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Characterization of cytotoxic necrotizing factor 1-producing *Escherichia coli* strains from faeces of healthy macaques

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Twenty-five (27 %) of 92 clinically normal macaques were found to have β-haemolytic *Escherichia coli* isolated from their faeces. Five of six isolates chosen for further characterization had multiple antibiotic resistance and were PCR-positive for cytotoxic necrotizing factor 1 (*cnf1*) with a demonstrated cytopathic effect *in vitro*. By repetitive element sequence-based PCR genotyping, genetic similarity was established for selected isolates. We believe this to be the first report of *E. coli* strains producing CNF1 in non-human primates.

INTRODUCTION

Escherichia coli strains that produce cytotoxic necrotizing factors (CNFs) belong to the pathotype necrotoxigenic *E. coli* (NTEC) and are associated with intestinal and extra-intestinal infections in both humans and animals (Kaper *et al.*, 2004). The majority of toxin-producing isolates produce either the chromosomally encoded CNF1 (Falbo *et al.*, 1993) or the plasmid-encoded CNF2 (Oswald *et al.*, 1989); more recently, a third type has been isolated from sheep and goats and named CNF3 (Orden *et al.*, 2007). CNF1 is a 115 kDa protein with lethal and necrotizing activity *in vivo*. *In vitro*, affected cells undergo multi-nucleation and membrane ruffling and form focal adhesions and actin stress fibres. The mechanism involves activation of Rho GTPases, a family of molecular switches with multiple cellular functions, resulting in reorganization of the actin cytoskeleton. CNF1 shares a pathogenicity island with α-haemolysin and P fimbriae (De Rycke *et al.*, 1999). CNF1-producing strains most notably cause urinary tract infections in humans. These strains have also been isolated from healthy and diseased animal species. The list includes weaned pigs (Tóth *et al.*, 2000) and dogs (Starcic *et al.*, 2002) with diarrhoea; cats (Féria *et al.*, 2001) and dogs (Johnson *et al.*, 2003; Siqueira *et al.*, 2009; Yuri *et al.*, 1998) with urinary tract infections; ferrets with diarrhoea and extra-intestinal infections (Marini *et al.*, 2004); and birds and mink with suspected colibacillosis (Rodriguez-Siek *et al.*, 2005) and colisepticaemia (Tibbetts *et al.*, 2003). NTEC-2 strains, although commonly reported in ruminants (De Rycke *et al.*, 1999), have also been reported in a very small percentage of human faecal *E. coli* isolates (Kadhum *et al.*, 2008). The objective of this study was to evaluate the presence of specific virulence genes and toxin activity in several β-haemolytic *E. coli* isolates cultured from a macaque colony used for neurobiology research.

Abbreviations: CNF, cytotoxic necrotizing factor; CPE, cytopathic effect; NTEC, necrotoxigenic *Escherichia coli*; rep-PCR, repetitive element sequence-based PCR.

METHODS

Animals. Macaques (*Macaca mulatta* and *Macaca fascicularis*) received from three US-based vendors in 2005–2006 received physical examinations and routine diagnostic evaluations during quarantine and at quarterly intervals. Animals were routinely pair-housed and maintained in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. They were fed specified amounts of primate chow (Purina Lab Diet 5038) twice a day and provided with water *ad libitum*. Housing conditions were maintained at 20–22 °C, 30–70 % humidity, 10–15 non-recirculated air changes h^{-1} and a light cycle of 12 h light : 12 h dark.

Microbiological analysis. Faecal samples were collected over a 2-year period from 2005 to 2006, either rectally or from cage pans, from a cohort of 92 clinically normal macaques (those received in quarantine as well as the established cohort of macaques actively being used in research). For those animals that were pair-housed and not isolated prior to sample collection, faeces were taken from both cage pans but it is not definitively known which animal the faeces came from, although it is likely that cage mates would both be affected. *E. coli* colonies were isolated on MacConkey lactose agar (Remel). For each isolate, a lactose-positive colony was streaked onto a sheep blood agar plate (Remel) and β-haemolysis was visualized directly on the blood agar plate. Several β-haemolytic colonies were then used to identify each isolate using API 20E strips (bioMérieux). Six isolates representing two macaque species, two vendors and four rooms in two animal facilities were chosen for further characterization.

Antibiotic susceptibility testing was performed for all six *E. coli* isolates using antimicrobial susceptibility discs (Remel for all discs except for the tetracycline disc, which was from Becton Dickinson)

for the following antibiotics: ampicillin 10 μg, amoxicillin/clavulanic acid 20/10 μg, cephalothin 30 μg, gentamicin 10 μg, trimethoprim/sulfamethoxazole 1.25/23.75 μg, tetracycline 30 μg and enrofloxacin 5 μg. A few bacterial colonies from a pure culture isolated on blood agar were transferred to tryptic soy broth and incubated for 1 h at 37 °C. The bacterial suspension was then plated on Mueller–Hinton agar (Remel) and incubated overnight at 37 °C. Following incubation, the diameters of inhibition zones were measured with a standard ruler.

Serotyping and PCR. Isolates were submitted on trypticase soy agar (Remel) to the *E. coli* Reference Center at Pennsylvania State University, PA, USA, for serotyping. Isolates underwent O- and H-typing as well as virulence testing for the selected toxins and adhesins heat-labile enterotoxin (LT), heat-stable enterotoxin a and b (STa and STb), Shiga-like toxin types I and II (*stx* I and *stx* II), intimin-γ (*eae*), and CNF1 and CNF2 (*cnf1* and *cnf2*) by PCR following the procedures outlined on the Reference Center's website (http://ecoli.cas.psu.edu). We performed additional PCRs for α-haemolysin (*hlyA*) and pyelonephritis-associated pilus (*papG* I, II and III) using previously published protocols and designated primers, as these genes share the same pathogenicity island as *cnf1* (Kadhum *et al.*, 2008). In addition, there are *E. coli* strains that produce both CNF and cytolethal distending toxin (CDT); therefore, a multiplex PCR using two pairs of *cdtB*-specific primers was performed (Tóth *et al.*, 2003).

CNF activity assays. HeLa cell culture experiments were conducted to correlate CNF activity with the presence of *cnf1*. After overnight incubation at 37 °C in Difco LB broth (Becton Dickinson), *E. coli* cultures were centrifuged for 3 min at 17 900 ***g*** at room temperature. Supernatants were filtered using a 0.2 μm Acrodisc syringe filter (Pall Gelman Laboratory) and stored at −80 °C until use. Bacterial pellets were resuspended in 1 ml PBS and stored at −80 °C for 15 min before sonication. Bacteria were sonicated for three 30 s pulses on ice with a 30 s cooling time in between using an Artek Sonic Dismembrator (model 150; Artek Systems). Sonicates were centrifuged for 3 min at 17 900 ***g*** at room temperature and syringe-filtered before storage at −80 °C until use. *E. coli* sonicates or supernatants from the six macaque isolates and a non-pathogenic laboratory *E. coli* strain (XL-1 Blue), as well as controls of PBS and Eagle's minimal essential medium (Sigma-Aldrich) supplemented with 10 % fetal calf serum, were added to HeLa cell monolayers and incubated for 72 h. HeLa cells (5.5×10^3) were plated in a Lab-Tek II Chamber Slide System and incubated at 37 °C, 6 % CO_2 for 3 h. Twelve microlitres of sonicate or supernatant was added to each chamber of HeLa cells and incubated at 37 °C, 6 % CO_2 for 72 h. Media chambers were removed from the slide system and the slides were stained using an eosin Y/azure A Diff-Quick procedure. Cytopathic effect (CPE), specifically multinucleation and cell enlargement, was visualized by light microscopy.

Genotyping by repetitive element sequence-based PCR (rep-PCR). High-resolution genotyping using a DiversiLab *Escherichia* DNA Fingerprinting kit based on the rep-PCR method of Versalovic *et al.* (1994) was carried out. DiversiLab software was used to compare the amplification patterns obtained and to analyse genetic similarity among the six tested isolates.

RESULTS

Microbiology results

Twenty-five (27 %) of 92 faecal samples were culture-positive for β-haemolytic *E. coli* on sheep blood agar plates. Serotyping and virulence factor testing results for six of these isolates are summarized in Table 1. Five serotypes were identified: O1 : H7, O88w : H7, O6 : H7, O6 : H1 and O2 : H1 (two isolates). All isolates were β-haemolytic and *hlyA*- and *papG*-positive (all isolates were *papG* I-negative and *papG* II-positive, and three of the six isolates were *papG* III-positive), and five of the six isolates were *cnf1*-positive. All other assays used to screen for virulence determinants were negative for all six isolates (LT, STa, STb, *stx1*, *stx2*, *eae*, *cnf2* and *cdtB*). Five of the isolates showed multiple antibiotic resistance (Table 2). The antibiotic to which the greatest number of isolates had resistance was cephalothin, a first-generation cephalosporin. Five of the isolates were resistant and one isolate had an intermediate zone of susceptibility. Five of the isolates were susceptible to enrofloxacin, and one isolate had an intermediate zone of susceptibility.

Table 1. Serotyping and virulence testing results

All isolates were β-haemolytic and negative for LT, STa, STb, *stx1*, *stx2*, *eae*, *cnf2* and *cdtB*.

Isolate no.	O type	H type	*cnf1*	*hlyA*	*papG*
06-2830	2	1	+	+	+
06-2832	2	1	+	+	+
06-2725	6	1	+	+	+
06-3320	6	7	+	+	+
06-2828	88w	7	+	+	+
06-2934	1	7	−	+	+

CNF1 activity shown *in vitro*

The five *cnf1*-positive *E. coli* isolates produced CPE (Fig. 1), except for 06-2725, which caused cell death at the 72 h time point. The *cnf1*-negative isolate, 06-2934, showed no CPE.

Genotyping by rep-PCR quantifies genetic similarity

Isolates 06-2828 and 06-2832 showed 97.2 % similarity by rep-PCR, whilst isolates 06-2725 and 06-2830 were

Table 2. Antibiotic susceptibility testing results

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cephalothin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; ENRO, enrofloxacin; S, susceptible; I, intermediate; R, resistant.

Isolate no.	AMP	AMC	CEF	GEN	SXT	TET	ENRO
06-2830	S	S	R	S	S	R	S
06-2832	S	S	R	S	S	S	S
06-2725	S	S	R	S	S	R	S
06-3320	S	S	R	S	S	R	I
06-2828	R	S	R	S	R	I	S
06-2934	S	S	I	R	R	R	S

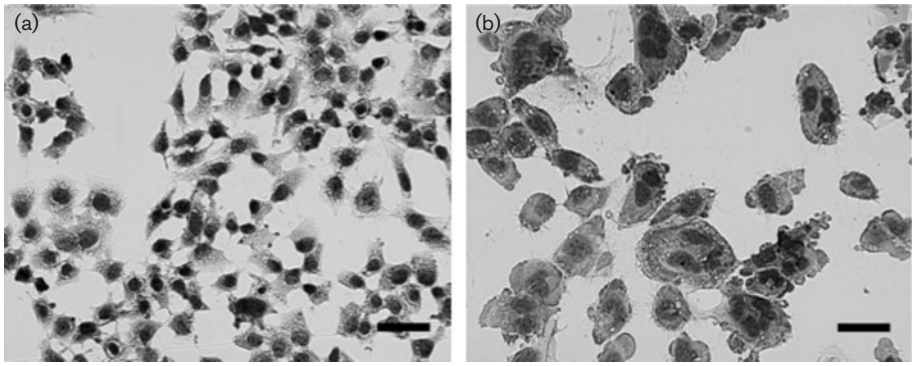

Fig. 1. CPE representative of CNF1, most notably multinucleation and cell enlargement, in HeLa cell monolayers after 72 h. (a) An untreated HeLa cell monolayer. (b) HeLa cell monolayer treated with a sonicate from a *cnf1*$^+$ isolate, 06-3320. Monolayers were stained with Diff-Quick and pictures were taken at the same magnification. Bars, 40 μm.

indistinguishable by rep-PCR (Fig. 2). Isolates 06-2934 and 06-3320 were clearly distinguishable by rep-PCR and showed no evidence of a genetic relationship.

DISCUSSION

To our knowledge, this is the first reported isolation of *cnf1*-positive *E. coli* in macaques. The *cnf1*-positive isolates had confirmed CNF1 activity *in vitro*, whilst the *cnf1*-negative isolate did not, showing that the *cnf1* gene was functional. Some *E. coli* strains also produce CDTs, although CDT typically causes mononucleation *in vitro* in contrast to the multinucleation seen with CNF (Kadhum *et al.*, 2008). Although our *in vitro* cell culture assays showed multinucleated giant cells typical of CNF, a *cdt* PCR was run to verify that these isolates were negative for the presence of *cdt*. The macaque isolates represent a heterogeneous population of CNF-1-producing *E. coli*, most likely due to the historical acceptance of animals positive for β-haemolytic *E. coli* into the research colony from quarantine. Our rep-PCR results suggest a common point source for several of the strains, as two of the rep-PCR patterns were represented by two isolates each. 06-2828 and 06-2832 were highly similar comparing rep-PCR results but had different antibiotic profiles and serotypes. 06-2725 and 06-2830 were indistinguishable by rep-PCR and had identical antibiotic susceptibility patterns, but differed in their serotype, having different O groups. Five of the six monkeys colonized with these *E. coli* strains came from the same vendor, were captive-bred of Chinese origin and entered our quarantine facility on the same day, although only four of these strains were *cnf1*$^+$. One monkey was acquired the following year from a different vendor, was wild-caught of Chinese origin and the *E. coli* strain isolated was *cnf1*$^+$. Their exact age was unknown but they were all young adult male rhesus macaques. They most likely entered our facility harbouring these *E. coli* strains, as these animals were housed in different holding rooms and were used by different investigators. Antibiotic susceptibility testing revealed that the *E. coli* isolates had several multidrug-resistance patterns. Despite this, a high percentage of isolates were susceptible to enrofloxacin. Enrofloxacin is a fluoroquinolone antibiotic commonly used to treat various diseases in non-human primates, for example as a prophylactic treatment for subclinical *Shigella* infections while in quarantine before shipment to

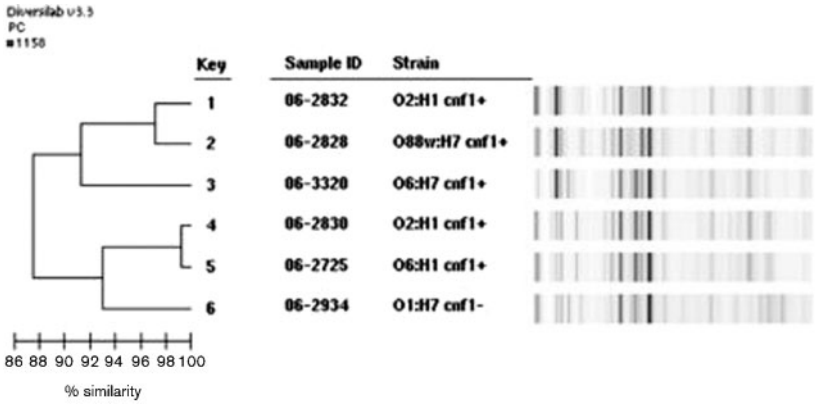

Fig. 2. Analysis of the six *E. coli* macaque isolates using a DiversiLab *Escherichia* DNA Fingerprinting kit and DiversiLab software. Isolates 06-2828 and 06-2832 showed 97.2 % similarity and may represent the same clone. Isolates 06-2725 and 06-2830 were indistinguishable and probably represent a second clone. Isolates 06-2934 and 06-3320 were clearly distinguishable and showed no evidence of a genetic relationship.

institutions (Fox *et al.*, 2001). The usage of antibiotics as prophylaxis or in the absence of culture and sensitivity results may have exacerbated the antibiotic resistance noted in these *E. coli* isolates. Indeed, *Shigella flexneri* strains isolated from monkeys housed in the Massachusetts Institute of Technology (MIT) colony have multiple antibiotic resistance, including enrofloxacin, which is plasmid-mediated (Martin *et al.*, 2008). The method of acquisition of antibiotic resistance in the *E. coli* isolates is unknown and requires further study. Irrespective of the mechanism of antibiotic resistance in the *E. coli* strains isolated and characterized in this study, in the absence of clinical signs and disease, our recommendation is to avoid antibiotic treatment, thereby limiting the potential for promoting antibiotic resistance. If an animal is clinically compromised, antibiotic therapy is recommended based on culture and sensitivity results.

CNF1-producing *E. coli* was isolated previously from ferrets housed at MIT (Marini *et al.*, 2004). Extra-intestinal *E. coli*-associated diseases are a clinically significant cause of morbidity and mortality in post-parturient jills and neonates. Similarly, these ferret *E. coli* isolates were all β-haemolytic, and α-haemolysin- and P fimbriae-positive, and were negative for other virulence factors tested. Another common feature between the ferret and macaque isolates was the somatic O-antigens, which were types 2 and 6. *E. coli* is a normal inhabitant of the gastrointestinal tract of macaques and several different serotypes of *E. coli* have been isolated from asymptomatic rhesus macaques (Schiff *et al.*, 1972). There are reports of *E. coli*-associated diarrhoeal disease in young chimpanzees and an orang-utan, with some of the chimpanzees developing peracute disease and dying within hours (McClure *et al.*, 1972). Tamarins also have mortality attributed to enteropathogenic *E. coli* infection (Potkay, 1992). Enteropathogenic *E. coli* was also reported as a common opportunistic pathogen causing diarrhoea and wasting in rhesus macaques infected with simian immunodeficiency virus (Mansfield *et al.*, 2001). In a preliminary report, clinically inapparent *E. coli* infections in squirrel monkey infants were attributed as the cause of peracute deaths, although these organisms were non-motile and did not produce enterotoxin. Colitis and septic meningitis were seen histopathologically (Scimeca & Brady, 1990). Although gastrointestinal disease has been attributed to pathogenic *E. coli* infections in these non-human primate species, toxin production was either not found or not evaluated, and the serotypes identified differed from those of the six *E. coli* isolates described in our study. As NTEC has been isolated from human and animal populations, and these isolates can be highly related on a molecular basis, there is concern about potential zoonotic spread (Mainil *et al.*, 1999). The presence of CNF1-producing *E. coli* strains in primates used in neurobiology emphasizes the need to use appropriate personnel protection and hygienic practices when handling these primates to minimize this zoonotic risk. In cases where zoonotic spread is suspected, molecular as well as epidemiological evidence are needed to substantiate animal-to-human transmission.

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