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SHORT COMMUNICATION

Porcine intestinal epithelial cell lines as a new in vitro model for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) infections result in large economic losses in the swine industry worldwide. The organism causes diarrhea by adhering to and colonizing enterocytes in the small intestines. While much progress has been made in understanding the pathogenesis of ETEC, no homologous intestinal epithelial cultures suitable for studying porcine ETEC pathogenesis have been described prior to this report. In the current study, we investigated the adherence of various porcine ETEC strains to two porcine (IPEC-1 and IPEC-J2) and one human (INT-407) small intestinal epithelial cell lines. Each cell line was assessed for its ability to support the adherence of *E. coli* expressing fimbrial adhesins K88ab, K88ac, K88ad, K99, F41, 987P, and F18. Wild-type ETEC expressing K88ab, K88ac, and K88ad efficiently bound to both IPEC-1 and IPEC-J2 cells. An ETEC strain expressing both K99 and F41 bound heavily to both porcine cell lines but an *E. coli* strain expressing only K99 bound very poorly to these cells. *E. coli* expressing F18 adhesin strongly bound to IPEC-1 cells but did not adhere to IPEC-J2 cells. The *E. coli* strains G58-1 and 711 which express no fimbrial adhesins and those that express 987P fimbriae failed to bind to either porcine cell line. Only strains B41 and K12:K99 bound in abundance to INT-407 cells. The binding of porcine ETEC to IPEC-J2, IPEC-1 and INT-407 with varying affinities, together with lack of binding of 987P ETEC and non-fimbriated *E. coli* strains, suggests strain-specific *E. coli* binding to these cell lines. These findings suggest the potential usefulness of porcine intestinal cell lines for studying ETEC pathogenesis.

Keywords: porcine, enterotoxigenic *Escherichia coli,* intestinal epithelial cells, adherence, pathogenesis

1. Introduction

Both porcine neonatal and post-weaning diarrhea (PWD) caused by enterotoxigenic *Escherichia coli* (ETEC) result in significant morbidity and mortality and are economically important diseases of pigs (Fairbrother *et al.*, 2005; Nagy and Fekete, 2005). The colonization of ETEC in the small intestine is primarily mediated by fimbria which confer to ETEC the ability to attach to receptors on the enterocytes. Secretory diarrhea associated with ETEC infection is mediated by any of several enterotoxins which include heat labile enterotoxin (LT), heat stable enterotoxin-a (STa), and enterotoxin-b (STb). The most common adhesins of porcine ETEC include K88 (F4) (Jones and Rutter, 1972), K99 (F5) (Moon *et al.*, 1977), 987P (F6) (Isaacson *et al.*, 1978), F18 (Imberechts *et al.*, 1996), and F41 (Morris *et al.*, 1982). Three serological antigenic variants of K88 fimbrae exist, K88ab, K88ac, and K88ad (Gaastra and Pederson, 1986), and K88ac is the most prevalent and clinically important variant ETEC strain isolated from diarrheic pigs (Fairbrother *et al.*, 2005; Francis *et al.*, 1998; Nagy and Fekete, 2005).

A number of cellular systems had been used to study the adherence of ETEC, including erythrocytes (Evans *et al.*, 1979), primary enterocytes (Knutton *et al.*, 1984), brush border vesicles (Baker *et al.*, 1997), and human tumor cell lines (Roselli *et al.*, 2006). None of these cellular systems are highly suitable for porcine ETEC pathogenesis studies. The objective of this study was to examine the adherence of various strains of ETEC to two porcine intestinal epithelial cell lines IPEC-J2 and IPEC-1 and to compare their adherence to the human intestinal epithelial cell line INT-407. The findings of this study indicate that IPEC-J2 and IPEC-1 cell lines are superior to the human intestinal cell line (INT-407) in that they support the adherence of most porcine ETEC strains.

2. Materials and methods

2.1. Bacterial strains

All *E. coli* strains used in this study are listed, and their phenotypes described in Table 1. These strains were cultured on 5% sheep blood agar (brain heart

Table 1. Bacterial strains used for the cell adhesion assay

E. coli strains	Relevant properties	References
263	Wild-type O8:K87:K88ab ETEC, LT+, STb+	(Moon *et al.*, 1968)
1476	Laboratory construct K12:K88ab[a]	(Baker *et al.*, 1997)
F962	K12: K88ab 5K, amp^r, pFM205	(Bakker *et al.*, 1992)
3030-2	Wild-type O157:K87:K88ac ETEC, LT+, STb+	(Francis and Willgohs, 1991)
K12:K88ac	Laboratory construct K12:K88ac[a]	(Baker *et al.*, 1997)
F783 = F963	K12:K88ac C600 amp^r, pDB88-102	(Bakker *et al.*, 1992)
1836-2	Wild-type K88ac+; LT–, ST-, *ast*A+	(Zhang *et al.*, 2006)
2534-86	Wild-type O8:K87:NM: K88ac LT-I+, STb+	(Moxley *et al.*, 1998)
WAM 2317	Nalidixic acid-resistant mutant of 2534-86, LT-I+, STb+	(Berberov *et al.*, 2004)
MUN 285	Mutant of WAM 2317, LT-I-	(Berberov *et al.*, 2004)
MUN 287	Nal^r Km^r, LT-I+ complemented strain	(Berberov *et al.*, 2004)
Morris	Wild-type O8:K87:K88ad ETEC, LT+, STb+	(Baker *et al.*, 1997)
K12:K88ad	Laboratory construct K12:K88ad[a]	(Baker *et al.*, 1997)
F291	K12:K88ad 5K, amp^r, pBad1	(Gaastra and Pederson, 1986)
2134	Wild-type, O157:H19:F18ac, 4P-, STa+, STb+	(Casey *et al.*, 1992)
K12:K99	Laboratory construct K12:K99[a]	(Moon *et al.*, 1977)
B41	Wild-type, O101:K99; F41; STa+	(Orskov *et al.*, 1975)
1194	Wild-type, O141:987P, STa+, STb+	(Mullaney *et al.*, 1991)
G58-1	Non-pathogenic wild-type, O101:K28:NM non-fimbriated, LT–	(Francis *et al.*, 1986)
711	K12:K88-negative parent of laboratory constructs.	(Baker *et al.*, 1997)

[a] Wild-type plasmid was introduced by conjugation.

infusion base), except K12:K99 which was grown on Essential Salt Medium (Francis *et al.*, 1982) supplemented with Eagle's essential amino acids and vitamins. The bacterial cultures were incubated for 18 h at 32 °C before use in the adherence studies.

2.2. Cell lines and culture conditions

The IPEC-J2 and IPEC-1 cell lines have been previously described (Lu *et al.*, 2002; Schierack *et al.*, 2006). These undifferentiated porcine intestinal epithelial cell lines were derived from jejunum and small intestine respectively, from un-suckled 1-day-old piglets. Neither of these cell lines was immortalized and therefore they represent a better model of normal porcine intestinal epithelium than transformed cell lines. Both IPEC-J2 and IPEC-1 cells were seeded on plastic cell culture flasks (25 cm^2, Corning, NY). These cell lines were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM)-Hank F12 (GIBCO, Invitrogen Corporation, Grand Island, NY) supplemented with 5% fetal calf serum (FCS; Atlanta Biologicals, Lawrenceville, GA), penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (ITS Premix; Sigma, St. Louis, MI), and 5 ng/ml of epidermal growth factor (EGF; Sigma), hereafter, referred as IPEC media. The stock culture of INT-407 (ATCC, CCL-6), a non-transformed human embryonic intestinal epithelial cell line, was obtained from American Type Culture Collection (ATCC, Manassas, VA). INT-407 cells were cultured and maintained in DMEM (GIBCO, Invitrogen) supplemented with 10% FCS, l-glutamine (2 μM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin (25 μg/ml) (Mediatech, Herndon, VA), hereafter, referred as DMEM-10. The cultures were maintained in a humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37 °C. After 4 to 5 days of culturing, all the three types of cultured cells became confluent. Cell monolayers were washed with phosphate buffered saline (PBS) and trypsinized with 1× trypsin-EDTA (Mediatech). The detached cells were pelleted at 200 × *g* for 5 min, re-suspended in antibiotic-free medium, and used for the adherence assays. The continuous cultures of epithelial cell lines were maintained by seeding culture flasks at 1:3 ratios at each passage.

2.3. Polarized IPEC-J2 cells

The IPEC-J2 cells were differentiated and polarized on collagen-coated permeable supports in serum-free medium. Two million IPEC-J2 cells harvested from confluent culture flasks (25 cm^2) were seeded on a 24-mm diameter (growth area 4.7 cm^2) collagen-coated transwell permeable support filter (3.0 μm pore size). Two ml of IPEC medium was added to the upper chamber while 3 ml of the medium was added to the bottom of wells in a 6-well transwell culture plate (Corning). The cells were maintained in serum-containing medium for 48 h and then switched to the same medium containing 10^{-7} M dexamethasone (Sigma) without FCS to minimize cell growth and enhance differentiation. The medium was changed every second day until cells showed features of polarization as judged by a significant increase in trans-epithelial electrical resistance (TER) (Schierack *et al.*, 2006). Cell cultures showing TER values between 2000 and 5500 Ω cm^2 were used for adherence assays.

2.4. Bacterial adherence assays

Fimbriae-mediated binding specificity of various *E. coli* strains was determined by a cell adhesion assay. The cell lines IPEC-J2 (passage 42–52), IPEC-1 (passage 25–30), and INT-407 (passage 12–16) were used for this study. The adherence assay procedure was described previously (Baker *et al.*, 1997). Briefly, *E. coli* were cultured overnight on 5% sheep blood agar plates at 32 °C. Bacterial cells were suspended in sterile PBS to achieve an optical density of approximately 1.0 at 520 nm. Fifty microliters (50 μl) of *E. coli* suspension was added to a plate well followed by addition of a suspension of porcine/human intestinal epithelial cells (~5 × 10^4 cells) in PBS containing 4% of D-Mannose (to prevent binding by Type I fimbriae if present). These suspensions were mixed for 20 min with rotational agitation at 140 rpm and then incubated for an additional 4 h at 4 °C. ETEC binding to epithelial cells was observed by phase contrast microscopy and bright field microscopy under oil (1000× magnification), and extent of bacterial binding to the epithelial cells was determined. The relative number of bacteria binding to epithelial cells was judged and rated (– = no adherent bacteria; + = few bound bacteria, ++ = several to a moderate number of bacteria bound; +++ = large num-

bers of bacteria bound). Each adherence assay was repeated three times or more with each cell line (8× with IPEC-J2, 3× with IPEC-1, and 4× with INT-407) to confirm the consistency of results. In some assays, in order to ascertain the viability of adherent ETEC, parallel bacterial suspensions were stained with Live/Dead® BacLight™ stain (Invitrogen, CA, USA) with live cells fluorescing green and dead cells fluorescing red. Bacterial adherence was observed by fluorescence microscopy using an AX70 fluorescence microscope (Olympus).

2.5. Green fluorescence protein-tagged E. coli

To enhance the visualization of bacterial adherence and confirm the findings obtained using unlabelled ETEC, the confluently binding strain B41 was transformed to express eGFP from plasmid pGLO (Bio-Rad, Hercules, CA). The eGFP gene is under the control of an arabinose-inducible promoter. Strain B41 was made chemically competent by standard methods (Sambrook *et al.*, 2001) and transformed by heat shock with pGLO as per the manufacturer's instructions. Transformants were selected by plating on an LB agar plate containing ampicillin (50 μg/ml) and arabinose (6 mg/ml), and incubated overnight at 37 °C. Successfully transformed colonies fluoresced green upon UV excitation. Transformants to be used in adherence assays were cultured overnight on sheep blood agar amended with arabinose (6 mg/ml). The adherence assay was performed as described above. Cells were viewed by confocal scanning laser microscopy (CSLM) using an Olympus Fluoview FV300 Laser Scanning Confocal Microscope System interfaced with an inverted-microscope (Olympus IX70) using Blue Argon (488 nm) and Green Helium Neon (543 nm) excitation lasers.

3. Results

Both porcine small intestinal epithelial cell lines bound a range of ETEC, albeit to varying degrees. Representative photomicrographs indicating the degree of adherent bacteria are shown in Figure 1A and

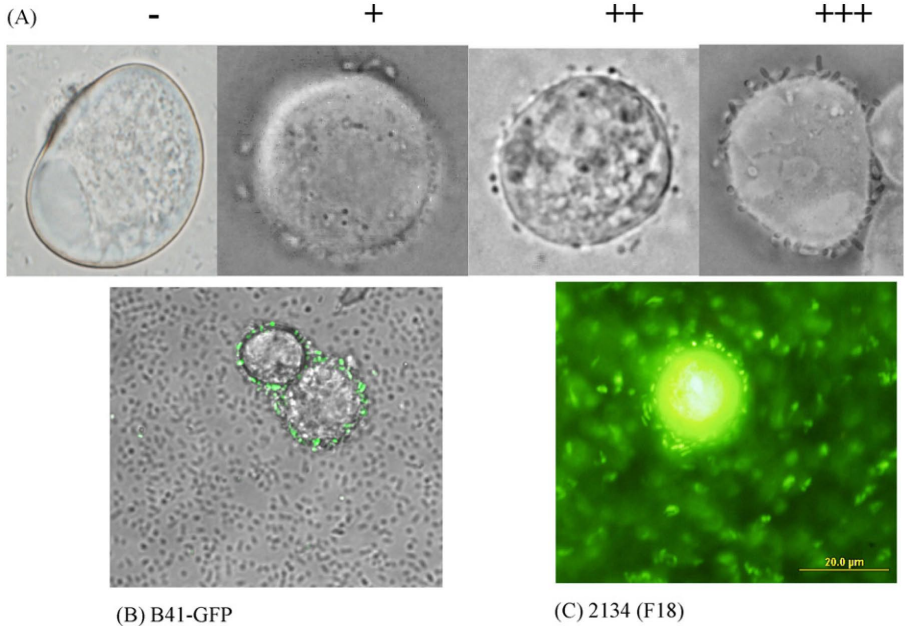

Figure 1. Adherence of *E. coli* strains to IPEC-J2 and IPEC-1 cells. (A) Shows the degree of *E. coli* binding to porcine intestinal epithelial cells. Final magnifications 1000×. −: No binding of bacteria; +: few bacteria bound per cell; ++: several bacteria bound per cell and +++: large numbers of bacteria bound per cell. GFP-transformed bacteria and BacLight dye were used to enhance the bacterial visualization. (B) The confocal image showed GFP-transformed bacterial (ETEC strain B41) attachment to IPEC-J2 cells. (C) ETEC F18 strain 2134 intensively bound to IPEC-1 cells. All bacterial cells attached to the IPEC-1 cell were live cells.

Table 2. Results of adherence assays with pig intestinal epithelial cell lines using ETEC and laboratory constructs

Strains	Adhesin	IPEC-J2 cells	IPEC-1 cells	INT-407 cells
263[a]	K88ab	++ or +++	+++	+
K12:K88ab	K88ab	+++	+++	+
F962	K88ab	+ or ++	+++	+
3030-2[a]	K88ac	+++	++ or +++	++
K12:K88ac	K88ac	+	++ or +++	+
F783	K88ac	+	++	–
1836-2[a]	K88ac	+	++	–
2534-86[a]	K88ac	++ or +++	++ or +++	++ or +++
WAM 2317	K88ac	++	++ or +++	++ or +++
MUN 287	K88ac	++ or +++	++ or +++	++ or +++
MUN 285	K88ac	++ or +++	+++	++ or +++
Morris[a]	K88ad	+++	+++	++ or +++
K12:K88ad	K88ad	+ or ++	+++	–
F291	K88ad	+ or +++	+++	–
1194[a]	987P	–	–	–
2134[a]	F18	–	+++	–
B41[a]	K99, F41	+++	+++	+++
K12:K99	K99	+	+	+++
G58-1[a]	None	–	–	–
711	None	–	–	–

[a] Wild-type strains are indicated in bold.

the results of adherence assays are summarized in Table 2. Wild-type *E. coli* strains 263, 3030-2, and Morris expressing K88ab, K88ac, or K88ad fimbriae strongly bound to both IPEC-J2 and IPEC-1 cells. *E. coli* strain B41 expressing both K99 and F41 fimbria heavily bound to all three epithelial cell lines (IPEC-1, IPEC-J2, and INT-407). The laboratory construct K12:K99 bound poorly to either IPEC-J2 or IPEC-1 cells when compared to the binding of the B41 strain, but heavily bound to INT-407.

The *E. coli* strain 2134 expressing F18 bound only to IPEC-1 cells. However, *E. coli* that expressed 987P failed to bind any of the cell lines. In addition, non-fimbriated *E. coli* strains G58-1 and 711 failed to bind cells of any of the three cell lines we examined. Polarization of IPEC-J2 cells did not result in the appearance of binding patterns that differed from those of non-polarized cells (data not shown).

Wild-type *E. coli* K88ac strain 3030-2 bound in greater abundance to IPEC-J2 cells than did laboratory constructs K12:K88ac and F783, which expressed the same fimbrial antigens. The K88ac-positive but toxin-negative wild-type strain 1836-2 showed very limited binding to both IPEC-1 and IPEC-J2 cells. However, wild-type K88ac strain 2534-86 and its nalidixic acid-resistant (NalR) mutant (WAM2317) and LT– mutant derivative of WAM2317 (MUN285) mutants adhered to both porcine cell types (IPEC-J2 and IPEC-1) and human cell line INT-407 with similar efficiencies (Table 2). Wild-type strains expressing K88ab and K88ad bound in abundance to IPEC-J2, but K88ab and K88ad laboratory constructs bound in small numbers to these cells. However, these wild-type strains and laboratory constructs bound equally to IPEC-1 cells.

Adherence assays with GFP-tagged bacteria (B41) were also conducted and their adherence to all the three cell lines was observed using confocal scanning laser microscopy (Figure 1B). Similar adherence patterns as observed with GFP-negative bacteria (Table 2) were detected. BacLight staining was used in the adherence assays with ETEC F18 strain 2134 to enhance the visualization of bacterial adherence and differentiate between live and dead bacteria. All the attached bacterial cells fluoresced green indicating that they were live (Figure 1C).

4. Discussion

In this study all three K88+ ETEC strains (K88ab, K88ac, and K88ad) adhered to both IPEC-J2 and

IPEC-1 cells, and the degree of ETEC adherence to these cells was strain-specific. Adhesin-negative *E. coli* strains G-58-1 and 711 did not adhere to any of three cell lines tested in this study. Furthermore, ETEC strains expressing K99 and 987P fimbria did not adhere to IPEC-J2 and IPEC-1 cells. Not surprisingly, most porcine ETEC strains bound better to porcine epithelial cells than to INT-407 cells. The K99+ ETEC strain was the exception to this general finding as it bound extensively to INT-407 and not to either porcine cell line. Overall, IPEC-1 cells were superior to IPEC-J2 cells in ETEC binding, both in terms of the types of adhesins that bound to these cells and the intensity of bacterial binding (Table 2). In general, the observations of this study suggest that IPEC-J2 and IPEC-1 strongly support the adhesion of porcine ETEC and may be a suitable model for studying some aspects of ETEC pathogenesis.

In this study, higher numbers of K88ac bacteria of wild-type strain 3030-2 bound to IPEC-J2 cells when compared to laboratory constructs. The reason for such a difference in binding was unclear and was not investigated. However, it is possible that fimbria are expressed in greater abundance on the wild-type strains compared to laboratory constructs. Another possibility is that there are other fimbriae or non-fimbrial adhesins involved in binding that vary in their presence among laboratory constructs and wild-type strains. Two different wild-type K88ac strains, 3030-2 and 2534-86, bound to all the three cell lines efficiently, supporting the reproducibility of the adherence assays and cell culture models. Recent studies from our laboratory have suggested that LT contributes to the colonization of K88+ strains in the intestines of piglets (Berberov *et al.*, 2004; Zhang *et al.*, 2006). ETEC K88ac strain 1836-2 which lacks LT and ST did not adhere efficiently to IPEC-J2 cells when compared to wild-type strain 3030-2. In contrast, ETEC 3030-2 and 1836-2 bound equally to IPEC-1 cells. ETEC K88ac strain 2534-86 and its LT– mutant (MUN285) bound to both porcine cell types with equal efficiencies. Therefore, further investigations are required to clarify the role of LT, if any in bacterial adherence. In this study, F18 ETEC strain 2134 bound to IPEC-1 cells but not to IPEC-J2 cells. This indicated that IPEC-1 cells might represent a suitable in vitro cellular model for studying the pathogenesis of F18 ETEC. To our knowledge, no other porcine intestinal epithelial cell line susceptible to ETEC F18 has been described. When polarized IPEC-J2 cells were used, the same *E. coli* binding patterns and intensities were displayed as observed with non-polarized cells suggesting that both non-polarized and polarized porcine intestinal cells are equally suitable for in vitro ETEC studies.

Each antigenic variant of K88, namely K88ab, K88ac and K88ad, exhibits a unique specificity to host receptors and various sugars are involved in the binding of different K88 variants to host receptors (Francis *et al.*, 1999; Jin and Zhao, 2000). Based on the phenotypic diversity of binding of three antigenic variants of K88 ETEC, six different phenotypes (A-E) of pigs have been described (Baker *et al.*, 1997; Bijlsma *et al.*, 1982). As both IPEC-J2 and IPEC-1 cells bound to all three antigenic variants of K88, both cell lines were probably derived from phenotype A pigs.

In summary, this study demonstrates that various porcine ETEC strains bind to IPEC-J2 and IPEC-1 in greater abundance than to INT-407 cells and both IPEC-J2 and IPEC-1 cells provide a biologically relevant in vitro model system for studying porcine ETEC-host intestinal epithelial cell interactions.

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