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Evaluation of heat-labile enterotoxins type IIa and type IIb in the pathogenicity of enterotoxigenic *Escherichia coli* for neonatal pigs

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ABSTRACT

Type II heat-labile enterotoxins (LT-II) have been reported in *Escherichia coli* isolates from humans, animals, food and water samples. The goal here was to determine the specific roles of the antigenically distinguishable LT-IIa and LT-IIb subtypes in pathogenesis and virulence of enterotoxigenic *E. coli* (ETEC) which has not been previously reported. The prevalence of genes encoding for LT-II was determined by colony blot hybridization in a collection of 1648 *E. coli* isolates from calves and pigs with diarrhea or other diseases and from healthy animals. Only five isolates hybridized with the LT-II probe and none of these isolates contained genes for other enterotoxins or adhesins associated with porcine or bovine ETEC. Ligated intestinal loops in calves, pigs, and rabbits were used to determine the potential of purified LT-IIa and LT-IIb to cause intestinal secretion. LT-IIa and LT-IIb caused significant secretion in the intestinal loops in calves but not in the intestinal loops of rabbits or pigs. In contrast, neonatal pigs inoculated with isogenic adherent *E. coli* containing the cloned genes for LT-I, LT-IIa or LT-IIb developed severe watery diarrhea with weight loss that was significantly greater than pigs inoculated with the adherent, non-toxigenic parental or vector only control strains. The results demonstrate that the incidence of LT-II appeared to be very low in porcine and bovine *E. coli*. However, a potential role for these enterotoxins in *E. coli*-mediated diarrhea in animals was confirmed because purified LT-IIa and LT-IIb caused fluid secretion in bovine intestinal loops and adherent isogenic strains containing cloned genes encoding for LT-IIa or LT-IIb caused severe diarrhea in neonatal pigs.

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1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) causes severe diarrhea with significant morbidity and mortality in neonatal calves, newborn and weaned pigs. The primary pathogenic mechanisms of ETEC include adherence to intestinal epithelial cells, mediated by surface adhesins called fimbriae or pili, and production of various combinations of different heat stable (ST) and heat-labile (HLT) enterotoxins (Nagy and Fekete, 2005). There are two heat-labile enterotoxin (HLT) families that are distinguished by differences in antigenicity and receptor binding specificities. Type II HLTs, including LT-IIa, LT-IIb and the recently described LT-IIc (Green et al., 1983; Guth et al., 1986b; Holmes et al., 1986; Nawar et al., 2010) have been isolated from cattle waste lagoons (Chern et al., 2004; Khatib et al.,

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2002), various foods, particularly beef (Cerqueira et al., 1994; Franco et al., 1991; Rasrinaul et al., 1988), as well as from calves, pigs and ostriches with diarrhea (Celemin et al., 1994, 1995; Nardi et al., 2005; Rigobelo et al., 2006; Salvadori et al., 2003; Seriwatana et al., 1988). Although Type II HLTs share many similarities with Type I HLT, the specific role of LT-II in ETEC pathogenesis and virulence in animals has not been established (Holmes et al., 1995).

The objectives of the studies reported here were to: (i) determine the incidence of LT-II genes in a collection of *E. coli* isolates from calves and pigs in the United States, (ii) determine the potential role of LT-IIa and LT-IIb in ETEC pathogenesis by evaluating purified LT-IIa and LT-IIb for eliciting fluid secretion in ligated intestinal loops in pigs, rabbits and calves, and (iii) determine the pathogenicity of isogenic adherent *E. coli* strains containing the cloned genes for LT-I, LT-IIa or LT-IIb by oral inoculation experiments employing a neonatal pig model.

2. Materials and methods

2.1. Colony blot hybridization

A collection of 1648 *E. coli* isolates from animals was examined for the incidence of LT-II genes using colony blot hybridization methods previously described (Mainil et al., 1986; Moseley et al., 1980). The LT-IIa probe was derived from a subclone of pCP3727 as a 750 bp H*ind*III-P*st*I fragment (Pickett et al., 1986). The LT-IIb probe was derived from a subclone of pCP4185 as a 780 bp N*he*I-P*st*I fragment (Pickett et al., 1989). Both probes included partial sequences encoding for both the A and B subunits.

2.2. Ligated intestinal loop experiments

LT-I, LT-IIa, and LT-IIb were purified, stored and assayed in Y1 adrenal cells as previously described (Guth et al., 1986b; Holmes et al., 1986). Cholera toxin (CT) was purchased from Sigma (St. Louis, MO). Intestinal loop experiments were conducted in four 6–9 week old pigs, six 9–12 week old rabbits and two calves that were 7 and 9 weeks old. The surgical procedures for these experiments have been previously described (Whipp, 1991). Briefly, the animals were anesthetized with halothane and the intestines were externalized. The lumen of the small intestine was rinsed with a 0.9% saline solution containing 1 mg/ml soybean trypsin inhibitor (Sigma). Loops in pigs started approximately 150 cm distal to the Ligament of Trietz and each loop was 4–8 cm in length separated by 2–5 cm interloops. In rabbits the loops were 10 cm in length separated by 3–5 cm inter-loops beginning approximately 10 cm from the Ligament of Trietz. In calves, purified enterotoxins were evaluated in the anterior jejunum in one calf and in the distal jejunum in the other calf using loops starting 90 cm and 1000 cm distal to the Ligament of Trietz respectively. Loops in pigs and calves were injected with 5 ml of 0.9% saline (control) or 5 ml of 4 μg/ml purified CT, LT-1, LT-IIa, or LT-IIb diluted in 0.9% saline. Rabbit loops were injected with 2 ml of each enterotoxin (4 μg/ml) or 2 ml of 0.9% saline as the control. All animals were treated with an analgesic for post-surgical discomfort (0.1 mg/kg butorphenol, IM). After 4 h incubation, the animals were euthanized (Sleep-Away, Fort Dodge Laboratories, Fort Dodge, IA) and the volume of fluid contents and length were measured for each loop. The results are expressed as the final volume per cm loop length minus the volume per cm of adjacent saline-injected control loops in the same animal. The fluid accumulation responses for each of the purified toxins were compared by ANOVA with Tukey-Kramer Multiple comparison test and $P < 0.05$ was considered significant (GraphPad InStat).

2.3. Cloning and strain construction

The LT-IIa and LT-IIb operons were cloned into the low copy number vector pK184 under control of their native promoters. The pK184 vector is derived from pACYC184 and contains a multiple cloning site, P15A origin of replication and a kanamycin resistance marker (Jobling and Holmes, 1990). The LT-IIa operon, in a 2.2 kb E*co*RI-K*pn*I fragment originally from pCP3727 (Pickett et al., 1986) was subcloned into pK184 to produce pTDC205.1 (LT-IIa). The LT-IIb operon in a 1.7 kb B*gl*II-*Hpa*I fragment originally from pCP4185 (Pickett et al., 1989) was derived from pTDC100 (T.D. Connell and R.K. Holmes, unpublished results), as a E*co*RI-K*pn*I fragment and cloned into pK184 to produce pTDC102.1 (LT-IIb). The cloned LT-I operon in EWD299 has been described (Dallas et al., 1979).

The LT-I (pEWD299), LT-IIa (pTDC205.1), LT-IIb (pTDC102.1) plasmids and the pK184 empty vector were electroporated into *E. coli* strain 226M. Strain 226M is a non-toxigenic mutant that has lost a plasmid encoding for enterotoxin STa but expresses F41 adhesin. Strain 226M was previously shown to colonize neonatal piglets at levels similar to its wild type parent without causing significant diarrhea or weight loss (Casey et al., 1998; Casey and Moon, 1990).

2.4. Oral inoculation of newborn piglets

Cesarean-derived, colostrum-deprived newborn piglets were used to compare the adherent, non-toxigenic parental strain 226M, with isogenic strains containing a plasmid clone expressing the LT-I, LT-IIa, or LT-IIb operon, or the empty cloning vector pK184. Neonatal pigs were housed in isolation at 35 °C and given an intraperitoneal injection of normal swine serum as a source of nutrition but they were not otherwise fed or given water. Piglets less than 8 h old were weighed and orally inoculated, via gavage, with 10^{10} CFU of one of the isogenic strains. At 18 h following inoculation, the pigs were examined for diarrhea, weighed and necropsied. Samples of ileum were collected and processed for bacteriology and histopathology as previously described (Casey et al., 1998; Casey and Moon, 1990). ANOVA with the Tukey-Kramer multiple comparisons test was used to compare weight loss and ileal colonization determined by plate counts (log-transformed) on LB agar with and without antibiotics. $P < 0.05$