum (FCS; Sigma) 10% and gentamicin (Sigma) 50 mg/L in Eagle's Minimal Essential Medium (MEM; Sigma). For the rumen, small pieces (*c.* 1 cm²) of tissue were prepared and the top layer of cells was scraped into pre-warmed Ringer's solution. The cell preparation was passed through a coarse mesh to remove excess tissue and the cell suspension was centrifuged at 100 *g* for 5 min. The pellet was resuspended in Accutase and the cells were digested for 60 min at 37°C. Cells were pelleted by centrifugation and resuspended in complete tissue-culture medium.

The trachea was cut into small rings (*c.* 5-mm lengths) and bathed in complete medium, whereas a cell suspension was prepared from the bovine kidney by the method described by Youngner [25]. All tissue preparations were placed in eight-well tissue-culture plates (Nunc) and incubated at 37°C at 95% humidity; the normal air phase was supplemented with CO₂ 5%. The primary cells were lightly trypsinised every second day to selectively remove fibroblasts. At the earliest opportunity the primary cells were detached enzymically from the tissue-culture plastic, suspended in cryopreservation medium (dimethyl-sulphoxide 8% FCS 92% and secured in liquid nitrogen. A seed stock of 10 vials was prepared from each cell type that had grown *in vitro*.

Cells

HEp-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury). Transformed and primary cell types were maintained in complete medium in 75-cm² flasks at 37°C in air with

CO₂ 5% and passaged twice weekly. To suspend, the cells were washed and then incubated in Accutase for 15 min at 37°C (CO₂ 5%). For confluent monolayers, 24-well tissue-culture plates were seeded with 1×10^5 cells/well and incubated for 2 days at 37°C (CO₂ 5%). Confluent monolayers contained (4–6) $\times 10^5$ cells/well.

Association and invasion assays

Tissue-culture assays were performed by the method described previously [26]. Briefly, duplicate plates containing confluent monolayers of cells were washed in Hanks's Balanced Salts Solution (HBSS; Sigma) and 1 ml of incomplete medium, containing NEAA 1%, L-glutamine 1% and EMEM 98% was added per well. The monolayers were infected with 10 μ l of the inoculum as described above containing *c.* 1×10^7 viable bacterial cells and the monolayers were incubated for 3 h at 37°C (CO₂ 5%). For the association assay, the monolayers were washed six times with HBSS before disrupting with Triton (Sigma) 1% v/v. Numbers of bacteria (cfu) were determined by plating on to nutrient agar. Association is reported as a combination of adhering and invading bacteria.

For invasion, the assay was performed as described for the association assay, but after allowing the bacteria to associate with the monolayer, wells were washed three times in HBSS before adding EMEM containing gentamicin 100 mg/L to kill bacteria external to the cells. Monolayers were incubated at 37°C (CO₂ 5%) for 2 h, washed twice, disrupted with Triton and the number of cfu was determined.

Association and invasion assays were performed three times (two wells per test). For statistical analyses, counts were transformed to their log₁₀ and analyses of variance (ANOVA) performed, followed by standard *t* tests.

Fluorescent actin staining

The fluorescent actin staining (FAS) patterns were investigated following the method described by Knutson *et al.* [27]. Bovine kidney, trachea, ileum, colon, rectum and HEp-2 cells were grown to confluence on coverslips as described previously [26] and were infected with 1×10^7 bacteria from an overnight culture of EHEC O157 VT⁻, EPEC O111 and *E. coli* K12. Monolayers were washed with HBSS, fixed with buffered formalin, permeabilised with Triton 0.1%, probed with a phalloidin-FITC conjugate and mounted on to microscope slides with DPX mountant. Slides were observed by fluorescence microscopy at $\times 1000$ magnification with a Zeiss light microscope (Axiovert 25) equipped with a CONTAX 167MT camera.

Scanning electron microscopy (SEM)

Confluent monolayers of bovine kidney, trachea, ileum, colon, rectum and HEp-2 cells were prepared on 13-mm coverslips within 24-well tissue-culture plates. Monolayers were infected with bacterial strains as described for the association and invasion assays and incubated for 3 h at 37°C (CO₂ 5%). Infected monolayers were washed four times with HBSS and replaced with incomplete medium for a further 3-h incubation. Monolayers were washed three times, fixed in glutaraldehyde 3% and observed by scanning electron microscopy as described previously [28].

Results

Development of primary bovine cell lines

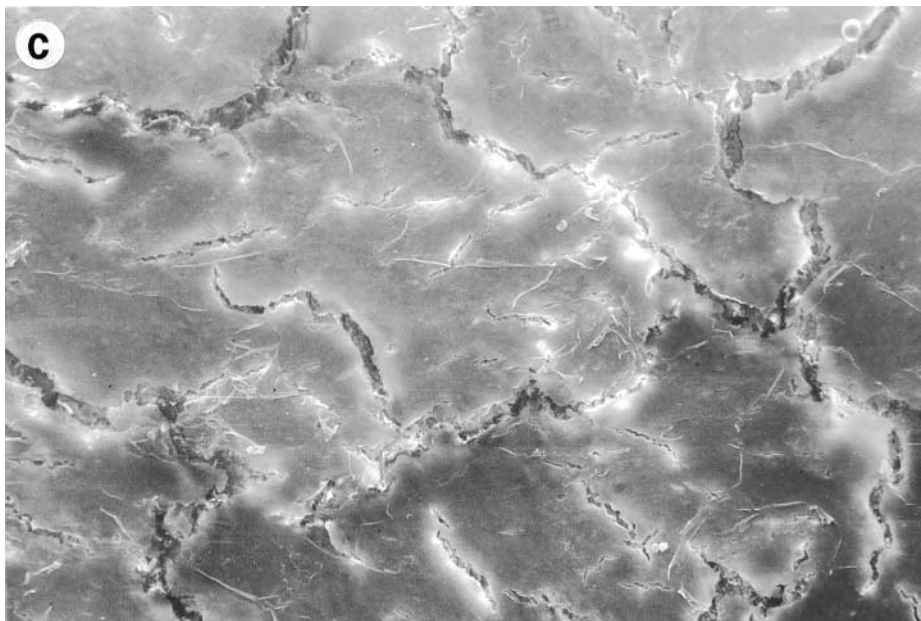
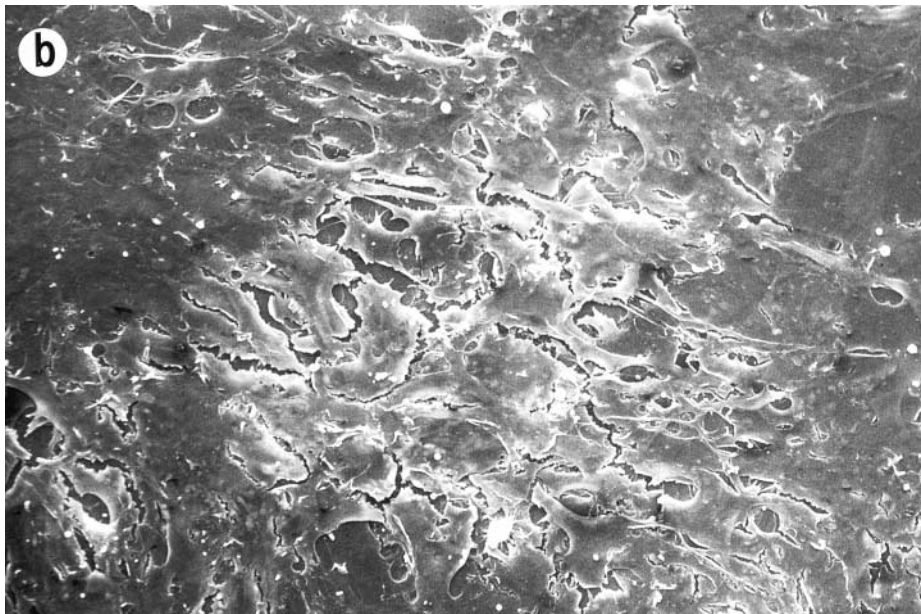
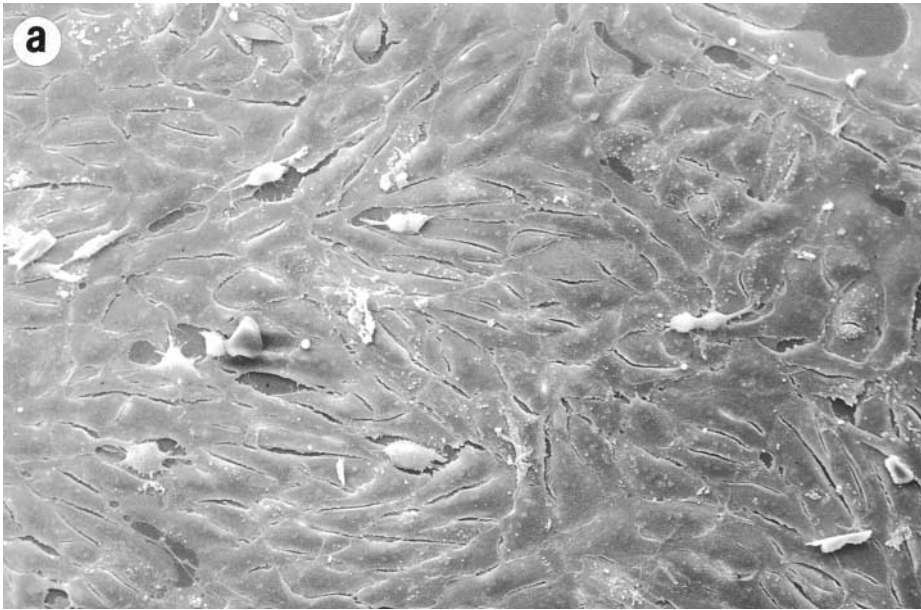
Islands of cells were observed microscopically within 7 days and these monolayers were expanded successfully into larger tissue-culture flasks. At the earliest opportunity, the primary cells were detached enzymically from the tissue-culture plastic and secured in liquid nitrogen. A seed stock of 10 vials was prepared for cells originating from the kidney, trachea, ileum, colon and rectum, but cells derived from the rumen were non-viable in tissue culture. Frozen cells were reconstituted successfully and growth characteristics of the cells and morphological observations suggested that these were 'epithelial-like' (Fig. 1).

Interaction of bacteria with cells

Association and invasion assays demonstrated that each of the three *E. coli* serotypes and *S. Typhimurium* interacted with each of the different bovine cell types. A similar trend was observed for the total number of bacteria associating for each bovine cell type (Fig. 2). *E. coli* K12 associated with lowest numbers – between 10- and 100-fold lower, depending on cell type, than EHEC O157:H7, which adhered with the highest numbers ($p < 0.05$). There were no statistical differences between the association of the EPEC O111 strain and the *S. Typhimurium* strain 3530 with bovine cells derived from the kidney, trachea or colon.

The numbers of bacteria associating with HEp-2 cells showed a similar trend. There were no significant differences between EPEC O111 and *S. Typhimurium* ($p < 0.354$), but differences were significant between the other strains ($p < 0.05$) whereby EHEC O157:H7 adhered with the highest numbers and *E. coli* K12 the lowest.

More than 10% of adherent *S. Typhimurium* invaded each of the bovine-derived cell types or HEp-2 cells, whereas in general $< 0.1\%$ of adherent EHEC O157:H7 and EPEC O111 invaded. *E. coli* K12 was shown to invade but at the limits of detection and, with the notable exception of bovine kidney cell, significantly



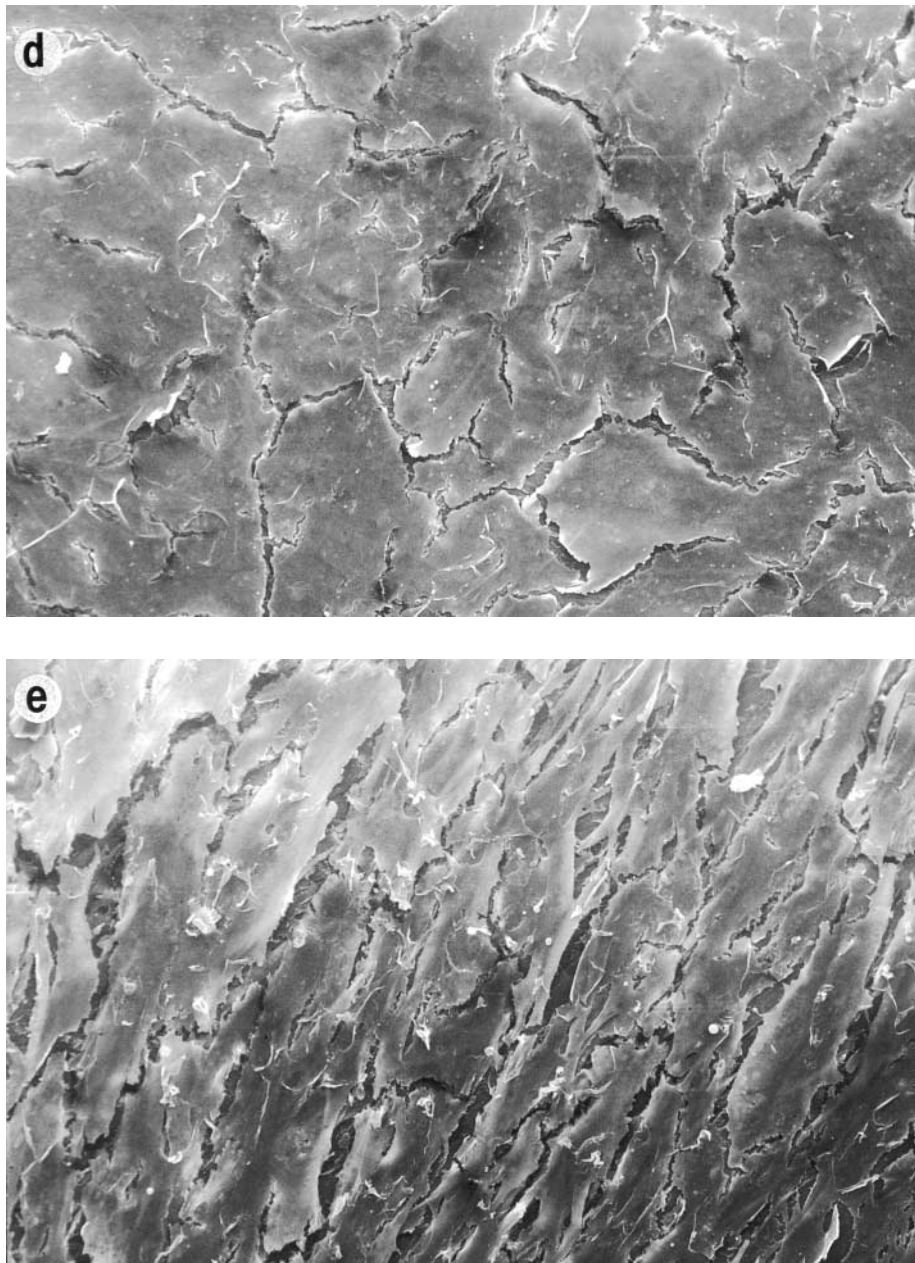


Fig. 1. Electron micrographs of bovine cells derived from (a) kidney, (b) trachea, (c) ileum, (d) colon and (e) rectum. Magnification $\times 280$.

less than the other bacteria ($p < 0.05$). For invasion with all cell types, the mean counts for *S. Typhimurium* were significantly higher ($p < 0.05$) than other bacteria.

Electron microscopical analyses of adherence

Localised adherence by EHEC O157:H7 and EPEC O111 was observed on cells derived from bovine kidney, trachea, ileum, colon and rectum and also the HEP-2 cells, whereas a diffuse colonisation pattern was observed with *E. coli* K12 and *S. Typhimurium* strain 3530 on all cell types. Examples of adherence pattern are shown in Fig. 3 and a description of the type of adherence pattern for each bacterial type with each cell type is listed in Table 1. Densely packed bacterial cell clusters or micro-colonies were formed by EHEC

O157:H7 on all cell types, but were more numerous on cells derived from the colon. Micro-colonies were formed by EPEC O111 on HEP-2 cells but not on primary bovine cells (Table 1). A shrinkage of the cells was observed by electron microscopy following localised adherence by EHEC O157:H7 and EPEC O111 (Fig. 3).

Cytoskeletal re-arrangements

Interactions of EHEC O157:H7 and EPEC O111 with HEP-2 cells caused a strong localised cytoskeletal re-arrangement induced by the polymerisation of actin filaments (Table 2, Fig. 4). With all primary bovine cells, cytoskeletal re-arrangements were observed upon interactions with EHEC O157:H7 and EPEC O111.

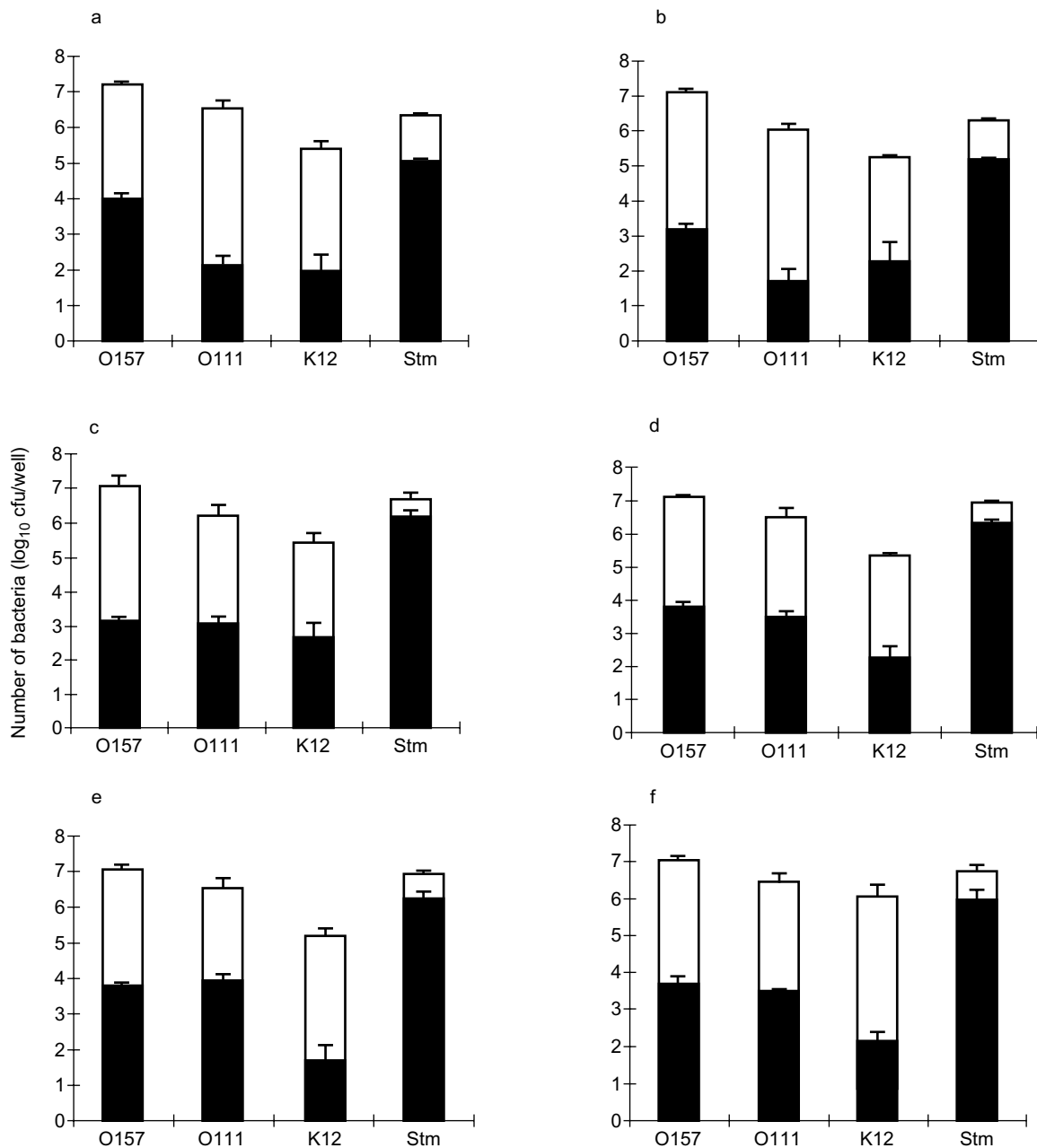


Fig. 2. Association with (□) and invasion of (■) (a) HEp-2 cells, and bovine primary cells derived from (b) kidney, (c) trachea, (d) ileum, (e) colon and (f) rectum, following infection with EHEC O157, EPEC O111, *E. coli* K12 and *S. Typhimurium* (Stm). Bars = SEM.

However, the intensity of the FAS reaction was greater with EHEC O157:H7 than for EPEC O111. For both EHEC O157:H7 and EPEC O111 the most intensive lesions were observed with HEp-2 cells. Lesions were not observed on primary bovine cells or HEp-2 cells following infection with *E. coli* K12 or *S. Typhimurium* strain 3530.

Discussion

The aim of this study to prepare bovine primary gastrointestinal epithelial-derived cells was achieved and the cells were used to assess adherence and invasion with several test bacteria, including EHEC

O157:H7. To the best of our knowledge this is the first report of the preparation of bovine primary gastrointestinal-derived cells for specific use in studies with EHEC. Other studies have focused primarily on the interaction between EHEC O157 and human-derived cell lines such as Henle, HeLa, HEp-2, T84, HCT-8 and HEL cells as reported by Tarr and Bilge [29]. Cattle are reservoirs of EHEC O157 and yet relatively little is understood of the nature of the interaction between EHEC O157 and bovine cells, especially as there is a need to understand the mechanisms of persistence in this host.

The morphology of cells derived from each of the five tissue types was consistent with epithelial cells and

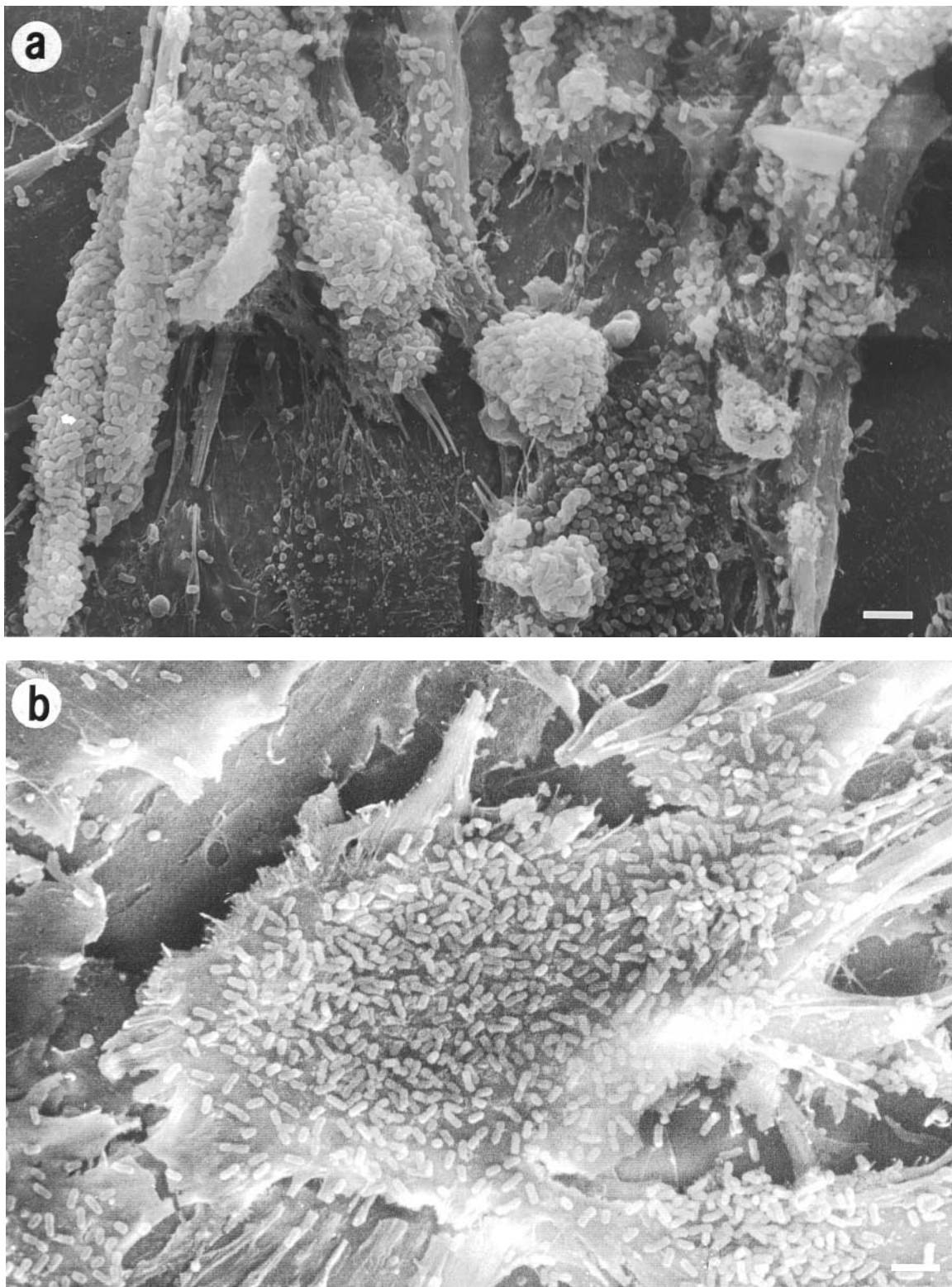


Fig. 3. Scanning electron micrographs showing the patterns of interaction of (a) EHEC O157:H7 strain NCTC 12900 and (b) EPEC O111 strain NM:B171 with primary bovine cells from the colon. Bars = 5 μ m.

microscopic examination taken in conjunction with growth characteristics of the primary cultures indicated that no fibroblastic cells were present in monolayers used for adherence and invasion assays. However, no quantifiable evidence was presented to demonstrate total absence of fibroblastic cells. Thus, it is debatable whether the potential presence of very low numbers of

fibroblastic cells may alter the interpretation of the observations.

No significant differences in either adherence or invasion by *S. Typhimurium* were observed between any of the five cell types. *S. Typhimurium* was considered an appropriate positive control serotype

Table 1. Summary of patterns of interaction of *E. coli* and *S. Typhimurium* with HEp-2 and bovine primary cells

Bacterial strain	Adherence to					
	Primary bovine cells					
	Kidney	Trachea	Ileum	Colon	Rectum	HEp-2
EHEC O157 strain NCTC12900	*LA	*LA	*LA	*LA	*LA	*LA
EPEC O111 strain NM:B171	LA	LA	LA	LA	LA	*LA
<i>E. coli</i> K12 strain DH5 α	DA	DA	DA	DA	DA	DA
<i>S. Typhimurium</i> strain 3530	DA	DA	DA	DA	DA	DA

LA, localised adherence; DA, diffuse adherence.

*Formation of densely packed micro-colonies.

Table 2. Fluorescent actin staining (FAS) of bovine primary cells and HEp-2 following interaction with *E. coli* and *S. Typhimurium*

Bacterial strain	FAS effects demonstrated in					
	Primary bovine cells					
	Kidney	Trachea	Ileum	Colon	Rectum	HEp-2
EHEC O157 strain NCTC12900	++	++	++	++	++	+++
EPEC O111 strain NM:B171	+	+	+	+	+	+++
<i>E. coli</i> K12 strain DH5 α	-	-	-	-	-	-
<i>S. Typhimurium</i> strain 3530	-	-	-	-	-	-

+++ , high intensity FAS lesions formed; ++ , medium intensity FAS lesions formed; + , low intensity FAS lesions formed; - , no FAS lesions formed.

because of its association with bovine calf diarrhoea [30]. Consistent with reports with other cells and cell lines, >10% of adherent *S. Typhimurium* invaded [31]. *E. coli* K12 strain DH5 α adhered less well than all other bacterial isolates tested and invasion was detectable, but only at the level of sensitivity of the methods used. *E. coli* K12 is regarded as non-invasive and it is possible that incubation for 3 h, as done in these studies, may be associated with invasion. This finding was either an artefact of the method or, under the conditions used, these cells did facilitate bacterial uptake or were susceptible to invasion by as yet unknown mechanisms.

Of the two pathogenic *E. coli* serotypes tested, the EHEC O157:H7 isolate adhered in significantly greater numbers to all bovine tissue types than the EPEC O111 isolate or the *S. Typhimurium* control strain. This suggests that EHEC O157:H7 may be particularly well adapted to adhere to bovine cells. However, considerable care must be taken in drawing such conclusions because these studies were performed with single isolates and the findings may not be representative of the various serotypes tested. Further studies with a library of O157:H7 isolates of different provenance in adherence and invasion assays are in hand to assess this possibility. Matthews *et al.* [32] demonstrated adherence and invasion of bovine MAC-T mammary secretory and MDBK kidney epithelial cell lines by 12 distinct EHEC O157:H7 strains. Thus, invasion of the bovine cells in this study was anticipated, albeit significantly lower by a factor of 3–4-fold than for *S. Typhimurium*. MDBK cells are

an established line and it would be of interest to compare the adherence and invasion patterns of the bacterial strains used in this study between these and the primary kidney cells derived here. Invasion, as in passing the gut barrier, has yet to be reported in any whole animal studies and invasion of primary cells by EHEC O157:H7 indicates a limitation in the use of these cells as a model. For example, primary cells were in an inappropriate physiological state *in vitro* and, therefore, susceptible to invasion. However, this should not invalidate their use for more detailed studies on the initial interaction between EHEC O157:H7 and bovine cells.

The localised adherence (LA) pattern of EPEC and EHEC has been characterised previously in established cell lines [27]. The present study demonstrated that EHEC O157 gave an intense FAS reaction on all bovine tissue types, and that adherence was typical with well-developed localised micro-colony formation with some additional diffuse adherence. Similar observations have been made regarding the interaction of EHEC O157:H7 and human cells [10, 33, 34]. In contrast, EPEC O111 gave an intense FAS reaction on HEp-2, but weaker intensity with bovine primary cells, and adherence was typically localised without micro-colony formation, except on HEp-2 cells only. The clear phenotypic differences observed between the EHEC and EPEC strains tested may support the conclusion that EHEC were better adapted to colonising bovine tissues than EPEC. Whilst this is an unsurprising conclusion, because EPEC are primarily human pathogens, the data also suggest that these

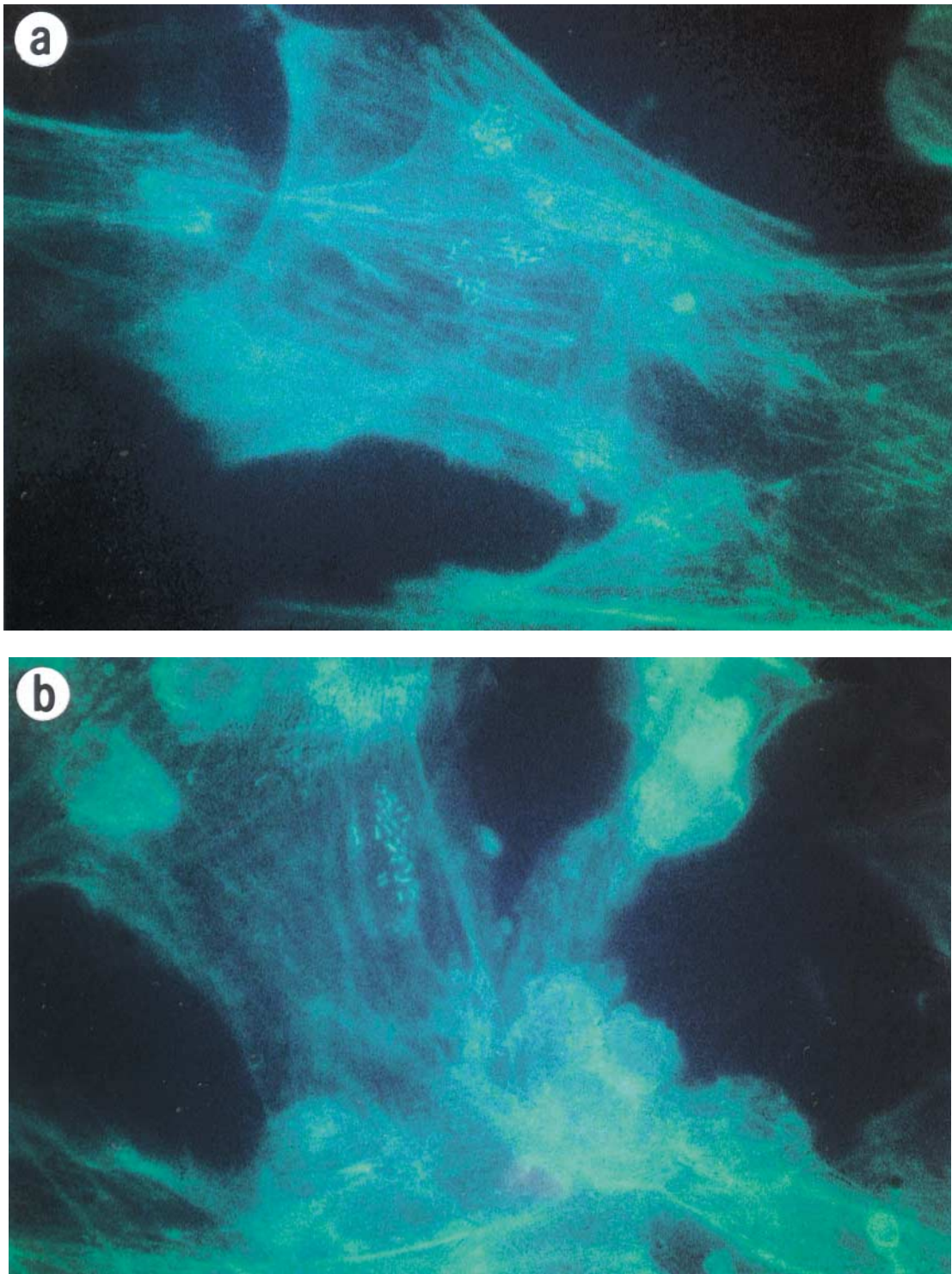


Fig. 4. Fluorescent actin staining of primary bovine cells from the colon following interaction with (a) EHEC O157:H7 strain NCTC 12900 and (b) EPEC O111 strain NM:B171. Magnification $\times 1000$.

primary cells may possess bovine-specific factors that enhance EHEC adherence.

Phillips *et al.* [35] demonstrated a cascade of complex events leading to intimate attachment of EPEC O111 and EPEC O127 to HEp-2 cells. These authors showed transient elaboration of microvillus-like projections (MLPs) emanating from the HEp-2 cells that appeared within 20 min of the addition of bacteria to the cells. By 60 min, the MLPs had confined and recruited

bacteria that would subsequently become bound intimately. This process was intimin dependent. Furthermore, these processes were not observed in similar experiments with CaCo cells. It would be interesting to test whether similar events occur in the interaction between EHEC O157 and bovine-derived cells. Adu-Bobie *et al.* [36] established that there are at least four intimin types (α , β , γ and δ) of which the variant C-terminal portion is associated with tissue tropism [37–39]. EPEC possess β -intimin and attach

preferentially to the mucosa of the small intestine, whereas EHEC possess γ -intimin and attach preferentially to the mucosa of the large intestine. However, no obvious differences in adherence of EHEC O157:H7 to bovine primary cell cultures correlated with this observation.

We conclude that adherence of EHEC O157:H7 to the bovine cells was, in part, dependent upon the locus for enterocyte effacement because actin re-arrangement was visualised by FAS, invariably at the site of micro-colony formation. This observation is compatible with the development of attaching and effacing lesions by EHEC O157:H7 in bovine colonic explant experiments [40]. However, there were diffusely adherent EHEC O157:H7 bacteria that were not associated with actin re-arrangement and the question arises as to what mechanism mediated this adherence. The present studies were not performed in the presence of mannose to block the type 1 fimbrial adhesin (FimH) activity. Although Durno *et al.* [41] suggested that type 1 fimbriae mediated mannose sensitive adherence of EHEC O157:H7 strain CL-49 to epithelial cells, Sajjan and Forstner [42] and more recently Enami *et al.* [43] showed that strain CL-49 was unique because of >40 other EHEC O157 strains tested none elaborated type 1 fimbriae *in vitro*. The EHEC O157:H7 strain used in the present study lacked type 1 fimbriae (data not shown). However, it remains unclear whether EHEC O157:H7 may express type 1 fimbriae *in vivo*. Both Bilge *et al.* [33] and Cockerill *et al.* [34] demonstrated that transposon mutants unable to elaborate O157 LPS were more adherent to HEp-2 cells than the wild-type. Therefore, it seems unlikely that either lipopolysaccharide or type 1 fimbriae played a role in adherence and it remains possible that EHEC O157:H7 adherence to bovine tissues may be mediated by both locus for enterocyte effacement-dependent and independent pathways, as previously suggested by Tarr and Bilge [29].

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