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Cytotoxic *Escherichia coli* strains encoding colibactin colonize 1 laboratory mice

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Abstract

Escherichia coli strains have not been fully characterized in laboratory mice and are not currently excluded from mouse colonies. Colibactin (Clb), a cytotoxin, has been associated with inflammation and cancer in humans and animals. We performed bacterial cultures utilizing rectal swab, fecal, and extra intestinal samples from clinically unaffected or affected laboratory mice. Fifty-one *E. coli* were isolated from 45 laboratory mice, identified biochemically, and selected isolates were serotyped. The 16S rRNA gene was amplified and sequenced for specific isolates, PCR used for *clbA* and *clbQ* gene amplification, and phylogenetic group identification was performed on all 51 *E. coli* strains. Clb genes were sequenced and selected *E. coli* isolates were characterized using a HeLa cell cytotoxicity assay. Forty-five of the 51 *E. coli* isolates (88 %) encoded *clbA* and *clbQ* and belonged to phylogenetic group B2. Mouse *E. coli* serotypes included: O2:H6, O−:H−, OM:H+, and O22:H−. Clb-encoding O2:H6 mouse *E. coli* isolates were cytotoxic *in vitro*. A Clb-encoding *E. coli* was isolated from a clinically affected genetically modified mouse with cystic endometrial hyperplasia. Our findings suggest that Clb-encoding *E. coli* colonize laboratory mice and may induce clinical and subclinical diseases that may impact experimental mouse models.

Keywords

Escherichia coli; colibactin; mice; disease; cytotoxic; cancer

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1. Introduction

Escherichia coli are commonly found in the gastrointestinal tract of humans and animals. Some *E. coli* strains are described as commensals whereas others as pathogenic. Commensal *E. coli* may be difficult to differentiate from extra intestinal pathogenic *E. coli* (ExPEC) which may be part of the normal flora and also be facultative pathogens [1 – 3]. Pathogenic strains are associated with disease, encode virulent determinants, and can be classified into pathogenic groups [4]. Recognized *E. coli* pathotypes include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adhering *E. coli* (DAEC) and adherent-invasive *E. coli* (AIEC) [4]. Atypical EPEC colonizes Norway rats and EPEC and EHEC have been isolated from laboratory rabbits [5, 6]. *E. coli* with adherent invasive phenotype has also been isolated from dogs with granulomatous colitis [7]. Furthermore, *E. coli* strains can be classified into phylogenetic groups (A, B1, B2, and D) and/or subgroups (A0, A1, B1, B22, B23, D1, and D2) which can be used for epidemiological investigations [8–10].

A genomic island called polyketide synthase or *pks*, which included colibactin (Clb) genes, was described in B2 Nissle 1917 and B2 *E. coli* strains associated with meningitis, septicemia, and urinary tract infection (ExPEC) as well as in other *Enterobacteriaceae* including *Klebsiella pneumoniae*, *Citrobacter koseri*, and *Enterobacter aerogenes* [11, 12]. In vitro characterization of *pks*-encoding *E. coli* demonstrated megalocytosis (or progressive enlargement of cells and nuclei without mitosis), activation of G2 checkpoint and induction of cell cycle arrest and DNA double-strand breaks [11]. Strains encoding other cyclomodulins (bacterial effectors and toxins that obstruct the cell cycle) including cytotoxic necrotizing factor (CNF), cytolethal distending toxin (CDT), and cycle inhibiting factor (Cif) are also able to induce megalocytosis [8, 13].

Historically, laboratory mice have been used as experimental *E. coli* infection models [14]. *E. coli* colonizing the intestine are often considered commensals in laboratory mice and rats, but have been implicated in cases of bacterial infections in laboratory mice [15, 16]. Previous studies also reported isolation of “enteropathogenic” *E. coli* in mice, and cecocolitis in immunodeficient mice associated with lactose negative *E. coli* [17, 18].

An *E. coli* isolate from wild-type mice raised in specific pathogen-free conditions induced inflammation of the cecum in interleukin-10 (IL-10) knockout (IL-$10^{-/-}$) mice after a 3-week monoassociation period [19]. This isolate, serotype O2:H6/41, phylogenetic group B2, and named NC101, was found to encode *pks* [20, 21]. Other studies using an IL-$10^{-/-}$ mouse model determined that colonization with a specific *E. coli* (O7:H7:K1) strain of phylogenetic group B2 was associated with intestinal inflammation [22]. Studies determined that monoassociation with NC101 promoted invasive carcinoma in IL-$10^{-/-}$ mice treated with azoxymethane (AOM) and the promotion effect was dependent on expression of the *pks* island [21].

The prevalence of specific *E. coli* strains in laboratory mouse colonies is unknown. Our laboratory animal facilities are routinely evaluated for microbiological contamination

utilizing a surveillance, sentinel program. This program, in addition to viral and parasitic screening, incorporates collection of rectal swab samples from surveillance mice for bacterial culture. Our current study focused on characterization of rectal *E. coli* isolates from surveillance mice as well as fecal or extra intestinal isolates from non-surveillance clinically affected and unaffected mice. We hypothesized that *E. coli* strains encoding virulence determinants such as *pks* colonize the gastrointestinal tract of laboratory mice naturally exposing these animals to the toxigenic effects of colibactin.

2. Materials and Methods

2.1 Murine samples, culture, and biochemical characterization

Rectal swabs were collected routinely as part of necropsy procedures performed on Crl:CD1(ICR) mice (CD1 mice) used as sentinels in a rodent health monitoring (surveillance) program encompassing International AAALAC-approved animal facilities managed by the Division of Comparative Medicine at the Massachusetts Institute of Technology. The surveillance program is approved by the MIT Committee on Animal Care (CAC). Mice used for the MIT surveillance program originate from a CD1 breeding colony established in a dedicated room (F3f) within a barrier in one of our facilities. This CD1 breeding colony is replaced with new CD1 stock from the commercial supplier every 2–3 years. The initial breeding stock of CD1 mice were tested by the supplier and the results were negative for viruses, parasites, and the following bacteria: *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Helicobacter spp., Klebsiella pneumoniae, Klebsiella oxytoca, Mycoplasma pulmonis*, *Pasteurella multocida, Pasteurella pneumotropica, Pseudomonas aeruginosa, Salmonella spp., Streptobacillus monilliformis*, beta hemolytic *Streptococcus spp*. (including Groups B and G), CAR bacillus, and *Clostridium piliforme*. Neither the supplier nor our institution excluded *E. coli* from CD1 mice prior to establishing the breeding colony used for our surveillance program. The breeding colony is routinely screened several times a year for selected murine viruses, parasites, *Helicobacter* spp., *Salmonella* spp., and *Citrobacter rodentium*.

In general, surveillance mice receive dirty bedding from other cages in the same mouse holding room and samples are obtained from surveillance mice to determine their exposure to infectious or adventitious agents. The infection status of surveillance mice in a particular holding room is used to represent the health status of that specific room. Four surveillance mice are housed per cage. At two months after being placed in a particular room, diagnostic tests are performed on two of the four surveillance mice. At four months after placement, the other two mice are euthanized and tested. Finally, at 6 months after placement, the first two mice are euthanized and tested. Necropsies are performed and rectal swab samples are enriched in Gram negative broth and cultured on Hektoen enteric agar, XLD agar, and MacConkey agar plates. *Salmonella* s p p. and *Citrobacter* s p p. screening is performed on fecal samples. Tracheal/lung washes, and nares flush samples are collected, enriched in tryptic soy broth (TSB) and cultured on chocolate agar and a split plate of blood agar and MacConkey agar. Serum is examined by ELISA for pathogenic murine viruses, *Mycoplasma* sp., and CAR bacillus. Other tests include mouse parvovirus PCR and parasite examination.

Fecal samples were also collected from the CD1 breeding colony, the CD1 commercial supplier, a different commercial supplier of mice, from mice that were not part of our surveillance program, and from tissues of clinically affected mice that were submitted for diagnostic necropsy. The clinically affected mice included a 9 month old female lamellipodin deficient mouse ($LPD^{-/-}$) [23, 24] that presented with dystocia and an enlarged right uterine horn; the uterine fluid and uterus wall were placed in freeze media (brucella broth and 20% glycerol) and cultured. In addition, an adult female "alpha V integrin$^{+/-}$; alpha v fl$^{/+}$; Tie 2, Cre$^{+/-}$" mouse on a C57BL/6 genetic background presented with lethargy and postpartum abdominal distention and was submitted for diagnostic tests and necropsy to our laboratory where a uterine culture was also performed. This mouse strain is deficient in αv integrin in tissues including endothelial cells and myeloid cells where the Tie2 promoter is expressed [25]. Fecal pellets were also collected from cages of selected mouse strains that belonged to resident colonies (non- surveillance) including interleukin 10 (IL-10) knockout (C57BL/6 background), C57BL/6J-*Apc*Min/J, $Lpd^{-/-}$, and 129Rag2 deficient mice.

Fecal pellets were deposited in tubes with sterile TSB, mashed with a sterile swab, and incubated overnight at 37°C. The next day, a sterile swab was dipped in the TSB culture tube and then plated on chromID™ CPS® agar plates (Biomérieux) [13]. *E. coli* colonies growing on CPS or MacConkey agar plates were identified by their characteristic pink to burgundy and pink, respectively. These colonies were collected with sterile plastic loops placed in sterile PBS in microfuge tubes and used for DNA extraction. In addition, cultures were characterized by API® 20 E (Biomérieux).

2.2 DNA extraction, PCR amplification, and sequencing

The Roche High Pure PCR Template Preparation Kit was used for bacterial DNA extraction. DNA concentration was measured using NanoDrop 2000c (Thermo). Primers were used for analyses of *pks* (*clbA* and *clbQ*) [8, 26], *cdtB*, phylogenetic group determination [27–29], genotyping by ERIC PCR [30], and 16S rRNA and *clbA* and *clbQ* gene sequencing (Table S1). Sequencing of 16S rRNA and *clbA* and *clb*Q genes was performed at QuintaraBio (Allston, MA) using primers 9F and 1541 or 9F and F16, IHAPJPN42, IHAPJPN4 6, IHAPJPN55, and IHAPJPN56, respectively (Table S1).

2.3 Serotyping

Ten *E. coli* isolates from surveillance mice and seven isolates from the CD1 breeding colony mice were submitted to the *E. coli* Reference Center at Penn State University for testing which included: O and H typing and analyses including PCR for heat-labile toxin (LT), heat-stabile toxin (STa and STb), Shiga-type toxin 1 and 2 (Stx1 and Stx2), intimin gamma (*eae*), and cytotoxic necrotizing factor 1 and 2 (*cnf1* and *cnf2*).

2.4 Cell culture of cytotoxicity assays

E. coli strains used for cytotoxicity assay included K12 (negative control), O18:K1 (K1 or RS218; a phylogenetic group B2 human neonatal meningitis ExPEC) (K12 and K1 were acquired from David B. Shauer's collection: catalog samples DBS 83 and DBS 91 respectively), V27 (acquired from *E. coli* Reference Center is a *cdt* positive control) [31], NC101 (Clb positive control acquired from Christian Jobin's Lab), and novel isolates M, F,

A2, A4, A5, A7, A8, A10, A11, A12, A13, A15, A21, A28, and A30 (all from mice in the current study). Selected tests also were performed on the *E. coli* strains used in the cytotoxicity assays included: API® 20 E for biochemical characterization and PCR for *clbA* and *clbQ*, phylogenetic groups, and *cdt*.

2.4.1 Cell culture assay for colibactin cytotoxicity—The cytotoxicity assay was performed as described previously with modifications [11]. HeLa S3 cells (ATCC CCL2.2) were grown and maintained in Eagle's Minimum Essential Medium (EMEM, ATCC) containing 10% Fetal Calf Serum (FCS, Sigma) and 1% Antibiotic-Antimycotic (Gibco) at 37 °C with 5% CO_2. 5×10^4 cells were seeded onto 24-well cell culture plates and incubated at 37 °C with 5% CO_2 for 24 hours. Overnight liquid cultures of *E. coli* strains were grown for 2 hours at 37 °C and then adjusted to O.D.600nm of 0.0025 and 0.01 in 1% FCS EMEM media, corresponding to a multiplicity of infection (MOI; is the number of bacteria per cell at the onset of infection) of 25 and 100, respectively. Following inoculation, plates were centrifuged at 200 g for 10 minutes to facilitate bacteria interaction and then incubated at 37 °C with 5% CO_2 for 4 hours.

Cells were then washed with EMEM and replaced with EMEM containing 10% FCS and 200 ug/mL gentamicin (Gibco). Following 72 hours incubation, plates were stained with Diffquick stain (Thermo Scientific). Cells were then inspected under a microscope for confluence and morphological changes. Images were captured with Zeiss Axiovert-10 microscope using Image Pro-Plus software version 7.0 at 20× magnification.

2.4.2 Cell culture assay for sonicate cytotoxicity—Overnight cultures of *E. coli* strains were grown for 2 hours at 37 °C and pelleted by centrifugation at 12000 rpm for 5 minutes. Supernatant was removed and samples were washed in 1 ml of PBS. Samples were pelleted again by centrifugation at 12000 rpm for 5 minutes. Pellets were re-suspended in 2 ml of PBS and then sonicated on ice using the following program: amplitude: 35; power: 7 watts; 30 second intervals for a total of 5 minutes with 1 minute breaks between intervals. Sonicate samples were centrifuged at 12000 rpm for 10 minutes at 4 °C to remove large debris. Supernatant was collected and then filter-sterilized through 0.2 μm filters. Total protein was quantified using the BCA assay (Thermo Fisher Scientific). 5×10^4 HeLa cells were seeded onto 24-well cell culture plates and incubated at 37 °C with 5% CO_2 for 24 hours. Cells were treated with 4 or 20 μg/mL total protein of crude bacterial sonicate for 72 hours. Plates were stained and microscopically analyzed for confluence and morphological changes as described above.

2.5 Histopathology and Peptic nucleic acid (PNA) fluorescent in situ hybridization (FISH)

Tissue sections of the reproductive tract from the two clinically affected mice ($LPD^{-/-}$ and "alpha V integrin$^{+/-}$; alpha v fl$^{/+}$; Tie 2, Cre$^{+/-}$") were fixed in 10% formalin, embedded in paraffin, sectioned at ~4 μm, and stained with hematoxylin and eosin. These mouse tissues were also prepared and utilized for PNA fluorescent in situ hybridization for *E. coli* identification utilizing *GNR Traffic Light™* PNA FISH® probe (Kindly provided by Mark J. Fiandaca) [5]. Incubation of slides with probe was performed in a hybridization oven at 55 °C for 1 hour and 45 minutes. Slides were examined using a Zeiss Axioskop 2 plus

microscope and image acquisition was performed using a QImaging-QIClick camera (QImaging, Surrey, BC, Canada).

3. Results

3.1 E. coli colonizes laboratory mice

A total of 51 *E. coli* isolates were cultured from the 45 mice surveyed in this study (see Table 1). Of the 51 *E. coli* isolates, 49 were obtained from newly arrived mice and those housed at MIT, and the other two isolates from a mouse housed at another research institution. Twenty-three of 51 (45%) isolates were obtained from CD1 surveillance mice housed in different rooms of six animal facilities at MIT (facilities: F1, F2, F3, F4, F5, F6) whereas 5 of 51 (10%) isolates were obtained from non- surveillance mice (excluding breeding colony and newly arrived mice) housed in different rooms of two animal facilities at MIT (F3 and F4) (Table 1). Seven of 51 (14%) *E. coli* isolates were cultured from the CD1 breeding colony. All surveillance and non-surveillance mice housed at MIT originated from animal facility rooms that only housed research mice, except one room in F4 (F4e), which housed wild-caught meadow jumping mice (*Zapus hudsonius*).

E. coli isolates obtained from CD1 surveillance mice were housed in: four rooms in F1 (isolates: M, E, F, G); Two rooms in F2 (A1, A2, A10); Six rooms in F3 (A3, A4, A5, A6, A7, A8, A9); Three rooms in F4 (A24, A27, A28, A29); Three rooms in F5 (A15, A20, A22, A23); and two rooms in F6 (A25 and A26). *E. coli* was also isolated from non-surveillance mice including: 129Rag2 deficient mice housed in one room in F4 (A11 and A12); C57BL/6-*Apc*^{Min} mice housed in one room in F3 (A13 and A14); and a clinically affected LPD$^{-/-}$ mouse housed in F4 (A21). *E. coli* isolates A8 and A13 were obtained from surveillance (CD1) and nonsurveillance (C57BL/6-*Apc*^{Min}) mice housed in the same room (F3e). *E. coli* isolate A9 was obtained from a surveillance mouse monitoring the CD1 breeding colony (F3f). In addition, two *E. coli* (A30 and A31) isolates and a *Streptococcus bovis* originated from a clinically affected "alpha V integrin$^{+/-}$; alpha v fl$^{/+}$; Tie 2, Cre$^{+/-}$" mouse housed at another research institution submitted to MIT for pathological evaluation.

No *E. coli* was detected on fecal culture plates from IL-10 knockout mice housed at MIT. Of the 51 isolates, the most common API code was 5144572 observed in 61% (31 of 51 isolates). Other API codes were 7144572 (5 of 51 isolates (10%), 5144552 [5 of 51 isolates (10%)], 4144572 [3 of 51 isolates (6%)], and 5344552 [3 of 51 isolates (6%)]. API codes observed in single isolates were 5144532 (2%), 6144572 (2%)], 5144272 (2%), and 5177572 (2%).

3.2 Surveillance and non-surveillance mouse isolates were genetically confirmed to be E. coli and encode colibactin genes

Selected mouse isolates were confirmed to be *E. coli* by 16S rRNA PCR amplification and sequencing. Sequences from selected mouse *E. coli* isolates including A2, A3, A11, A21, A21-2 (A21 for confirmation), A30, and A31 were compared to sequences of *E. coli* W and *E. coli* ATCC 25922 in the GenBank database (CP002967.1 and X80724.1 respectively) and found to be more than 99% similar confirming that mouse isolates were *E. coli*.

Forty-five of 51 (88%) isolates analyzed by PCR for Clb genes (*clbQ* and *clbA*) were positive (Table 1; Fig. 1). The partial *clbQ* gene sequences of our *E. coli* isolates (including A2, A3, A5, A6, A7, A8, A9, A10, and A12) had 99% identities with the *clbQ* gene of *E.coli* strain IHE3034 (Genbank Number AM229678.1). The Clb genes (*clbA* and *clbQ*) of isolates M and F were also confirmed by sequence analysis and Blast search. None of the DNA from *E. coli* isolates tested were positive for *cdt, eae*, or *stx1*.

3.3 Clb-encoding mouse E. coli isolates belong to phylogenetic group B2

All *E. coli* isolate DNA samples positive for Clb genes (45 of 45; 100%) were phylogenetic group B2. Those negative for both Clb genes were either phylogenetic group B2 (1 of 6; 17%) or B1 (5 of 6; 83%) (Table 1; Fig. 2).

3.4 Possible transmission of E. coli between non surveillance mice and surveillance mice

E. coli isolates A8 and A13 obtained from mice housed in the same room shared similar DNA amplification patterns and this amplification pattern was also observed in DNA from other mouse *E. coli* isolates (Fig. 3).

3.5 CD1 breeding colony is colonized with Clb-encoding E. coli and is a source of E. coli for surveillance mice

E. coli isolated from the feces of 7 of 10 (70%) cages in the CD1 breeding room, tested positive by PCR for *clbQ* and *clbA*, and belonged to phylogenetic group B2.

3.6 Serotyping and analyses of other virulence factors

Thirteen of 17 (76%) *E. coli* isolates from surveillance and non- surveillance mice were determined to be serotype O2:H6, including isolate A13. The other 4 isolates were identified as other serotypes including: OM:H+ (one isolate: A4), O22:H− (one isolate: A11), and O −:H− (two isolates: A30 and A31). None of these 17 isolates were positive for LT, Sta, Stb, *stx1*, *stx2*, *eae*, *cnf1* or *cnf2*.

3.7 Clb-encoding mouse E. coli isolates are cytotoxic

Representative *E. coli* isolates from surveillance and non-surveillance mice were tested using cell culture assays to confirm cytotoxic activity predicted by the PCR results. Colibactin activity was verified by transiently infecting HeLa cells with live bacteria for 4H followed by a 72H incubation in gentamicin-containing media. At both MOI 25 and 100, all novel Clb-encoding *E. coli* strains tested caused contact-dependent megalocytosis, with morphology and frequency consistent to that of NC101 (colibactin positive control) (Fig. 4). Compared to the other Clb-encoding strains, A12 appeared to cause milder colibactin cytotoxicity at MOI 100 and 25; however, megalocytic cells were present in the A12 preparation at MOI 100 and 25. The human strains V27 and RS218 also induced colibactin-like megalocytosis. HeLa cells infected by the strains PCR-negative for the colibactin genes were indistinguishable from those treated with the media and K12 negative controls). Treatment with 4 or 20 μg/mL total protein of crude bacterial sonicate for 72H did not affect HeLa cell morphology and viability, therefore excluding expression of cytolethal distending toxin (CDT), cytotoxic necrotizing factors (CNFs), and other sonicate-detectable virulence

factors by these novel mouse isolates (data not shown). Characterization of the *E. coli* strains used in the cytotoxicity assays are summarized in Table S2.

3.8 Colonization by Clb-encoding E. coli varies depending on the commercial mouse supplier

Ten CD1 mice from the same source as those of our breeding colony were ordered from two different barriers (five from each barrier) at the commercial supplier. Two shipments of C57BL/6 mice obtained from the vendor with CD1 mice colonized with clb-encoding *E. coli* were also screened for *E. coli*. In addition, five Swiss Webster (Tac:SW) mice were ordered from a different commercial supplier, as well as a shipment of C57BL/6 mice. Although Swiss Webster and CD1 mice are not the same stock, they are genetically related and SW mice have been previously used for surveillance in our laboratory animal facilities [32, 33]. Fecal samples were collected from these mice immediately upon arrival and cultured. *E. coli* was isolated from the feces of all ten mice that arrived from the commercial supplier that provided our CD1 mice. From one commercial barrier, Clb-encoding *E. coli* were isolated from all five mice, and in the other barrier, Clb-encoding *E. coli* were isolated from three of five mice. In the newly arrived C57BL/6 mice from the same commercial vendor from which we received the CD1 mice, Clb-encoding *E. coli* was isolated from 1 of 1, and 2 of 3 mice from each of the 2 shipments, respectively. In contrast, no Clb-encoding *E. coli* were isolated from any of the five Swiss Webster mice from the second commercial vendor. In addition, 3 newly arrived C57BL/6 mice from this same vendor were negative for fecal *E. coli*.

3.9 Histopathological evaluation

The endometrium and endometrial stroma of the $LPD^{-/-}$ mouse were infiltrated with low numbers of inflammatory cells and lesions were consistent with cystic endometrial hyperplasia. A pks^{+} *E. coli* was isolated from this case of endometrial hyperplasia, but whether the organism played a role in producing this lesion is unknown. The “alpha V $integrin^{+/-}$; alpha v $fl^{/+}$; Tie 2, $Cre^{+/-}$” mouse exhibited necrotizing suppurative metritis and intralesional and intraluminal bacteria consistent with pyometra (Fig. 5). Although an *E. coli* strain was isolated from this mouse, it didn’t contain *clbA* or *clbQ* based on our PCR assay. The *E. coli* FISH assay was positive, indicating that *E. coli* colonized in affected tissue. Whether this organism induced the pyometra is not known.

4. Discussion

E. coli infection in laboratory mice has been previously reported [15–19, 22]. However, a survey including biochemical, genetic, and phenotypic characterization of mouse *E. coli* isolates has not been performed. The experimental characterization of *E. coli* NC101 in $IL\text{-}10^{-/-}$ mice and the natural and experimental characterization of O7:H7:K1 in $IL\text{-}10^{-/-}$ mice indicated the pathogenic potential of Clb-encoding *E. coli* in susceptible animals [19, 21, 22, 34]. Our current study was undertaken to characterize *E. coli* and investigate the relative prevalence of Clb-encoding *E. coli* in laboratory mouse colonies. We found that the majority of the mouse *E. coli* isolates encoded two Clb genes (*clbA* and *clbQ*) and selected Clb-encoding isolates induced megalocytic colibactin-like cytotoxicity to HeLa cells *in*

vitro. Also, most of the mouse *E. coli* isolates in our study were phylogenetic group B2, a group that has been previously documented in laboratory mice [22]. Furthermore, all of our Clb-encoding mouse isolates were phylogenetic group B2. B2 phylogenetic group-associated traits that can predict extraintestinal virulence in a mouse model [35]. In addition, some of our isolates were serotype O2:H6 which is the same serotype as that of *E. coli* NC101 [20]. The O2 serotype is found in avian pathogenic *E. coli* (APEC) and the O2:H6 serotype has been identified in Shiga toxin-producing *E. coli* [28, 36, 37]. Importantly, serotype O2 in humans is associated with UTIs and bacteremia [38].

The role of Clb-encoding *E. coli* has been explored in human colorectal cancer and investigated in different types of mouse models including IL-$10^{-/-}$ mice treated with azoxymethane (AOM), C57BL/6J-*Apc*^{Min}/J mice, mice treated with AOM and dextran sodium sulfate (DSS), and in nude mice with xenographs [13, 21, 39, 40]. These studies have established a role of Clb-encoding *E. coli* in inflammation and cancer. The increased in *E. coli* growth and colonization may be due to inflammation generated nitrate, as *E. coli*, a facultative anaerobe (unlike obligate anaerobes which are the major colonizers of the lower bowel), can produce energy through the use of nitrate, S-oxides and N-oxides, as terminal electron acceptors for anaerobic respiration and thus outcompete obligate anaerobes [41]. Our finding that mouse *E. coli* isolates in the current study were cytotoxic to cells *in vitro* suggest that mouse Clb-encoding *E. coli* have the potential to induce clinical or subclinical disease in laboratory animals and confound experimental results. Although we cannot exclude the possibility that mouse *E. coli* isolates may encode *cif*, and therefore induce megalocytosis in vitro, *cif* is mainly found in EPEC and EHEC, and our preliminary studies did not detect *eae* or *stx1* in selected mouse isolates [13, 42].

In a mouse model of septicemia and lethality, subcutaneous or intraperitoneal inoculation of mice with human *E. coli* isolates has been used to investigate relative extraintestinal virulence; however, in this model, colonization of mice with *E. coli* prior to experimental inoculation has not been reported [1, 2, 43]. In addition, some *E. coli* infection models in mice and rats have relied on the use of streptomycin treatment prior to inoculation [39, 44, 45]. However, others have found that mouse *E. coli* isolates are resistant to antibiotics [15]. Importantly, natural colonization with *E. coli* may contribute to colonization resistance to experimental *E. coli* infection [14, 46]. Clb appears to impact host survival by exacerbating lymphopenia during sepsis and can also affect oral tolerance and immune responses [43, 47]. Therefore, the *E. coli* colonization status of laboratory mice should be investigated prior to experimental infection. Specifically, colonization of mice with Clb-encoding *E. coli* should be determined prior to using NC101 or other Clb-encoding *E. coli* in experimental studies. The source of Clb- encoding *E. coli* in mice in this study originated from commercially reared mice colonized with enteric Clb-encoding *E. coli* strains. Studies in humans suggest that ExPEC are part of the intestinal flora and have the potential to become pathogenic in disease conditions [1, 2, 48]. Vertical transmission of Clb-producing *E. coli* has been demonstrated in Wistar rats [45]. *E. coli* may also be transmitted in laboratory rodents via *E. coli* contaminated bedding [49].

ExPEC infection is associated with urosepsis, bacteremia, and neonatal meningitis in humans [8, 31, 50]. We confirmed the Clb-producing nature of *E. coli* neonatal meningitis

isolate K1 (RS218) and also determined that the V27 human *E. coli* urosepsis isolate is a Clb-producer [31, 50]. *E. coli* isolates from human prostatitis cases have been characterized as ExPEC and some encode Clb [51]. *E. coli* urogenital tract infection has also been reported in animal species. Infection of dogs with *E. coli* results in pyometra/cystic endometrial hyperplasia and *E. coli* from cattle experimentally induces pelvic inflammatory disease in mice and cattle [52, 53]. It would be interesting to determine if these *E. coli* are also Clb-encoding strains. In mice, natural infection with *E. coli* has been associated with urogenital lesions, pneumonia, and septicemia [16]. In our study, one Clb-encoding isolate was associated with cystic endometrial hyperplasia and another isolate, although not positive for Clb, was recovered from a mouse with pyometra. Our findings represent an opportunity to develop experimental mouse models to investigate the pathogenic potential of colibactin positive *E. coli*, which naturally colonizes the intestines of mice.

Our characterization of *E. coli* strains from laboratory mice indicates that commercially available mice are colonized with Clb-encoding cytotoxic, potentially pathogenic *E. coli*. Our initial survey of other mouse strains indicated that they too can be colonized with Clb-encoding *E. coli*. The wild caught meadow jumping mice had *E. coli* isolated from their feces, and 2 of 3 mice were negative by PCR for *clbA* and *clbQ;* however, the third jumping mouse had a pks^+ *E. coli* isolated (data not shown). The epidemiology and virulence determinants of mouse *E. coli* strains should be investigated further as well as their impact in mouse models of inflammation, cancer, and/or immunity. These studies will determine if pks^+ *E. coli* confounds research studies, and if so, arguments made to exclude these *E. coli* strains from specific pathogen-free laboratory mouse colonies, should be articulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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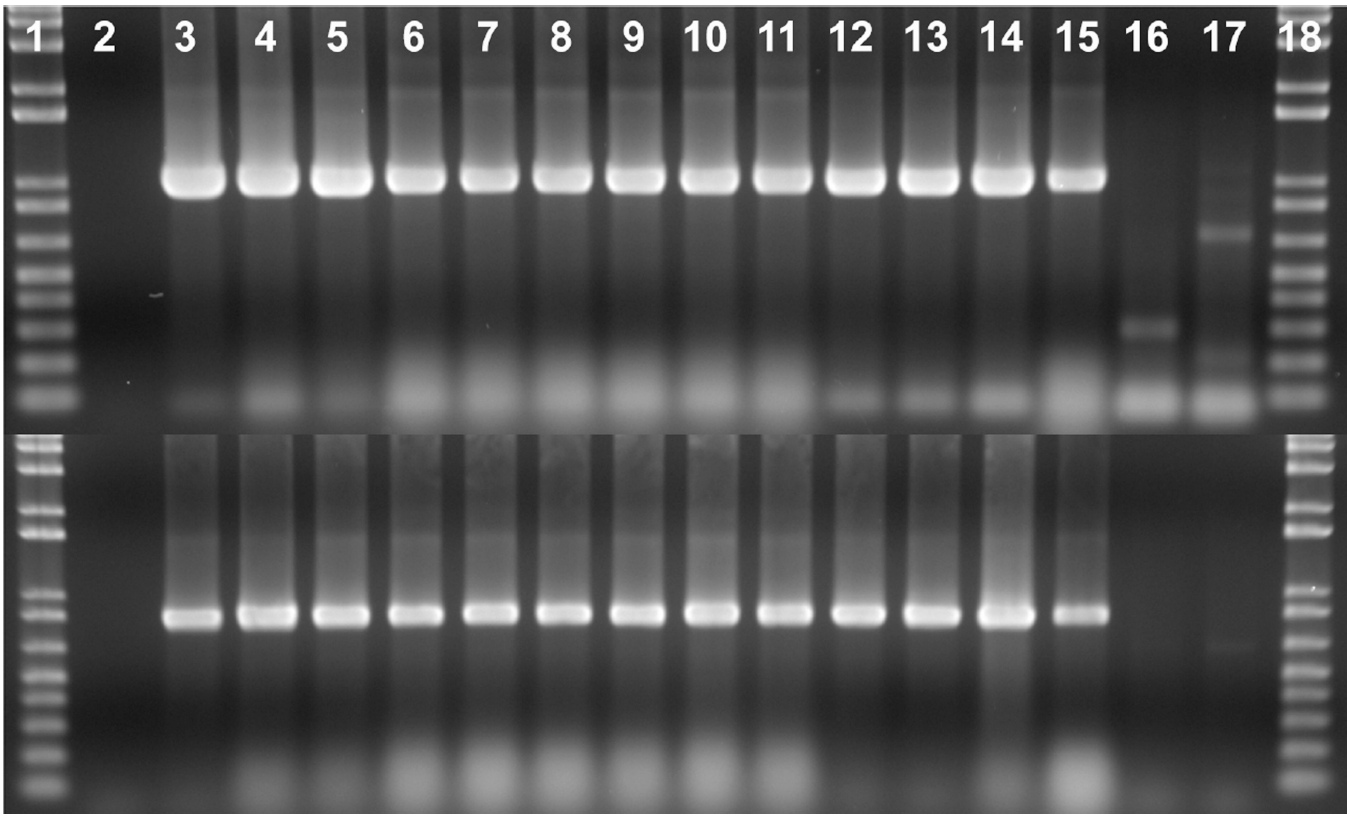

Fig. 1.

Amplification of *clb*A and *clb*Q in DNA from mouse *E.coli* isolates. Top row: *clb*A gene, bottom row: *clb*Q gene. Lane 1 and lane 18, 1 Kb plus molecular marker; lane 2, no DNA control; lane 3, NC101 (positive control); lane 4, M; lane 5, F; lane 6, A8; lane 7, A13; lane 8, BC1; lane 9, BC2; lane 10, NA1; lane 11, NA2; lane 12, NA4; lane 13, NA5, lane 14, NA9; lane 15, NA10; lane 16, A11; lane 17, A30.

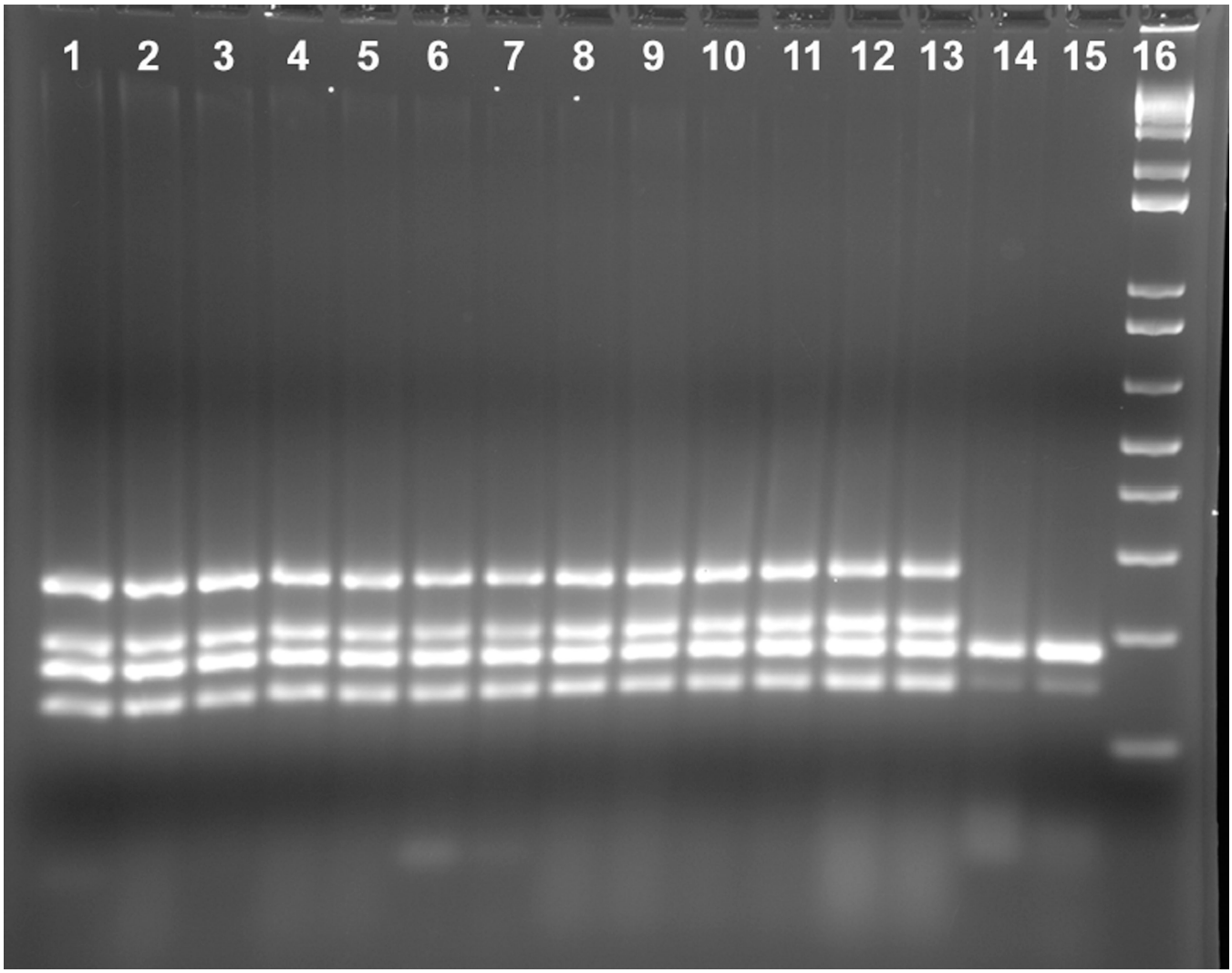

Fig. 2.

Phylogenetic group determination of *E.coli* : lane 1, NC101 (positive control); lane 2, M; lane 3, F; lane 4, A8; lane 5, A13; lane 6, BC1; lane 7, BC2; lane 8, NA1; lane 9, NA2; lane 10, NA4; lane 11, NA5, lane 12, NA9; lane 13, NA10; lane 14, A11; lane 15, A30; lane 16, 1 Kb plus molecular marker.

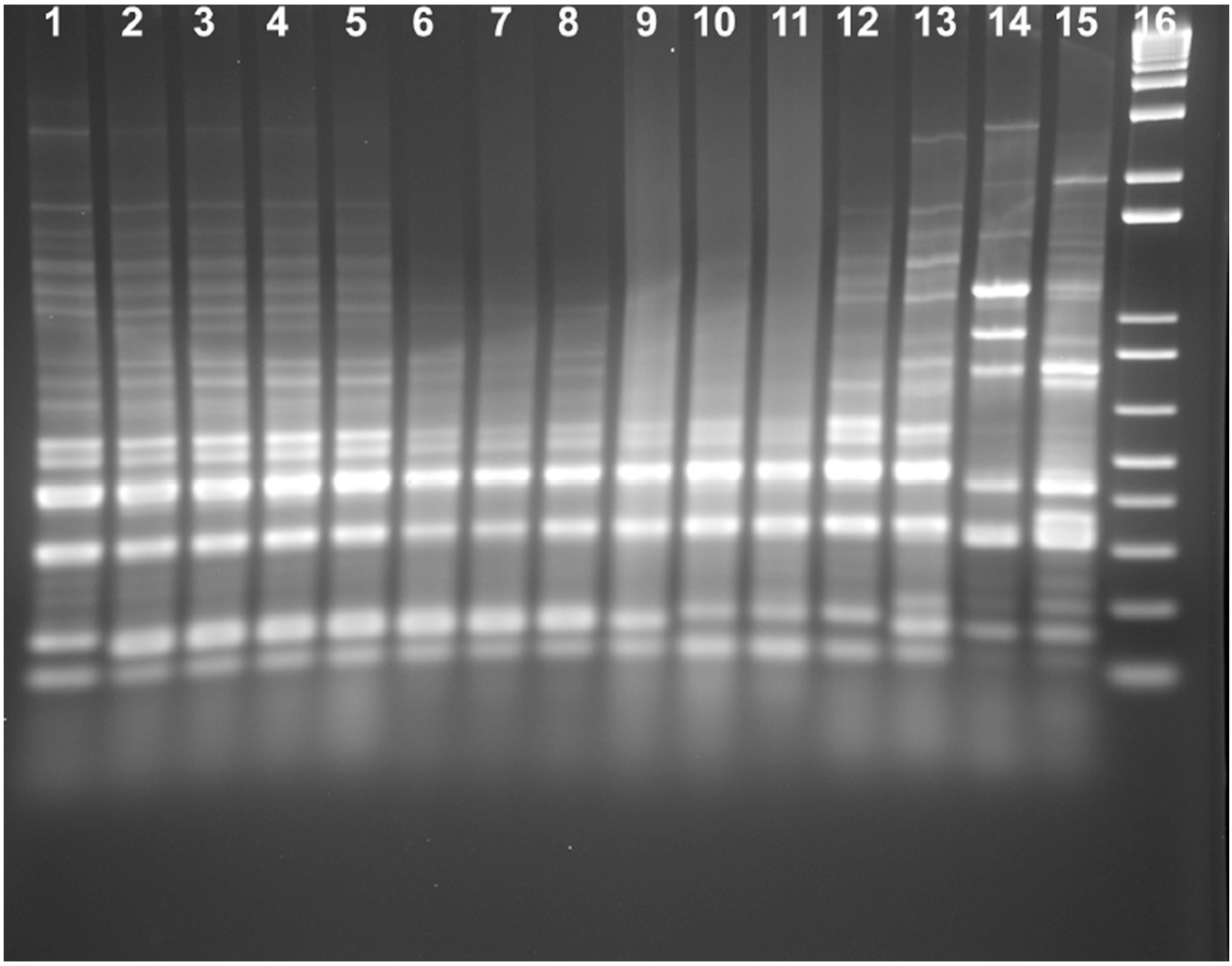

Fig. 3.

Genotyping of *E.coli* DNA using ERIC PCR: lane 1, NC101 (positive control); lane 2, M; lane 3, F; lane 4, A8; lane 5, A13; lane 6, BC1; lane 7, BC2; lane 8, NA1; lane 9, NA2; lane 10, NA4; lane 11, NA5, lane 12, NA9; lane 13, NA10; lane 14, A11; lane 15, A30; lane 16, 1 Kb plus molecular marker.

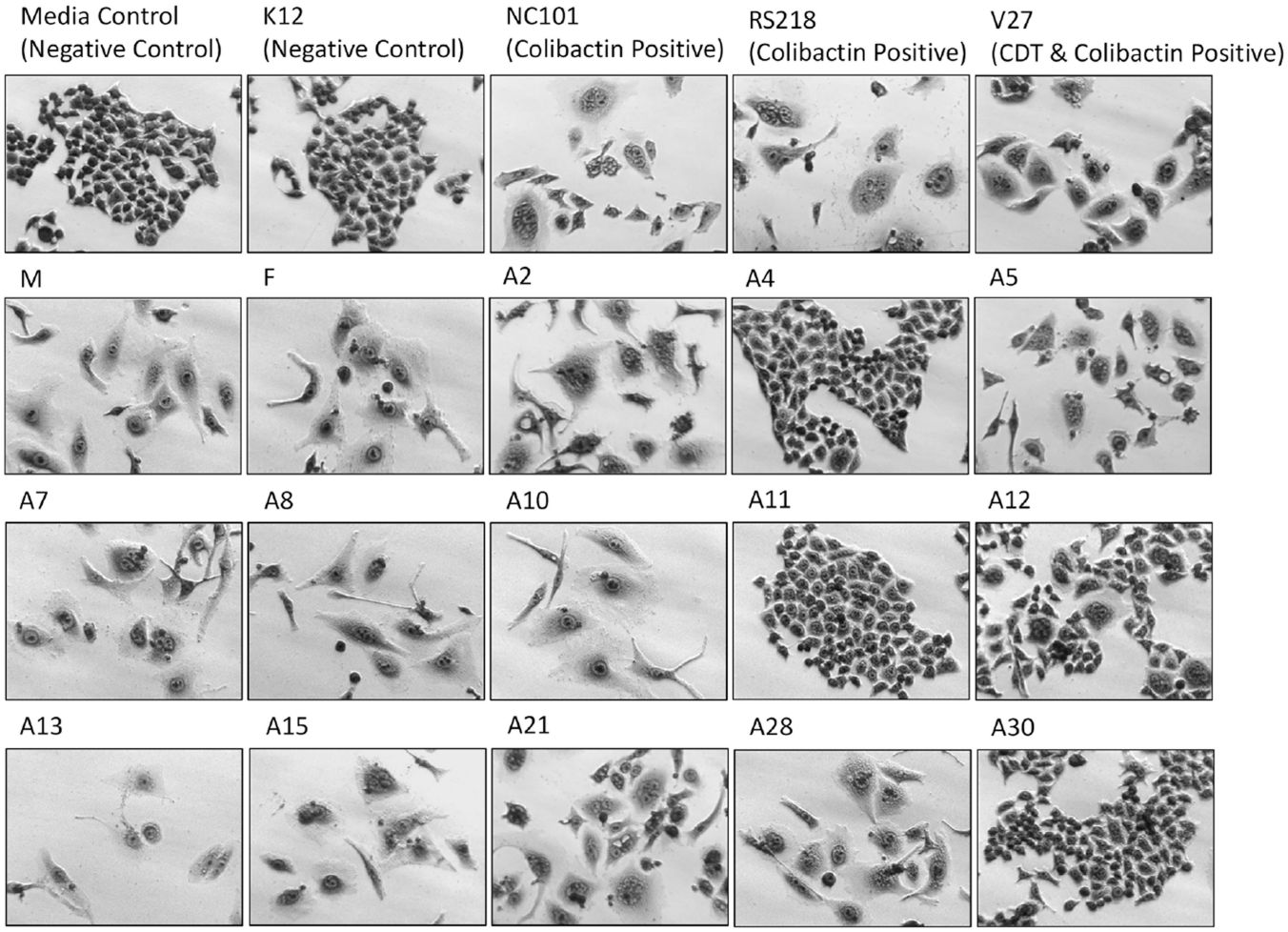

Fig. 4.

Cell culture assay for colibactin cytotoxicity. HeLa cells were inoculated with *E. coli* at a multiplicity of infection (MOI) of 100 for approximately 72 hours. Megalocytosis (enlargement of the cell body and nucleus) was observed in cells infected with colibactin (Clb)-encoding mouse *E. coli* isolates M, F A2, A5, A7, A8, A10, A12, A13, A15, A21, and A28, but not with clb-negative mouse *E. coli* isolates A4, A11, and A30. NC101 is a Clb-encoding mouse E. coli isolate (positive control). K12 is a *clb* negative *E. coli* (negative control). V27 is a cytolethal distending toxin (Cdt)- encoding human *E. coli* isolate from a urosepsis patient. RS218 (K1) is a human *E. coli* isolate from human neonatal meningitis. The V27 and RS218 human strains also induced colibactin-like megalocytosis. Images were taken at 20× magnification.

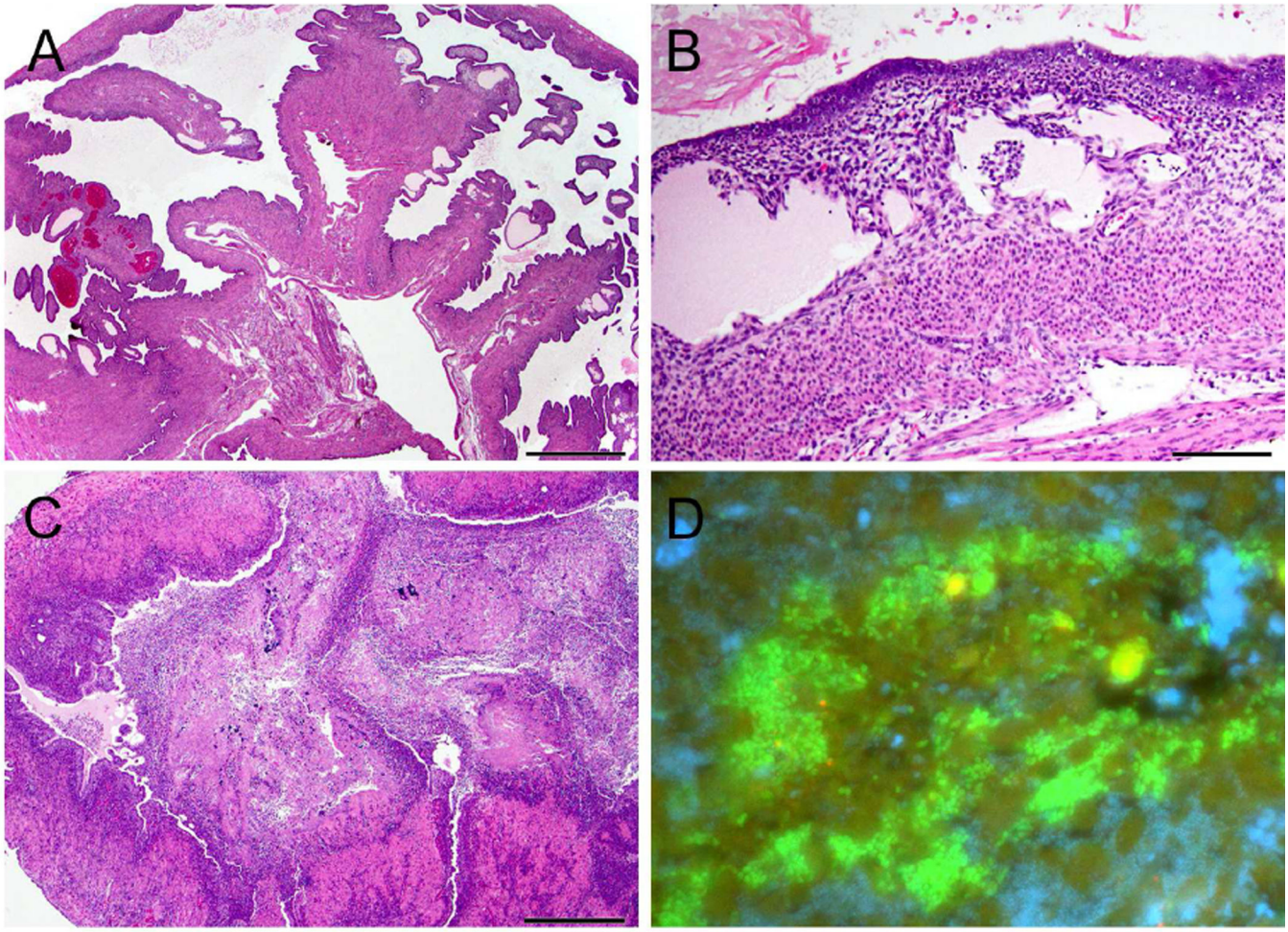

Fig. 5.

Histopathological lesions in clinically affected mice infected with *E. coli* and diagnosed with cystic endometrial hyperplasia (panels A and B are from a lamellipodin deficient female mouse infected with colibactin-encoding *E. coli*) and pyometra (panels C and D are from an "alpha V integrin$^{+/-}$; alpha v fl$^{/+}$; Tie 2, Cre$^{+/-}$" female mouse infected with colibactin negative *E. coli*). A, Severe cystic endometrial hyperplasia with angiectasis of the endometrial stroma; B, Granulocytes and mononuclear cells infiltrating the endometrium and endometrial stroma in low numbers; C, Intraluminal cellular debris and necrotizing suppurative metritis in a mouse with pyometra; D, Peptic nucleic acid fluorescent in situ hybridization using an *E. coli* specific green probe. Green fluorescent *E. coli* bacteria appear to aggregate in luminal and affected areas of the uterus. Cell nuclei are stained blue (DAPI). Scale bars: A, 1 mm (2×); B, 100 μm (20×); C, 500 μm (4×). D, no scale bar (×100).

Table 1

Characterization of *Escherichia coli* isolates from laboratory mice.

Isolate ID (Serotype)	Facility (F#) and room (letter)	Mouse (strain, stock, or line)	Surveillance (S) or non surveillance (NS)	Culture sample	API code	Phylogenetic group	*clbA*	*clbQ*	*cdtB*	CYT
M	F1a	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	+
E	F1b	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	NT
F	F1c	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	+
G	F1d	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	NT
A1	F2a	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	NT
A2[a] **(O2:H6)**	F2b	CD1(ICR)	S	Rectal swab	7144572	B2	+	+	Neg.	+
A3[a] **(O2:H6)**	F3a	CD1(ICR)	S	Rectal swab	5144272	B2	+	+	Neg.	NT
A4[a] **(OM:H+)**	F3a	CD1(ICR)	S	Rectal swab	5144552	B2	Neg.	Neg.	Neg.	Neg.
A5[a] **(O2:H6)**	F3b	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	+
A6	F3c	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	NT
A7	F3d	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	+
A8	F3e	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	+
A9	F3f[b]	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg.	NT
A10	F2a	CD1(ICR)	S	Lung	5144572	B2	+	+	Neg.	+
A11[a] **(O22:H−)**	F4a	129Rag2 deficient	NS	Feces	4144572	B1	Neg.	Neg.	Neg.	Neg.
A12[a] **(O2:H6)**	F4a	129Rag2 deficient	NS	Feces	5144572	B2	+	+	Neg.	+
A13[a] **(O2:H6)**	F3e	C57BL/6-*Apc*^{Min}	NS	Feces	5144572	B2	+	+	Neg.	+
A14	F3e	C57BL/6-*Apc*^{Min}	NS	Feces	5144532	B2	+	+	Neg.	NT
A15	F5a	CD1(ICR)	S	Nares	7144572	B2	+	+	Neg.	+
A20	F5b	CD1(ICR)	S	Rectal swab	7144572	B2	+	+	Neg.	NT

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Isolate ID (Serotype)	Facility (F#) and room (letter)	Mouse (strain, stock, or line)	Surveillance (S) or non surveillance (NS)	Culture sample	API code	Phylogenetic group	*clbA*	*clbQ*	*cdtB*	CYT
A21[a] **(O2:H6)**	F4b[c]	LPD−/−	NS	Uterine fluid and wall	5144572	B2	+	+	Neg.	+
A22	F5a	CD1(ICR)	S	Rectal swab	7144572	B2	+	+	Neg.	NT
A23	F5c	CD1(ICR)	S	Rectal swab	7144572	B2	+	+	Neg.	NT
A24	F4c	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg	NT
A25	F6a	CD1(ICR)	S	Rectal swab	4144572	B2	+	+	Neg	NT
A26	F6b	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg	NT
A27	F4d	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg	NT
A28	F4e	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg	+
A29	F4e	CD1(ICR)	S	Rectal swab	5144572	B2	+	+	Neg	NT
A30[a] **(O−:H−)**	Other[d]	"alpha V integrin $^{+/-}$; alpha v $fl^{/+}$; Tie 2, $Cre^{+/-}$"	NS	Uterine fluid and wall	4144572	B1	Neg.	Neg.	Neg.	Neg.
A31[a] **(O−:H−)**	Other[d]	"alpha V integrin $^{+/-}$; alpha v $fl^{/+}$; Tie 2, $Cre^{+/-}$"	NS	Uterine fluid and wall	6144572	B1	Neg.	Neg.	Neg.	NT
BC1 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC2 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC3 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC4 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC5 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC6 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
BC7 (O2:H6)	F3f	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
NA1	F3g	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT
NA2	F3g	CD1	NS	Feces	5344552	B2	+	+	Neg.	NT
NA3	F3g	CD1	NS	Feces	5144572	B2	+	+	Neg.	NT

Isolate ID (Serotype)	Facility (F#) and room (letter)	Mouse (strain, stock, or line)	Surveillance (S) or non surveillance (NS)	Culture sample	API code	Phylogenetic group	*clbA*	*clbQ*	*cdtB*	CYT
NA4	F3g	CD1	NS	Feces	5344552	B2	+	+	Neg.	NT
NA5	F3g	CD1	NS	Feces	5344552	B2	+	+	Neg.	NT
NA6	F3g	CD1	NS	Feces	7144572	B2	+	+	Neg.	NT
NA7	F3g	CD1	NS	Feces	5144552	B2	+	+	Neg.	NT
NA8	F3g	CD1	NS	Feces	5144552	B2	+	+	Neg.	NT
NA9	F5a	C57BL/6	NS	Feces	5144572	B2	+	+	Neg.	NT
NA10	F5d	C57BL/6	NS	Feces	5144572	B2	+	+	Neg.	NT
NA11	F5d	C57BL/6	NS	Feces	5144552	B1	Neg.	Neg.	Neg.	NT
NA12	F5d	C57BL/6	NS	Feces	5177572	B2	+	+	Neg.	NT
NA13	F5d	C5/BL/6	NS	Feces	5144552	B1	Neg.	Neg.	Neg.	NT

CYT, colibactin cytotoxicity assay; +, positive by PCR or cytotoxic; Neg., negative by PCR or not cytotoxic; NT, not tested;

[a], serotyping performed by *E. coli* Reference Center. These samples were also negative by PCR for: LT (heat-labile toxin), Sta and Stb (heat stable toxin), *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *eae* (intimin gamma), *cnf1 and cnf2* (cytotoxic necrotizing factors 1 and 2);

[b],breeding colony location;

[c], this LPD$^{-/-}$ mouse was housed in various locations prior to the indicated location.

[d], "Other" indicates an animal facility not at MIT. BC, breeding colony; NA, newly arriving from vendor. The following pairs of isolates are from the same mouse: A1 and A10; A15 and A22; A28 and A29; A30 and A31; NA10 and NA11; NA12 and NA13.