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Characterization of Hemolytic *Escherichia coli* Strains in Ferrets: Recognition of Candidate Virulence Factor CNF1

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Diseases associated with *Escherichia coli* infection are the subject of renewed interest due to emerging conditions such as hemolytic uremia syndrome. A collection of 15 strains of beta-hemolytic *E. coli* was isolated from diarrheic feces and diseased tissues of ferrets. All 15 strains were positive in specific PCR assays for the presence of *hlyA*, *pap1*, and *cnf1*. Seven of the *cnf1*-positive isolates were tested and shown to have a cytopathic effect on HeLa cell monolayers. The pathogenesis of these strains warrants future study.

Cytotoxic necrotizing factor 1 (CNF1) is a 115-kDa protein toxin produced by uropathogenic and some other *Escherichia coli* strains which have been designated necrotoxigenic *E. coli* (NTEC) strains (2, 7, 23). In humans, CNF1-producing *E. coli* isolates have been implicated in extraintestinal infections, especially urinary tract disease and meningitis. NTEC strains cause at least 80% of uncomplicated urinary tract infections and most nosocomial urinary tract infections (18). A distinct set of virulence determinants distinguish these strains from commensal strains of *E. coli* found in the colon and feces; additional factors such as *ibeA* (invasion of brain endothelium) may be required to produce meningitis (16). Strains belonging to a limited number of O serogroups possess P fimbriae, iron acquisition systems, toxins such as hemolysin (Hly) and CNF1, capsule formation, and serum resistance (1, 14). These factors presumably allow NTEC to evade host urinary tract defense mechanisms. In animals, *cnf1*-positive *E. coli* strains have been isolated from dogs with diarrhea and urinary tract infections (17, 28), cats with urinary tract infections (11), piglets with diarrhea and edema disease (12), cattle with both extraintestinal infections and diarrhea (3, 6), and healthy animals of the aforementioned species. In cattle, the related toxin CNF2 is especially prevalent among *E. coli* isolates (6).

CNF1 is a member of a family of bacterial toxins that includes the dermonecrotizing toxin of *Bordetella bronchiseptica* and CNF1 of *Yersinia pseudotuberculosis.* It interferes with cellular signaling by causing the constitutive activation of Rho-GTPases. Activation leads to actin cytoskeleton changes which manifest as stress fiber formation, an increased number of focal adhesions, membrane ruffling with subsequent macropinocytosis, and the formation of multinucleate cells in cell culture systems (9, 21, 26). Later intoxication events include the activation of cell motility, filopodium formation, and enhanced internalization of bacteria (8, 10, 15, 29).

Many *E. coli*-associated extraintestinal diseases, including gangrenous mastitis, pyometra, vaginitis, pyelonephritis, omphalophlebitis, and septicemia, have been reported for the ferret (13). Most common among these is a severe mastitis characterized histopathologically by coagulative and liquefactive necrosis of the mammary tissues and adjacent fat, muscle, and skin (20). During the course of clinical investigations, our laboratory archived *E. coli* isolates from diarrheic feces and diseased tissues (mammary gland, uterus, and brain) from ferrets over a 10-year period. The purposes of this study were to characterize these *E. coli* isolates and to identify candidate virulence determinants as a necessary first step toward elucidating the pathogenesis of these infections.

Animals. Ferrets (*Mustela putorius furo*) were purchased from a commercial vendor (Marshall Farms, North Rose, N.Y.) as time-pregnant jills (i.e., jills ordered at a specific time during gestation) or were born at our institution from such jills. Vendor jills were vaccinated for canine distemper and rabies and received type C botulism toxoid and bacterins of *Pseudomonas aeruginosa* and *B. bronchiseptica*. Jills born in-house were vaccinated for distemper. Animals were housed in an AAALAC International accredited animal facility in modified rabbit caging made of stainless steel (24 by 30 by 27 in.) or plastic (27 by 26 by 17.5 in.). Jills with litters were provided a nest box and nesting material; all ferrets were provided a cloth sleeping tube. Cage board was provided within the cage and was changed daily. Ferrets were fed an admixture of ferret chow (high-density chow 5L14; PMI, St. Louis, Mo.) and canned cat food (Science Diet feline kitten growth; Hill's, Topeka, Kans.); water was provided ad libitum. Pregnant and postparturient jills were maintained with 14 h of light and 10 h of dark per day; the temperature and humidity were controlled (68 to 74°F and 40 to 65%, respectively), and there were 12 nonrecirculated air changes per h.

Microbiology. Fifteen isolates were collected from each animal from the following sites: milk ($n = 5$), feces ($n = 5$), vagina ($n = 2$), urine ($n = 1$), blood ($n = 1$), and brain ($n = 1$). These *E. coli* isolates from clinical cases were isolated by plating on MacConkey lactose agar (Remel, Lenexa, Kans.). Lactose-positive colonies were then plated on sheep blood agar (Remel). The pattern of hemolysis was determined by

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direct visual observation. Isolates were identified by the use of API strips (bioMerieux, Hazelwood, Mo.). Individual isolates of *E. coli* were frozen at −70°C in *Brucella* broth containing 15% glycerol. Subcultures were inoculated into Trypticase soy broth (Remel) and shipped to the *E. coli* reference laboratory of the Gastroenteric Disease Center of Pennsylvania State University (State College). Isolates were subjected to O and H antigen serotyping as well as select adhesin and toxin gene assays by PCR. O and H serotyping was performed as described by the testing laboratory web site (http://ecoli.cas.psu.edu/procedures.htm). Examinations for select virulence factors were performed by PCRs using 3 μl of template DNA, a 0.5 μM concentration of each primer (Integrated DNA Technologies Inc., Coralville, Iowa), a 0.18 mM concentration of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, 0.4 U of *Taq* DNA polymerase (PGC Scientific, Gaithersburg, Md.), 50 mM Tris (pH 8.3), 250 mg of bovine serum albumin/ml, 2% sucrose, and 0.1 mM Cresol Red. PCRs were performed in a RapidCycler instrument (Idaho Technologies Inc., Salt Lake City, Utah). The cycling conditions consisted of initial denaturing at 94°C followed by 30 cycles of denaturation at 94°C, annealing at 55 or 48°C, and extension at 74°C. The amplified products were electrophoresed in 1% agarose gels at 200 V for 1 h, stained with ethidium bromide, and visualized under UV light. Positive samples were identified based on the presence of bands of appropriate sizes compared to positive control strains (strain B41 for *sta*, strain 80–2575 for heat-labile enterotoxin and *stb*, and strain 43895 for *stx1* and *stx2* [Gastroenteric Disease Center of Pennsylvania State University]). The primer sequences and thermocycling conditions used were from published methods for *sta* and *stb* (25), for heat-labile enterotoxin (27), and for *stx1* and *stx2* (31). Examinations of additional virulence factors were performed via PCRs with previously reported conditions and primers (4, 18).

Cytopathic effect on HeLa cell monolayers. Bacterial sonicates or supernatants of seven ferret *E. coli* isolates and one nonpathogenic laboratory strain of *E. coli* without detectable *cnf* DNA homology or CNF activity, DH5α (Invitrogen, Carlsbad, Calif.), were evaluated for cytopathic effects on HeLa cell (CCL-2) monolayers. Five of the isolates were from clinical cases of mastitis (97–1214, 92–834, 93–1626, 98–1362, and 94–2593), one was from a urine culture (92–336), and one was from a rectal culture (02–0042). After overnight growth in Lennox broth (American Bioanalytical, Natick, Mass.), the bacteria were pelleted for 10 min at 2,040 × *g* at room temperature. The supernatants were filtered through a 0.2-μm-pore-size syringe filter (Pall Corporation, Ann Arbor, Mich.) and stored at −80°C. The pellets were resuspended in 1 ml of phosphate-buffered saline and disrupted by six 30-s pulses on ice with a Virsonic 50 sonicator (Virtis, Gardiner, N.Y.). Sonicates were cleared of debris by centrifugation for 3 min at 16,000 × *g* at room temperature and were frozen at −80°C until use.

For assays of cytopathic effect, 6×10^3 HeLa cells were plated on 13-mm-diameter glass coverslips in 24-well plates in Dulbecco's modified Eagle's medium (Mediatech, Inc., Herndon, Va.). The cells were incubated at 37°C in 5% CO_2 for 3 h. Twelve microliters of either supernatant or sonicate was added to the HeLa cell monolayers, which were then incubated at 37°C in 5% CO_2 for 72 h. The coverslips were stained with an eosin-azure B-based stain (Diff-Quik staining kit; Dade Behring, Newark, Del.) and mounted for visualization by light microscopy.

TABLE 1. Serotypes of NTEC strains[a]

Sample no.	Source	O type	H type
92–336	Ferret urine culture	2	4
02–0042	Ferret rectal culture	2	4
91–1918	Ferret rectal culture	2	4
01–3543	Ferret vaginal culture	2	4
94–28	Ferret brain culture	2	4
94–2593	Ferret milk culture	4	5
93–337	Ferret blood culture	4	−
93–1626	Ferret milk culture	4	−
93–178	Ferret rectal culture	4	−
95–5266	Ferret rectal culture	6	−
98–1330	Kit rectal culture	6	−
97–1214	Ferret milk culture	6	−
93–187	Ferret vaginal culture	6	−
92–834	Ferret milk culture	6	−
98–1362	Ferret milk culture	6	−

[a] Serotyping was performed at Pennsylvania State University. All samples were positive for *cnf1*, *hly*, and *pap1*, and all samples were negative for *cnf2*, heat-labile enterotoxin, *sta*, *stb*, stx_1, stx_2, and *eae*. All samples exhibited beta-hemolysis.

High-resolution genotyping. The genomic DNA of each ferret *E. coli* strain was evaluated by high-resolution genotyping according to the method of Versalovic et al. (30). Briefly, repetitive element-PCR (Rep-PCR) takes advantage of interspersed repetitive elements that are found throughout the bacterial genome. Rep-PCR amplification patterns can provide strain-level discrimination. REP1R and REP2 (30) were used as primers with puRe *Taq* Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, N.J.) and with 100 ng of genomic DNA as a template. Samples were amplified under cycling conditions of 95°C for 1 min, 40°C for 1 min, and 65°C for 8 min, with a final extension time of 16 min. The products were separated in 1% agarose gels, stained with ethidium bromide, and visualized with an Image Station 1000 (Eastman Kodak Co., Rochester, N.Y.). DNA profiles were quantitatively analyzed with GelCompar II software, v. 2 (Applied Maths, Kortrijk, Belgium). Similarity coefficients were calculated by use of the Pearson correlation and were clustered by the unweighted pair group method with arithmetic means.

Microbiology results. The results of serotyping and the identification of virulence factors are summarized in Table 1. All 15 isolates were beta-hemolytic *E. coli* and contained α-hemolysin (*hlyA*), *cnf1*, and the P pilis (*pap1*). Isolates were negative for several other virulence factors, including heat-labile enterotoxin, *st*, *cnf2*, and *eae*. Serotypes included O2:H4 (five isolates), $O6:H^-$ (six isolates), $O4:H^-$ (three isolates), and O4:H5 (one isolate).

Ferret NTEC strains have CNF1 activity. In order to determine if *cnf1* sequence homology was correlated with CNF1 activity, we treated subconfluent HeLa cell monolayers with sonicates and culture supernatants from seven ferret NTEC strains. After 72 h of incubation, HeLa cells that were treated with sonicates exhibited changes that were characteristic of CNF1 activity. Treated cells were larger than untreated control cells, and >80% of the treated cells had multiple nuclei (Fig. 1). In addition, evidence of nuclear fragmentation was apparent in some of the treated cells. Sonicates of all seven ferret

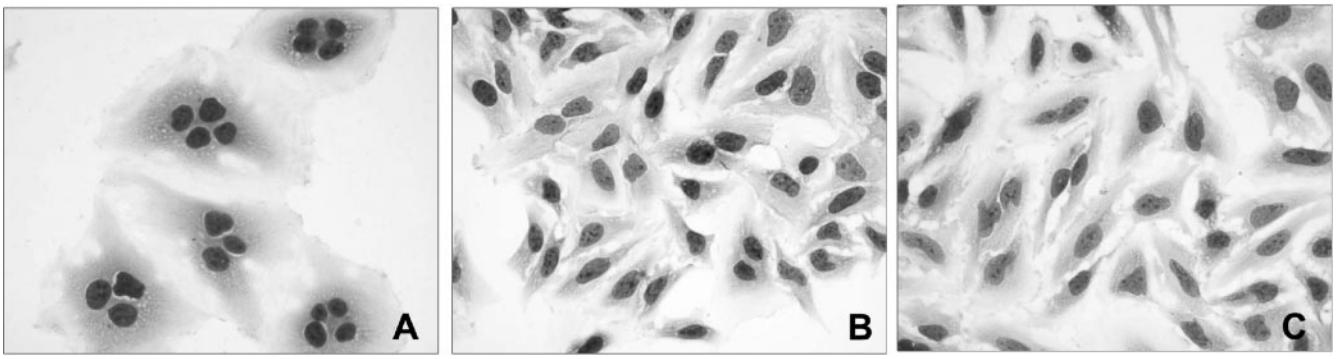

FIG. 1. Ferret NTEC strains exhibit CNF1-mediated cytopathic effects. Subconfluent monolayers of HeLa cells were treated with sonicates of ferret NTEC strains. After 72 h, the cells exhibited distention and >80% were multinucleated. Nuclear fragmentation was also apparent in some cells. Representative monolayers of HeLa cells treated with a sonicate of ferret NTEC strain 02–0042 (A) and a sonicate of the nonpathologic strain DH5α (B) and an untreated control monolayer (C) are shown at the same magnification after being stained with Diff-Quik.

NTEC strains had comparable cytopathic effects on HeLa cell monolayers 72 h after treatment. Weaker activities were observed when HeLa cells were treated with culture supernatants (data not shown).

High-resolution genotyping confirms serogroup-based relationship of ferret NTEC strains. To confirm the relationship of ferret NTEC strains based on O and H serotyping, we performed PCR amplification of genomic DNAs, using primers that were complementary to interspersed repetitive elements (Rep-PCR). Three groups of ferret NTEC strains could be distinguished based on their Rep-PCR amplification patterns (Fig. 2). The human O4:K6 strain J96, three ferret O4:H$^-$ strains, one ferret O4:H5 strain, and two ferret O6:H$^-$ strains made up the first group (top seven strains). The second group (middle four strains) were all O6:H$^-$ strains. The third group (bottom five strains) were all O2:H4 strains. Despite the limited genetic diversity among the ferret NTEC strains, the strains did not stratify as a function of the date of isolation. Strains comprising the first genotype, made up of O4 and O6 strains, were isolated between 1993 and 1997. The O6:H$^-$ strains comprising the second genotype were isolated between 1992 and 1998. The O2:H4 strains comprising the third genotype were isolated between 1991 and 2002. Interestingly, the O6:H$^-$ strains 93–187 and 97–1214 appeared to be more

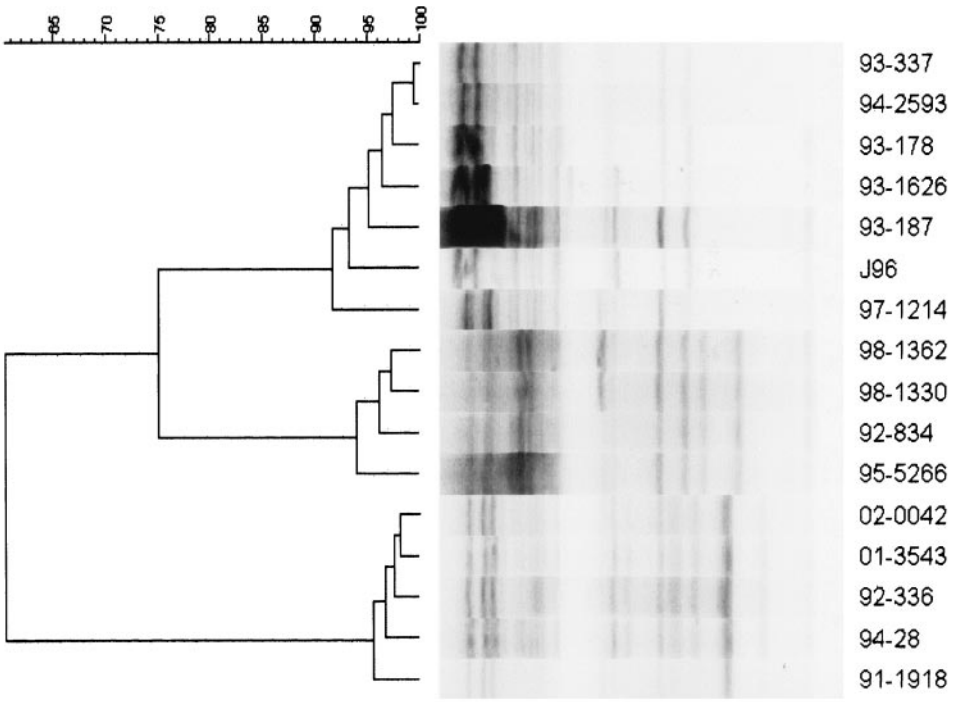

FIG. 2. Dendrogram showing similarities of Rep-PCR genotypes of ferret NTEC strains, representing five O4 strains, six O6 strains, and five O2 strains. Three groups of strains could be distinguished. From top to bottom, the strains are grouped as follows: three ferret O4:H$^-$ strains, one ferret O4:H5 strain, two ferret O6:H$^-$ strains, and the human O4:K6 strain J96 (top); four ferret O6:H$^-$ strains (middle); and five ferret O2:H4 strains (bottom).

closely related to the O4:H$^-$ strains (89 to 96% similarity) than to the other O6:H$^-$ strains (69 to 88% similarity).To our knowledge, this study is the first to document *cnf1*-positive isolates of *E. coli* from diseased ferrets. Necrotizing suppurative mastitis is an important disease of the lactating jill and potentially results in neonatal death due to its effects on the dam (20). Ophthalmia neonatorum and neonatal septicemia are other *E. coli*-associated diseases that can reduce fecundity (13). *E. coli* strains have also been cultured from the urine, kidneys, vagina, brain, tail abscesses, and feces of ferrets with or without diarrhea. Hemolytic *E. coli* strains from ferrets can be classified as urosepsis strains, uropathogenic *E. coli*, or necrotoxigenic *E. coli* (NTEC). NTEC may be the most appropriate term for these *E. coli* strains. Extraintestinal diseases associated with *cnf1*-positive *E. coli* have also been demonstrated in other species.Uropathogenic organisms contain many virulence factors, including bacterial adhesins, toxins, iron acquisition systems, and host defense avoidance mechanisms (1, 24). In the study reported here, representatives of all of these categories of virulence factors (with the exception of iron acquisition systems) were identified. All 15 ferret isolates were positive for P pili by PCRs using a pair of primers designed to amplify an internal fragment of *papC* (19) The human O4:K6 strain J96 contains unlinked loci encoding two distinct P pili, pyelonephritis-associated pili (*pap*) and Pap-related sequence (*prs*) (22). *pap* and *prs* cannot be distinguished by primers because the two loci are identical except for the gene encoding the adhesin (*papG* and *prsG*). Further characterization of P pili in ferret NTEC strains is needed, but a preliminary characterization of O6 strains suggested that they express Prs, while O2:H4 strains may express Pap (data not shown). The existence of *pap*, *hly*, and *cnf1* in urosepsis strains is evidence of the presence of pathogenicity islands (1). Additional characterizations of *pap* and *hly*, along with challenge studies using isogenic mutants, will provide insight into the contribution of these virulence factors to disease.The ferret may be a promising model for evaluations of the contribution of *cnf1* and other genetic factors to *E. coli*-associated virulence. Ferrets are carnivores, are easily maintained and manipulated in a vivarium, and are of an adequate size (approximately 1 kg) to perform longitudinal studies with the ability to obtain multiple samples. Another advantage over existing models is that the ferret is the natural host for several coliform diseases. In the clinical realm, extraintestinal *E. coli*-associated diseases of the ferret are major causes of morbidity and mortality in postparturient jills and in neonates (5, 20). *cnf1* or other virulence genes of these *E. coli* isolates may also be appropriate targets for vaccine development. Finally, comparisons of fecal and extraintestinal strains of *pap*-, *hly*-, and *cnf1*-positive *E. coli* from ferrets may provide insights into the critical genetic and proteomic differences required by these organisms for selecting ecological niches and their subsequent ability to elicit disease.

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