Pathogenicity of an Enterotoxigenic Escherichia coli Hemolysin (hlyA) Mutant in Gnotobiotic Piglets†

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Pigs infected with hemolytic F4+ strains of enterotoxigenic Escherichia coli often develop septicemia secondary to intestinal infection. We tested the hypothesis that inactivation of hemolysin would reduce the ability of F4+ enterotoxigenic E. coli to cause septicemia in swine following oral inoculation. Inactivation of the hemolysin structural gene (hlyA) did not decrease the incidence of septicemia in the gnotobiotic piglet model.

Escherichia coli is an important cause of diarrheal disease and extraintestinal infections in animals and humans (26). Strains that produce enterotoxins (enterotoxigenic E. coli [ETEC]) are among the most important causes of diarrhea in neonatal swine and cattle (20, 45) and are a common cause of septicemia in preweaned swine (8). E. coli bacteria that cause septicemia express virulence determinants that mediate their spread from the intestine to extraintestinal sites and that mediate bacterial persistence and proliferation in these locations (8). Putative virulence determinants include fimbrae, polysaccharide capsules, O-antigen capsules, lipopolysaccharide, aerobactin, hemolysin, and other cytotoxins (8). Fimbrae and hemolysin are the most important virulence factors that distinguish bacteremia-causing isolates from fecal control strains in human diabetic patients (3). The introduction of hemolysin genes into E. coli bacteria increases the virulence of these strains when inoculated into the peritoneal cavity or bloodstream in laboratory rodents (7, 33, 42). Hypothetically, hemolysin might increase virulence by increasing the availability of iron in the absence of aerobactin (27), mediating toxic effects on leukocytes and other nucleated cells (43), potentiating the effects of endotoxin (43), and mediating serum resistance (31). However, despite evidence suggesting that hemolysin increases the virulence of E. coli, there is little information regarding its role in vivo (43).

It has been shown that hemolytic ETEC strains naturally cause extraintestinal disease in preweaned and weaned swine, manifested clinically by sudden death and pathologically by lesions caused by endotoxic shock (9, 23). These strains most often belong to serogroups O8, O149, and O157 and are gene probe positive for hemolysin, K88 (F4) fimbrae, heat-labile enterotoxin I (LT-I), and heat-stable enterotoxin b (STb) (9, 23, 24). Experimental inoculation of gnotobiotic piglets with hemolytic strains of E. coli causes bacteremia and lesions identical to those seen in naturally occurring cases of hemolytic ETEC-induced endotoxic shock (9, 14, 16, 23, 24).

The results of these and other experiments using animal models and the association of hemolytic E. coli with extraintestinal disease in swine led us to hypothesize that inactivation of hemolysin would decrease the ability of F4+ ETEC to cause secondary bacterial septicemia in this species. We used the neonatal gnotobiotic piglet as a model because it is naturally susceptible to infection by F4+ ETEC and is suitable for the study of E. coli invasion (15, 16, 19). The objectives of this study were (i) to generate a stable nonhemolytic mutant from a hemolytic F4+ ETEC strain by gene replacement and (ii) to compare the virulence of the hemolytic and nonhemolytic isogenic strains in gnotobiotic piglets.

Bacterial strains. The bacterial strains and plasmids used in this study are shown in Table 1. ETEC strain 2534-86 serotype O8K87NM:F4ac was isolated from the small intestine of a 2-week-old pig with enteric colibacillosis and secondary bacte-

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Construction of hlyA deletion mutant. A nonhemolytic mutant of strain 2534-86 was constructed as follows (Fig. 1). Plasmid pWAM1097 contains hlyCA from E. coli urinary tract isolate J96, cloned in pUC19 (39). Plasmid pWAM1661 was constructed by removing PmeI-NaeI(hlyA2306–3197) in pWAM1097 and replacing it with a Km r cassette (aphA) from pUC4K (38, 39). This hlyCA region containing the Km r cassette was cloned into the XbaI site of pCVD442 and transformed into S17-1pir to yield donor strain WAM1746. WAM1746 was mated to a spontaneous nalidixic acid-resistant mutant of strain 2534-86, designated WAM2317. Bacterial matings were done at 37°C for 9 to 12 h on 0.45-μm-pore-size filters on Luria-Bertani (LB) agar containing 100 mM sodium citrate. Mating mixtures were subcultured overnight at 37°C on blood agar plates containing nalidixic acid (50 μg/ml) and kanamycin (50 μg/ml) (BA-NAL-KM plates). Five transconjugant colonies with decreased hemolytic zones on BA-NAL-KM plates were selected from the mating and pooled into one culture. This culture was incubated with aeration at 37°C for 5 h, pelleted, and plated for counterselection on LB agar plates containing 5% sucrose, nalidixic acid, kanamycin, and no NaCl (LB-NAL-KM-sucrose). After a 16-h incubation at 28°C, isolated colonies on the LB-NAL-KM-sucrose plates were patched onto LB plates containing 100 μg of ampicillin per ml and onto BA-NAL-KM plates. One nonhemolytic Ap′ colony, designated WAM2335, was selected for further examination. A liquid hemolysis assay (2) with dilutions made in 20 mM CaCl₂ detected no hemolytic activity in culture supernatant fluids of WAM2335, whereas the peak activity of WAM2317 was approximately 200 50% lysis hemolytic units/ml.

Southern blot analyses of PvuII digests of WAM2317 genomic DNA probed for hlyCABD contained hybridizable fragments of 8.5 and 6.2 kb (Fig. 2A). In contrast, digests of WAM2335 probed for hlyCABD contained hybridizable fragments of 6.2, 6.0, and 3.0 kb (Fig. 2A). The 3.0-kb fragment also hybridized to the aphA gene probe (Fig. 2B). Southern blot analyses of HindIII digests of WAM2335 genomic DNA contained hybridizable bands for LT-I and STb. Expression of F4 fimbrial antigen in WAM2335 was demonstrated by an indirect immunofluorescence assay (data not shown).

Gnotobiotic piglet experiments. Seven gnotobiotic piglets from one litter were derived by closed hysterotomy and reared in sterile isolator units according to standard procedures (18). When the piglets were 8 days old, swab samples from the nasal cavity and rectum and blood samples from the orbital sinus were obtained from each piglet under anesthesia (20 mg of tiletamine hydrochloride-zolazepam hydrochloride [Telazol; Fort Dodge Labs, Fort Dodge, Iowa] per kg of body weight [12, 40]). When the piglets were 9 days old, four were inoculated orally with WAM2335 and three were inoculated orally with WAM2317. Piglets in both groups were inoculated by instilling 1.0 × 10⁸ CFU into the milk replacer. After inoculation, the piglets were examined every 2 to 4 h for decreased rectal temperature, anorexia, vomiting, diarrhea, depression, and a moribund condition. Pre- and postinoculation blood samples, the latter when piglets were moribund or at 65 h postinoculation, were collected in sterile blood collection tubes for bacterial culture.

Rectal and nasal swab specimens and ileal, liver, and lung
specimens were cultured aerobically and anaerobically by standard procedures on sheep blood agar (5% sheep blood in Trypticase soy agar; Remel Labs, Lenexa, Kans.). Blood samples were cultured aerobically and anaerobically in commercial brain heart infusion broth culture vials (Septi-Check; BBL Labs, Cockeysville, Md.). Bacterial isolates were identified by standard procedures. Clinical parameters and mean colony counts per gram of ileal mucosa were compared by using Student’s t test (34).

Specimens of duodenum, jejunum, ileum, spiral colon, thymus, lung, liver, spleen, kidney, and brain were collected at necropsy, fixed in 10% neutral buffered formalin, processed for histopathology by standard procedures, and examined by light microscopy. Additional sections were stained by an immunohistochemical procedure (28) with rabbit polyclonal anti-O8 antiserum (E. coli Reference Center, The Pennsylvania State University). One block of paraffin-embedded ileum from a piglet inoculated with WAM2335 was further processed and examined with a transmission electron microscope (model 201; Philips, Eindhoven, The Netherlands). The F4 adherence phenotype of each piglet was determined by a brush border adherence assay (1).

All piglets became depressed, anorectic, and diarrheic after inoculation. All piglets developed diarrhea by 22 h postinoculation, and it persisted until euthanasia or death (38 to 65 h postinoculation). Three of four piglets inoculated with WAM2335 and one of three piglets inoculated with WAM2317 became hypothermic and moribund at 36 to 42 h postinoculation (P < 0.10).

Cultures of preinoculation rectal and nasal specimens from three piglets inoculated with WAM2335 and one piglet inoculated with WAM2317 yielded Staphylococcus spp. The preinoculation blood samples from all piglets were sterile. Cultures of the ileal specimens collected at necropsy yielded the respective E. coli inoculum strain from each of the piglets. Mean CFUs of WAM2335 and WAM2317 per gram of ileal mucosa were not significantly different (P > 0.05). WAM2335 was isolated from the liver, lung, and blood of three of four inoculated piglets. WAM2317 was isolated from the lungs of the three inoculated piglets but from the liver and blood of only one of these piglets (P > 0.10).

All seven piglets were determined to be of F4 adherence phenotype A (i.e., susceptible to F4ab, F4ac, and F4ad organisms) in the brush border adherence assay. Histologically, coliform bacteria were adherent to the small intestinal villous epithelium in all piglets inoculated with WAM2335 and WAM2317. In addition, they were within cytoplasmic vacuoles in two of four piglets inoculated with WAM2335 and in two of three piglets inoculated with WAM2317. Immunostained sections of small intestine from the same blocks of tissue had O8-positive bacteria in these locations (Fig. 3). Stomach, small intestine, spiral colon, liver, lung, kidney, and brain from three of four piglets inoculated with WAM2335 and from one of three piglets inoculated with WAM2317 were hyperemic and contained microthrombi (P > 0.10). The thymic cortices from all piglets had fragmented nuclei and cellular debris.

Ultrastructurally, WAM2335 organisms were adhered to the apical surfaces of villous enterocytes and within cytoplasmic

FIG. 2. Southern hybridization analysis of PvuII digests of genomic DNA of negative control WAM1808 (lanes 1), recipient strain WAM2317 (lanes 2), and nonhemolytic mutant strain WAM2335 (lanes 3) and XbaI digest of plasmid DNA of donor strain WAM1746 (lanes 4). Horseradish peroxidase-labeled probes for hlyCABD (7.0-kb AvaI fragment of pWAM04) (A) and the kanamycin resistance gene aphA (1.2-kb BamHI fragment of pUC4K) (B) were used. Numbers at right are molecular size markers.

FIG. 3. Light micrograph of jejunum of a gnotobiotic piglet inoculated with hemolytic E. coli strain WAM2317. O8-positive bacteria in villous enterocytes (arrows) are stained red; staining was done by the immunohistochemical staining technique. Bar = 10 μm.

FIG. 4. Transmission electron micrograph of ileum from a piglet inoculated with WAM2335. (A) WAM2335 is adhered to the apical surface of a villous enterocyte. Bar = 500 nm. (B) WAM2335 is within cytoplasmic vacuoles of a villous enterocyte. Bar = 2 μm.
Numerous bacteria are shown within a large cytoplasmic vacuole in an entero-memt on a conjugatable suicide plasmid. The occurrence of a *E. coli* tobiotic piglet inoculated with nonhemolytic mutant piglets inoculated with the Hly experienced the outcome (21) and may explain why only one of three its effects. Animal-to-animal variation also may have influenced the ability of *E. coli* to survive in the bloodstream by mediating resistance to complement and phagocytes and by serving as an alternative mechanism for the acquisition of iron in the absence of aerobactin (31, 43). Further studies with the enterically infected tobiotic piglet model could address the effects of hemolysin on extraintestinal infection. These include competition experiments with Hly and Hly organisms, studies of the effects of lower inoculum levels, studies addressing the time of onset and level of bacteremia, and studies of the effects of indigenous enteric bacteria (e.g., *Lactobacillus* spp. and *Streptococcus faecium*).

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