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Inverse relationship between heat stable enterotoxin-b induced fluid accumulation and adherence of F4ac-positive enterotoxigenic *Escherichia coli* in ligated jejunal loops of F4ab/ac fimbria receptor-positive swine

Joseph Erume [a,1], Prageeth Wijemanne [a], Emil M. Berberov [d,2], Stephen D. Kachman [b], Daniel J. Oestmann [a,3], David H. Francis [c], Rodney A. Moxley [a,*]

[a] School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0905, USA
[b] Department of Statistics, University of Nebraska-Lincoln, Lincoln, NE 68583-0905, USA
[c] Department of Veterinary Science, South Dakota State University, Brookings, SD 57007-1396, USA
[d] Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan S7N 5E3, Canada

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ABSTRACT

Heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* (ETEC) increases bacterial adherence to porcine enterocytes in vitro and enhances small intestinal colonization in swine. Heat-stable enterotoxin-b (STb) is not known to affect colonization; however, through an induction of net fluid accumulation it might reduce bacterial adherence. The relationship between fluid accumulation and bacterial adherence in jejunal loops inoculated with ETEC strains that produce LT, STb, both, or neither toxin was studied. Ligated jejunal loops were constructed in weaned Yorkshire pigs in two independent experiments (Exp. 1, $n = 5$, 8-week-old; Exp. 2, $n = 6$, 6–8-week-old). Each pig was inoculated with six F4ac$^+$ *E. coli* strains: (1) LT$^+$, STb$^+$ parent (WAM2317); (2) STb$^-$ (Δ*estB*) mutant (MUN297); (3) MUN297 complemented with STb (MUN298); (4) LT$^-$ STb$^-$ (Δ*eltAB* Δ*estB*) mutant (MUN300); (5) MUN300 complemented with LT (MUN301); and (6) 1836-2 (non-enterotoxigenic, wild-type). Pigs were confirmed to be K88 (F4)ab/ac receptor-positive in Exp. 2 by testing for intestinal mucin-type glycoproteins and inferred to be receptor-positive in both Exp. 1 and 2 based on histopathologic evidence of bacterial adherence. Strains that produced STb induced marked fluid accumulation with the response (ml/cm) to WAM2317 and MUN298 significantly greater than that to the other strains ($P < 0.0001$). Conversely, bacterial adherence scores based on immunohistochemistry and CFU/g of washed mucosa were both lowest in the strains that expressed STb and highest in those that did not. For the two experiments combined, the Pearson correlation coefficient (R) between fluid volume (ml/cm) and log CFU per gram was −0.57021 ($P < 0.0001$); $R^2 = 0.3521$ ($n = 197$). These results support the hypothesis that enterotoxin-induced fluid accumulation flushes progeny organisms into the lumen of the bowel, thereby increasing the likelihood of fecal shedding and transmission of the pathogen to new hosts.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains expressing K88 (F4) fimbria, heat-labile enterotoxin (LT) and heat-stable enterotoxin-b (STb) are common causes of fatal enteric colibacillosis in swine (Berberov et al., 2004). Colonization is dependent upon the binding of fimbrial

* Corresponding author. Tel.: +1 402 472 8460; fax: +1 402 472 9690.
E-mail address: rmoxley1@unl.edu (R.A. Moxley).
[1] Present address: College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P.O. Box 7062, Kampala, Uganda.
[2] Present address.
[3] Present address: 2234 Grainger Parkway, Lincoln, NE, USA.

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adhesins to specific heritable receptors on the apical surfaces of enterocytes (Francis et al., 1998). Enterotoxins, by definition, induce net secretion in ligated intestinal segments (Sears and Kaper, 1996), and are the main virulence factors responsible for diarrhea in ETEC infections. Induction of diarrhea is thought to facilitate the transmission of enteric pathogens to new hosts (Tauschek et al., 2002).

In addition to secretion, LT has been shown to promote bacterial adherence and intestinal colonization, suggesting that different enterotoxins might provide a balance between adherence and flushing into the gut lumen. In studies using F4ab/ac receptor-positive pigs and F4ac$^+$ isogenic constructs, significantly higher small intestinal colonization levels were detected as a result of LT (Berberov et al., 2004; Zhang et al., 2006) but not STb production (Erume et al., 2008; Zhang et al., 2006). Compared to parent and complemented strains, an F4ac$^+$ LT$^-$ mutant had reduced adherence to porcine enterocytes in vitro, and exogenously supplied LT enhanced adherence (Johnson et al., 2009).

In the present study, using isogenic F4ac$^+$ strains that vary in their production of LT and/or STb, we demonstrate an inverse relationship between STb-induced fluid accumulation and bacterial adherence using ligated jejunal assays in weaned F4ab/ac fimbrial receptor-positive swine. These results support the hypothesis that enterotoxin-induced fluid accumulation flushes progeny organisms into the lumen of the bowel, thereby increasing the likelihood of fecal shedding and transmission to new hosts.

2. Materials and methods

2.1. Bacterial strains and culture

Only *E. coli* strains were used in this study. Strain 2534-86 (O8:K87:NM) is wild-type, porcine-origin, F4ac$^+$, LT$^+$, STb$^+$, and enteroaggregative *E. coli* heat-stable toxin-1 (EAST1)$^+$ (Berberov et al., 2004). Strain WAM2317 is a spontaneous nalidixic acid-resistant (Nal^r) mutant of 2534-86 (Berberov et al., 2004). STb$^-$ mutant (Δ*estB*) MUN297, STb$^+$ complemented mutant (Δ*estB*) MUN298, LT$^-$ mutant (Δ*eltAB*) MUN299, LT$^-$ STb$^-$ mutant (Δ*eltAB* Δ*estB*) MUN300, and LT$^+$ complemented mutant (Δ*eltAB* Δ*estB*) MUN301 are isogenic derivatives of WAM2317 (Erume et al., 2008). Strain MUN302 is DH5α, LT$^+$ with *eltAB* cloned into pBR322 (Erume et al., 2008). Strains G58-1 (O101:K28:NM; Baker et al., 1997) and 1836-2 (O8:H4, F4ac$^+$; Zhang et al., 2006) are wild-type, porcine-origin, LT$^-$ and STb$^-$. Strains 1476 (F4ac$^+$) and 711 (F4$^-$) are K12 derivatives that, respectively, contain or do not contain plasmids expressing F4ac fimbria (Baker et al., 1997). MUN303 is K-12 strain DH5α containing pBR322 (Erume et al., 2008), and was used as a negative control for LT production in GM1-ELISA tests. ETEC reference strain H10407 (O78:H11, LT$^+$, STa$^+$) was originally isolated from a human patient with severe cholera-like disease in Bangladesh and was used as a positive control for LT secretion in GM1-ELISA (Evans and Evans, 1973).

2.2. Genotypic characterization of pigs for mucin-4 gene polymorphism

A polymerase chain reaction (PCR)-*Xba*I restriction fragment length polymorphism (RFLP) assay for detecting a polymorphism in intron 7 of the *mucin-4* gene (Jensen et al., 2006) was used to predict the F4ab/ac receptor (F4ab/acR) status of pigs prior to including them in intestinal loop assays in the second experiment. This *mucin-4* polymorphism test was done on 250 ng of DNA isolated by the Chelex-100 method (Walsh et al., 1991) from EDTA-stabilized blood samples.

2.3. Intestinal loop assays

Two independent experiments were conducted. In the first experiment, five 8-week-old, 6.3 ± 1.7 kg, weaned purebred Yorkshire pigs non-genotyped for *mucin-4* polymorphism were used. Intestinal loops were inoculated with one of six different strains, including WAM2317, MUN297, MUN298, MUN300, MUN301, and 1836-2. In the second experiment, six 6–8-week-old, 15.8 ± 4.2 kg, weaned specific-pathogen-free purebred Yorkshire pigs were used. Pigs used in the second experiment were pairs of littermates from each of three litters and selected from a group of 25 pigs screened by the *mucin-4* polymorphism test. One pig from each litter (a total of three) was homozygous and each of the three respective littermates were heterozygous for the *mucin-4* polymorphism-related F4ab/acR. Loops in the second experiment were inoculated with the same six strains as those in the first experiment and, additional loops were inoculated with (1) cell-free supernatants from strain WAM2317 that were heated at 65 °C for 15 min (WAM2317SH) to inactivate LT toxin; (2) cell-free supernatants from strain WAM2317 that were non-heated-treated (WAM2317SN); or (3) cell-free supernatants from strain MUN297 that were non-heat-treated (MUN297S).

Bacterial inocula for both experiments were grown in BBL™ Brain Heart Infusion broth containing 2% casamino acids (BHI-CA) overnight at 37 °C with moderate shaking (225 rpm). To prepare cell-free supernatants for inocula, 72 h BHI-CA broth cultures supplemented with lincomycin (90 μg/ml) and polymyxin B (100 μg/ml) were grown as previously described (Chapman and Swift, 1984). Following the 72 h incubation at 37 °C, the cells were centrifuged at 1200 × *g* for 10 min and the supernatants were filter-sterilized. Based on culture supernatant materials prepared retrospectively by these same methods, the LT concentration of the WAM2317 and MUN297 cell-free supernatants was estimated by GM1-ELISA to be 318.5 and 328.6 ng/ml, respectively. Further, using a bicinchoninic acid assay, the protein concentrations of the WAM2317 and MUN297 cell-free supernatants were determined to be 2.97 and 3.51 mg/ml, respectively.

For each experiment, pigs were ear tagged upon arrival, weighed, subjected to a health screen, and housed in an isolation room. The pigs were ad libitum-fed a non-medicated ground pig feed especially formulated for young growing pigs, and were given 3–7 days for acclimation to the new feed and environment. Twelve hours prior to surgery, the pigs were fasted but given free