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ORIGINAL ARTICLE

Analysis of inflammatory cytokine expression in the urinary tract of BALB/c mice infected with *Proteus* (*P.*) *mirabilis* and enteroaggregative *Escherichia* (*E.*) *coli* (EAEC) strains

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Abstract

This study aimed to analyze the proinflammatory cytokine mRNA expression in the urinary tract of BALB/c mice infected with bacterial strains with uropathogenic potential. Groups of four 6-week-old female BALB/c mice were intraurethrally inoculated with 5×10^7 colony-forming units (CFU) of *P. mirabilis* ATCC29906, EAEC O42, *P. mirabilis* RTX339, or sterile saline (control group) and then sacrificed at 0, 2, 4, 7, or 10 days post-infection (p.i.). Samples were cultured to determine the CFU/mL in urine or CFU/g in the bladders and kidneys. Cytokine expression (tumor necrosis factor (TNF)-α and interleukin (IL)-1β, -6, and -8) was evaluated in the target organs using real-time PCR and immunohistochemistry; histology was examined with hematoxylin and eosin staining. The results are presented as the means and standard deviations and were compared using one-way ANOVA, with $p < 0.05$ indicating significant differences. Bacteriuria was not detected in the infected groups; bacterial colonization occurred in the target organs at all time points, but was higher in mice infected with EAEC O42 or *P. mirabilis* RTX339 at 7 days p.i. The expression of all cytokine mRNAs was seen, but only the levels of the IL-8 protein increased in situ at 7 days p.i. in the *P. mirabilis* RTX339 and EAEC O42 groups in both organs. Morphological alterations, observed in all of the infected groups, were more prominent in the EAEC O42 and *P. mirabilis* RTX339 groups. The findings provide insights into the uropathogenicity and inflammatory cytokine expression in the urinary tract of mice infected with three previously untested bacterial strains.

Keywords *Proteus mirabilis* · Enteroaggregative *Escherichia coli* · Ascending urinary tract infection · Urinary tract inflammation

Introduction

Urinary tract infections (UTIs) are leading cause of morbidity and hospitalizations among at-risk populations. Clinically, UTIs are categorized as uncomplicated and complicated. Uncomplicated UTIs are commonly detected in immunocompetent women during the fertile period. Complicated UTIs are observed in long-term catheterized patients and in subjects with structural abnormalities of the urinary tract (Kumar et al. 2015; Tandogdu and Wagenlehner 2016). UTIs refer to infections in any part of the urinary tract that is normally sterile, including the kidneys, ureters, bladder, and urethra. Anatomically, UTIs are classified according to location: cystitis (bladder), pyelonephritis (kidneys), and urosepsis (a

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systemic infection from UTI) (Kumar et al. 2015; Tandogdu and Wagenlehner 2016). UTIs are predominantly caused by multidrug-resistant uropathogenic *Escherichia* (*E.*) *coli* (UPEC) and *Proteus* (*P.*) *mirabilis* strains (Tandogdu and Wagenlehner 2016). Currently, the multidrug-resistant diarrheagenic pathotype of enteroaggregative *E. coli* (EAEC) is recognized as an etiological agent of UTIs (Hebbelstrup Jensen et al. 2014).

A presumable step in EAEC pathogenicity is the colonization of tissue surfaces, which has been shown in vitro in human cancer-epithelial cell lines derived from the bladder and colon infected with EAEC O42 (Boll et al. 2013; Yáñez et al. 2016). Epithelial colonization appears to entail the interaction of EAEC components, such as aggregative adherence fimbria (AAF), with host receptors, such as fibronectin (an extracellular matrix protein) and MUC1 (an epithelial transmembrane mucin) (Boll et al. 2017; Yáñez et al. 2016). During colonization, EAEC components, such as AAF, flagellin, and plasmid-encoded toxin (Pet), trigger the proinflammatory response characterized by interleukin (IL)-1β, -6, -8, and tumor necrosis factor (TNF)-α production, as well as neutrophil recruitment, as reported in models of epithelial cell infection in vitro (Boll et al. 2012; Harrington et al. 2005; Rocha-Ramírez et al. 2016; Steiner et al. 2000). Mechanisms underlying the generation and expression of proinflammatory mediators appear to involve the activation of signaling pathways triggered by the interaction of EAEC antigens with host receptors, such as toll-like receptors (TLRs), as described in vitro (Goyal et al. 2010; Khan et al. 2004; Sanchez-Villamil et al. 2016).

The uropathogenic enterobacterium *P. mirabilis* has many types of fimbria (mannose-resistant *Proteus*-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), and uroepithelial cell adhesin (UCA) fimbria) that promote colonization and biofilm formation, as evidenced in the murine model of ascending UTI (Jansen et al. 2004; Massad et al. 1994; Pellegrino et al. 2013; Zunino et al. 2003). The ability of *P. mirabilis* to generate biofilms, flagellin, and urease is related to the expression and/or production inflammatory ILs and chemokines, as observed in cultures of infected cells in vitro (Fusco et al. 2017; Umpiérrez et al. 2013) and in the model of ascending UTI in CBA/J mice (Armbruster et al. 2017).

Inflammation is a mechanism of innate immunity that is a double-edged sword, as it is essential for pathogen elimination and exerts deleterious effects on the functional and structural integrity of the urinary tract (Ambite et al. 2016). The inflammatory cytokine response in the urinary tract following *P. mirabilis* infection in CBA/J mice has recently been documented (Armbruster et al. 2017), but data on this response to a UTI caused by EAEC are unavailable. In vivo studies provide substantive insights into the host-pathogen interactions that result in the inflammatory response in the urinary tract induced by pathogenic bacteria. Thus, the goal of this study was to analyze the induction of proinflammatory cytokine mRNA expression by bacterial strains that have not previously been tested in the model of ascending UTI in BALB/c mice.

Materials and methods

Animals

Six-week-old female BALB/c mice were provided ad libitum access to food (Laboratory Rodent Diet 5001 LabDiet, Saint Louis, MO, USA) and water and maintained in a noiseless room with a 12:12-h light/dark cycle (lights on at 7 am) at 20 °C and relative humidity 55% in the animal facilities of "Unidad de Produccion y Experimentación de Animales de Laboratorio, (UPEAL) Universidad Autónoma Metropolitana, Unidad Xochimilco". The protocol (No. 111) was approved by the "Comité Interno para el Cuidado y Uso de Animales de Laboratorio (CICUAL)" under the Mexican federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA), Mexico).

Bacterial strains

The bacterial strains assayed in this study included the EAEC O42 prototypic wild strain isolated from a child with watery diarrhea (Hebbelstrup Jensen et al. 2014), *P. mirabilis* RTX339 strain isolated from a hospitalized patient with recurrent UTIs (Gutiérrez-Lucas et al. 2012), and *P. mirabilis* ATCC29906 strain. Bacterial strains were cultured on MacConkey agar (Cat. No. 7112 MCD LAB, Estado de Mexico, Mexico) at 37 °C for 24 h. Bacterial purity was confirmed morphologically by Gram staining (Cat No. 541, Hycel, Mexico City, Mexico) and biochemically using an API20 system (Biomeriaux, Ciudad de Mexico, Mexico).

Each bacterial strain was cultured overnight in 50 mL of Luria broth at 37 °C and then centrifuged at 12,100*g* 4 °C, 20 min (Beckman Coulter Avanti Centrifuge model J-301, Brea, California, USA). The bacterial cells were washed once by suspending the pellets in sterile phosphate buffer (PBS; pH 7.2), centrifuged, and resuspended in sterile PBS as described above. Bacterial suspensions were diluted 10× in sterile PBS, and 100 μL of each 10× dilution was plated on MacConkey agar plates and incubated overnight at 37 °C. Isolated colonies were counted to adjust the colony-forming units (CFU) of the bacterial suspensions to 5×10^7 CFU/mL as described in a previous study (Hagberg et al. 1983). For infections, 5×10^7 CFU were suspended in 50 μL of Luria broth.

Protocol for establishing an ascending urinary infection in BALB/c mice

The experimental model of infection in BALB/c mice was established using previously reported protocols (Hagberg et al. 1983; Moayeri et al. 1991), with some modifications. Groups of four 6-week-old BALB/c female mice were anesthetized with an intraperitoneal injection of a mixture containing 10 mg/kg body mass xylazine and 60 mg/kg body mass ketamine (both from Pisa Agropecuaria, Tula Hidalgo, Mexico). Afterwards, the bladder of each mouse was emptied through gentle rubbing and a gel lubricant (Lubrigel®, Diporsa SA, Ciudad de Mexico, Mexico) was applied to the urethral cavity before infection. A sterile catheter (22 Gx1, BD Systems, Sandy, Utah, USA) fitted to a tuberculin syringe was inserted into the urinary tract (7 mm in length) to deliver the bacterial suspension containing 5×10^7 CFU/50 μL. Gentle massage was applied to mice to enable the contact of bacterial inoculum with the walls of the bladder.

Sample collection

At 0, 2, 4, 7, and 10 days p.i., mice were euthanized with isoflurane before collecting blood samples via cardiac puncture. Sampling was conducted at all time points under sterile conditions and included the collection of urine, bladder, and kidneys (right and left lobules). Urine samples (100–500 μL) and pre-weighed portions of bladders and kidneys were processed for culture assays. The bladder and kidneys were divided in portions (i) to analyze the proinflammatory cytokine response using quantitative real-time polymerase chain reaction (RT-PCR), (ii) to visualize the presence of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) using immunohistochemistry, and (iii) for a histological examination with hematoxylin and eosin staining.

Sample cultures

Organs were homogenized according to infection protocols described for *P. mirabilis* (Armbruster et al. 2017; Jansen et al. 2004) and EAEC (Boll et al. 2013). Briefly, individual portions of bladders and kidneys were completely homogenized in sterile PBS (pH 7.2) using a sterile pestle (Cat No. 47747-358, VWR International, Philadelphia, USA) fitted to a manual homogenizer (Cat No. 47747-370 VWR). The resulting tissue homogenates and urine samples were serially diluted 10× with sterile PBS, and 100 μL of each dilution was plated on MacConkey agar. After an overnight incubation at 37 °C, isolated colonies were counted to evaluate CFU/mL in urine or CFU/g in bladders and kidneys.

Real-time polymerase chain reaction

Total RNA was extracted from portions of tissue samples with Tri-reagent® (Cat. No. TR118, Molecular Research Center, Inc., Cincinnati, USA) according to the manufacturer's instructions. Total RNA was used as template for cDNA synthesis with M-MLV reverse transcriptase (Cat. No. M170A, Promega, Madison Michigan, USA).

For quantitative RT-PCR analyses, reaction mixtures for each cytokine assayed and the actin control were prepared in microtubes (Cat. No. 311-08-051, Axygen, Union City, California, USA) containing 1 μL of cDNAs (from bladder or kidney), 15 μL of Master Mix (Cat. No. A150306 Ampliqon, Odense M, Denmark), 0.5 μL each of forward and reverse primers (Table 1), 0.5 μL of Evagreen (Cat No. 31000, Biotium, Freemont, California, USA), 2.2 μL of sterile injectable water, and 0.3 μL of Rox (Cat No. R4526, Sigma Saint Louis, Missouri, USA). The Rox reference dye was included according to the technical instructions to run the RT-PCR assays in a real-time PCR cycler (Rotor Gene 6000, Qiagen, Valencia, California, USA) using the following thermocycling conditions: (1) denaturation at 50 °C for 2 min and 95 °C for 10 min; (2) 45 cycles of 95 °C for 15 s, 60 °C for 45 s, and 72 °C for 15 s; and (3) a melting curve analysis from 75 to 94 °C. Data were analyzed with RotorGene 6000 1.7 software (Qiagen) to determine the threshold cycle (Ct) values. The relative mRNA expression was calculated according to the comparative 2^-delta-delta Ct method. Actin was used as housekeeping gene.

Histological examination

Sections of bladders and kidneys were fixed with formaldehyde, embedded in paraffin, and thereafter transversally cut into 5 mm-thick slices. Slices were stained with hematoxylin and eosin for histological examinations. Other slices were sequentially deparaffinized with xylene, xylene, absolute

Table 1 Primer sequences of cytokines for the quantitative reverse transcriptase polymerase chain reaction

Primers	Sequence
IL-8 forward	5′-TGCATGGACAGTCATCCACC-3′
IL-8 reverse	5′-ATGACAGACCACAGAACGGC-3′
IL-6 forward	5′-CCCCAATTTCCAATGCTCTCC-3′
IL-6 reverse	5′-CGCATCAGGTTTGCCGAGTA-3′
IL-1β forward	5′-TGCCACCTTTTGACAGTGATG-3′
IL-1β reverse	5′-TGATGTGCTGCTGCGAGATT-3′
TNF-α forward	5′-GATCGGTCCCCAAAGGGATG-3′
TNF-α reverse	5′-TTTGCTACGACGTGGGCTAC-3′
Actin forward	5′-CTAAGGCCAACGGTGAAAAG-3′
Actin reverse	5′-ACCAGAGGCATACAGGGACA-3′

ethanol, 96% ethanol, and 70% ethanol and water prior to immunohistochemistry.

Immunohistochemistry

Immunohistochemistry assays were performed using the tissue staining kit (CTS006, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Tissue samples were incubated for 2 h at 37 °C with primary antibodies against CXR1/IL-8 RA (NBP2-16043, Novus Biologicals, Littleton, Colorado, USA), IL-6 (NB600-1131, Novus Biologicals) IL-1β (SC-1349, Santa Cruz Biotechnology, Houston, TX, USA), and TNF-α (SC-1250, Santa Cruz Biotechnology). After washes with sterile PBS (pH 7.2), samples were treated with 3-amino-9-ethylcarbazole (AEC) until the desired intensity was observed, after which the samples were counterstained with Mayer's hematoxylin (Cat MFCD00078111, Sigma-Aldrich, Saint Louis, Missouri, USA). Tissue samples were observed at 40× magnification with an optical microscope (DM1000, Leica-Biosystems, Wetzlar, Germany).

Statistical analysis

Experiments were repeated twice, unless stated otherwise, and data are reported as the means and standard deviations (SD). Comparisons of data from multiple groups were analyzed using one-way ANOVA with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA), and differences among groups detected with a post hoc Tukey test were regarded significant at $p < 0.05$.

Results

Bacterial infection

The evaluation of bladder samples (Fig. 1) revealed higher bacterial counts in mice infected with either EAEC O42 or *P. mirabilis* RTX339 than in the uninfected control on all days p.i. and the *P. mirabilis* ATCC29906-infected group on days 2, 4, and 7 p.i. Significant differences in bacterial loads between EAEC O42 and *P. mirabilis* RTX339 were not observed at any time point after the infection.

In the kidneys (Fig. 1), higher bacterial loads were observed in mice infected with *P. mirabilis* ATCC29906 on day 4 p.i., EAEC O42 on days 2, 7, and 10 p.i., or *P. mirabilis* RTX339 on days 2, 4, and 7 p.i. than in uninfected mice. Comparisons among the infected mice indicated higher bacterial loads in mice infected with either EAEC O42 or *P. mirabilis* RTX339 than in mice infected with *P. mirabilis* ATCC29906 on days 2 and 7 p.i. In mice infected with a *P. mirabilis* strains, bacterial loads were higher than in mice infected with EAEC O42 on day 4 p.i., whereas mice infected with EAEC O42 presented higher bacterial loads than mice infected with either *P. mirabilis* strain on day 10 p.i. No other differences were observed.

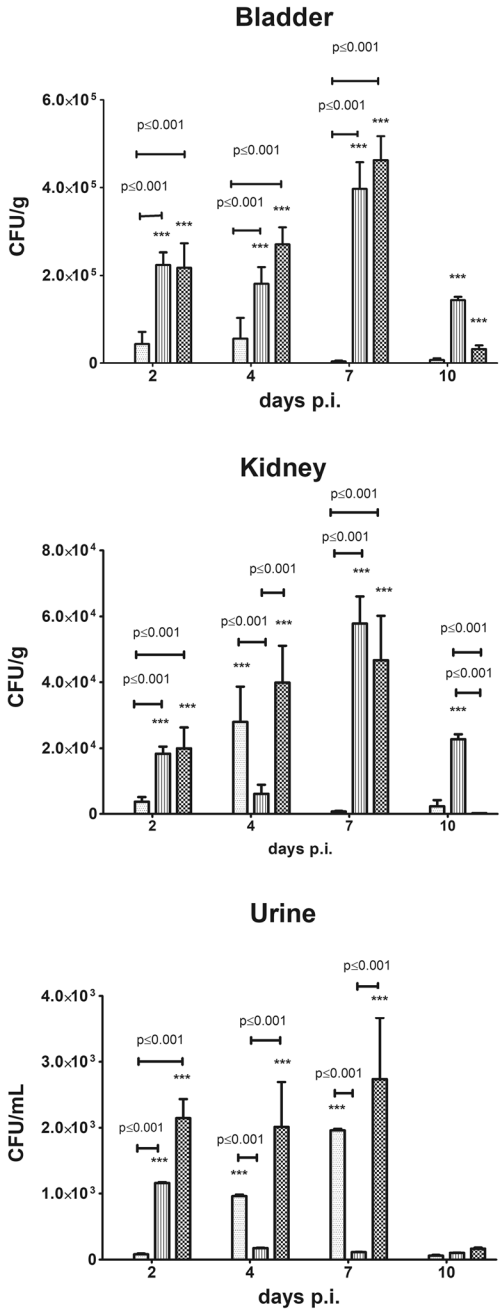

Fig. 1 Colony forming units (CFU) in the bladder, kidney, and urine samples from female BALB/c mice that were □ uninfected or infected with ⁝⁝ *P. mirabilis* ATCC29906, ||| EAEC O42 or ▩ *P. mirabilis* RTX339 and then sacrificed on days 2, 4, 7, and 10 p.i. Data are presented as the means ± standard deviations (SD). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ above the columns indicate significant differences compared with the uninfected group, and p values above the bars indicate differences among the infected groups

According to the urine bacterial counts (Fig. 1), the urine samples from all groups of infected mice contained less than 1×10^4 CFU/mL, a cutoff for bacteriuria (Hannan et al. 2010). However, compared with the uninfected control group, higher bacterial loads were observed in mice infected with *P. mirabilis* ATCC29906 on days 4 and 7 p.i., EAEC O42 on day 2 p.i., or *P. mirabilis* RTX339 on days 2, 4, and 7 p.i. Comparisons among the infected groups indicated higher bacterial loads in mice infected with either EAEC O42 or *P. mirabilis* RTX339 than in mice infected with *P. mirabilis* ATCC29906 on day 2 p.i., without differences between these strains. Higher bacterial loads were observed in mice infected with the *P. mirabilis* strains than in mice infected with EAEC O42 on days 4 and 7 p.i. without differences between these strains on those days.

Cytokine mRNA expression

Among the assayed groups, higher levels of the TNF-α mRNA were detected in the bladder of mice infected with *P. mirabilis* ATCC29906 on days 2, 4, and 10 p.i. or with *P. mirabilis* RTX339 on day 7 p.i. (Fig. 2). In the kidney, higher relative levels of the TNF-α mRNA were measured in EAEC O42-infected mice on day 7 p.i. or in mice infected with *P. mirabilis* RTX339 on day 10 p.i. (Fig. 2).

In the bladder, higher levels of the IL-1β mRNA were detected in mice infected with *P. mirabilis* ATCC29906 on day 4 p.i. or with *P. mirabilis* RTX339 on day 7 p.i. than in the other groups (Fig. 3). In the kidney, higher levels of the IL-1β mRNA were detected in *P. mirabilis* ATCC29906-infected mice on days 4 and 7 p.i. and in the EAEC O42-infected group on day 10 p.i. than in the other groups (Fig. 3).

An analysis of bladder samples from all groups revealed higher levels of the IL-8 mRNA in mice infected with *P. mirabilis* RTX339 on day 7 p.i. or with EAEC O42 on day 10 p.i. (Fig. 4). In the kidneys, higher levels of the IL-8 mRNA were measured in mice infected with *P. mirabilis* RTX339 on day 2 p.i. and with EAEC 042 on day 10 p.i. than in the other groups. Finally, in groups infected with either EAEC O42 or *P. mirabilis* RTX339, higher levels of the IL-8 mRNA were detected on day 7 p.i. than in the other groups, without differences between groups (Fig. 4).

Comparisons among all groups revealed higher expression of the IL-6 mRNA in the bladder tissues from mice infected with EAEC O42 on days 2, 4, and 10 p.i. or with *P. mirabilis* RTX339 on day 7 p.i. (Fig. 5). In the kidneys, higher levels of the IL-6 mRNA were detected in mice infected with EAEC O42 on days 2, 4, and 10 p.i. than in the other groups. Moreover, in mice infected with either EAEC O42 or *P. mirabilis* RTX339, higher expression of the IL-6 mRNA was detected on day 7 p.i. than in the other groups (Fig. 5).

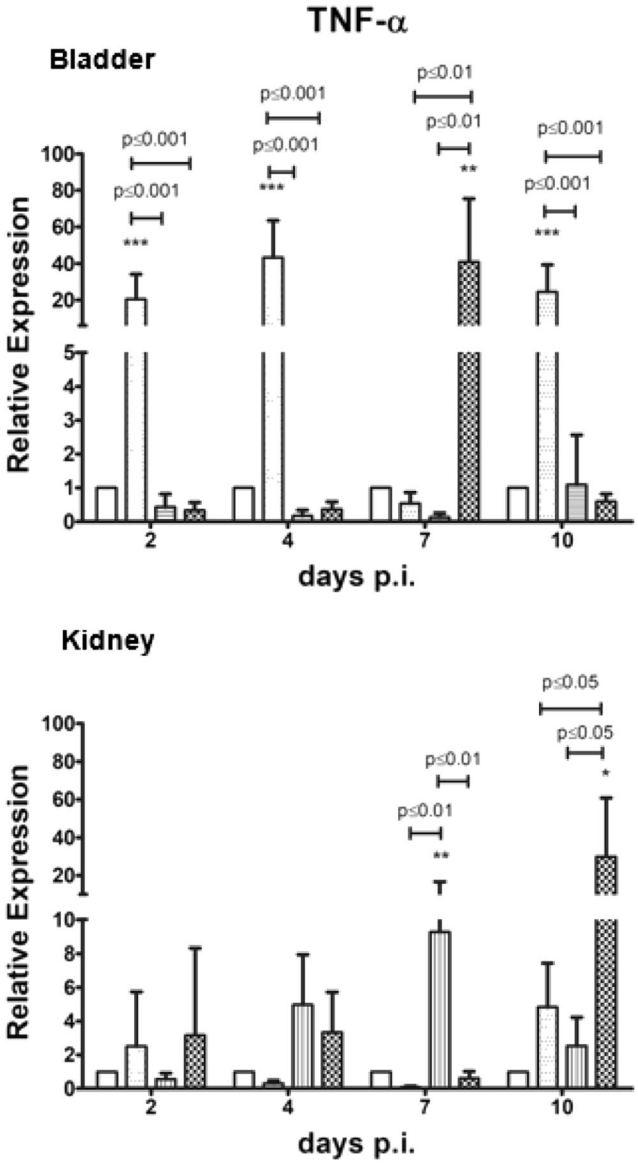

Fig. 2 Relative expression of the TNF-α mRNA in bladder and kidney samples from female BALB/c mice that were □ uninfected or infected with ⁝⁝ *P. mirabilis* ATCC29906, ||| EAEC O42 or ▩ *P. mirabilis* RTX339 and sacrificed on days 2, 4, 7, and 10 p.i. Data are presented as the means ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ above the columns indicate significant differences compared with the uninfected group and *p* values above the bars indicate differences among the infected groups

Immunohistochemical staining for cytokines

The expression of proinflammatory cytokines was assessed using immunohistochemistry at all time points. However, IL-8 was only detected on day 7 p.i. among all the cytokines evaluated. IL-8 staining was prominently visualized in both the bladders and kidneys of mice infected with either *P. mirabilis* RTX339 or EAEC O42. In contrast, faint IL-8 staining was only detected in the bladder of the *P. mirabilis* ATCC29906-infected group (Fig. 6).

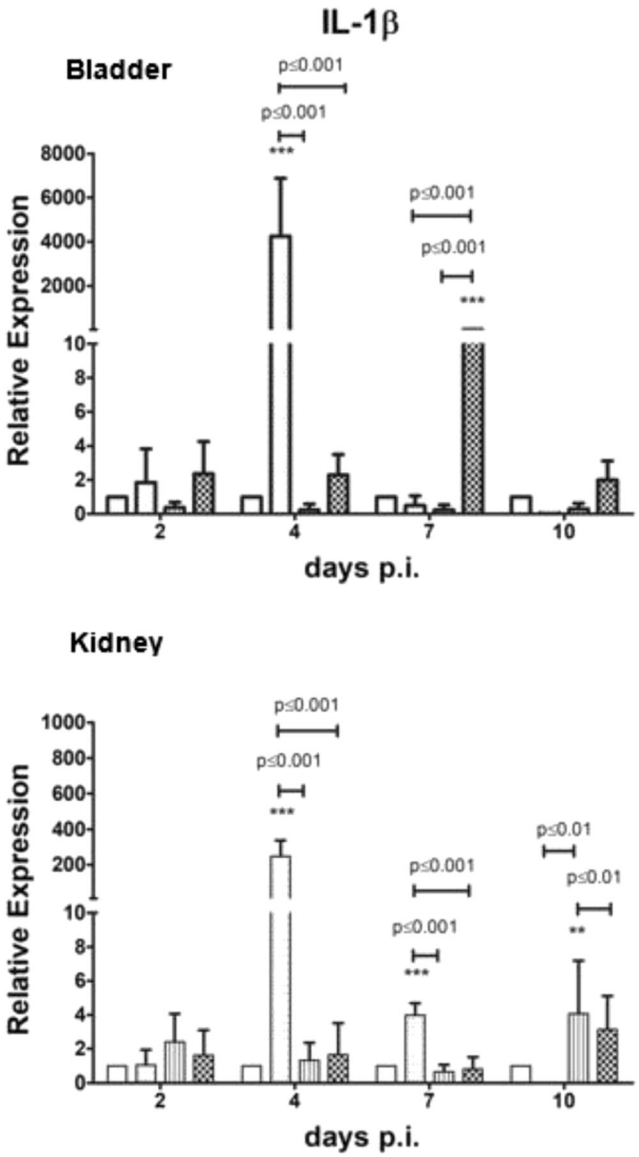

Fig. 3 Relative expression of the IL-1β mRNA in the bladder and kidney samples from female BALB/c mice that were □ uninfected or infected with ⁝⁝ *P. mirabilis* ATCC29906, ||| EAEC O42 or ▩ *P. mirabilis* RTX339 and then sacrificed on days 2, 4, 7, and 10 p.i. Data are presented as the means ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ above the columns indicate significant differences compared with the uninfected group, and p values above the bars indicate differences among the infected groups

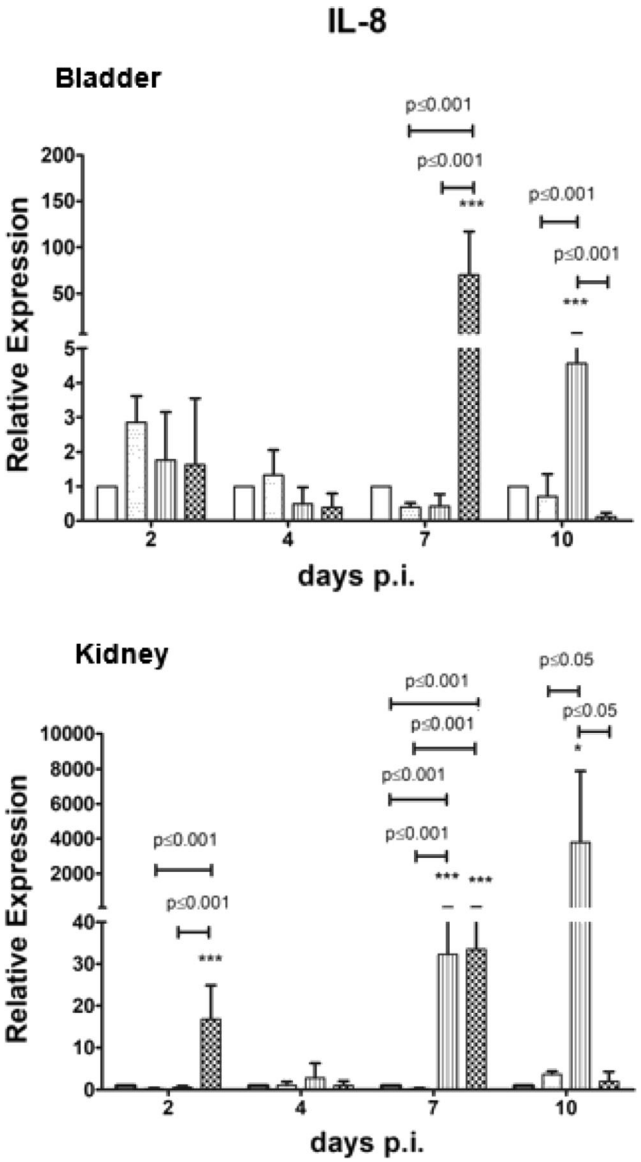

Fig. 4 Relative expression of the IL-8 mRNA in the bladder and kidney samples from female BALB/c mice that were □ uninfected or infected with ⁝⁝ *P. mirabilis* ATCC29906, ||| EAEC O42 or ▩ *P. mirabilis* RTX339 and sacrificed on days 2, 4, 7, and 10 p.i. Data are presented as the means ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ above the columns indicate significant differences compared with the uninfected group and p values above the bars indicate differences among the infected groups

Histological examination

Histological examinations performed on day 7 p.i. indicated that all bacterial strains altered the normal architecture of the bladder compared with the uninfected group; prominent morphological changes were observed in tissues infected with EAEC O42 and *P. mirabilis* RTX339 compared with *P. mirabilis* ATCC29906. In the kidneys, a prominent loss of morphological integrity was observed following the EAEC O42 and *P. mirabilis* RTX339 infections, while swelling and an enlargement of the renal tubules was observed in mice infected with *P. mirabilis* ATCC 29906 (Fig. 6).

Discussion

The inflammatory cytokine response has been described in the urinary tract following a *P. mirabilis* infection in vivo (Armbruster et al. 2017), whereas this response to an EAEC infection has not yet been addressed. To our knowledge, this

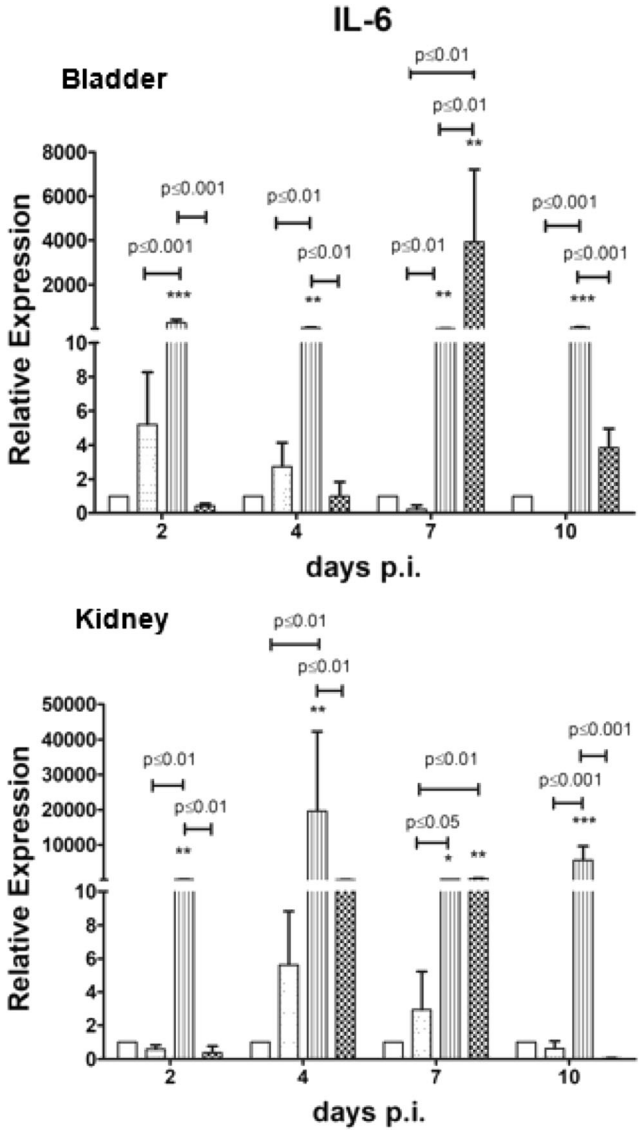

Fig. 5 Relative expression of the IL-6 mRNA in the bladders and kidneys from female BALB/c mice that were □ uninfected or infected with ⁝ *P. mirabilis* ATCC29906, ||| EAEC O42 or ▩ *P. mirabilis* RTX339 and sacrificed on days 2, 4, 7, and 10 p.i. Data are presented as the means ± SD. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ above the columns indicate significant differences compared with the uninfected group and *p* values above the bars indicate differences among the infected groups

study is the first to analyze the bacterial colonization and mRNA expression of proinflammatory cytokines in the urinary tracts of mice infected with three bacterial strains that were previously untested in the model of ascending UTIs.

An overall analysis of the findings did not reveal bacteriuria in mice infected with any bacterial strain, although bacterial loads were counted in the bladder and/or kidneys as analyzed target tissues. Bacterial urinary clearance may result from excretion and/or phagocytosis, as suggested in a previous study (Hagberg et al. 1983). In the present study, the *P. mirabilis* RTX339 strain isolated from patients with recurrent UTIs was tested. Therefore, the bladder acts a reservoir for uropathogenic bacteria causing chronic infections that are intermittently cleared via the urine without causing bacteriuria (Schilling et al. 2002).

In the present study, bacterial strains showed a divergent pattern of persistence in the bladder and kidney at each time point, suggesting their ability to adapt and proliferate, evidencing their uropathogenic capacities. In addition, based on the data, the contributions of unknown components underlie the bacterial uropathogenicity. Virulence factors do not always determine the bacterial persistence, as documented in assays of infection with EAEC mutant strains in vitro (Boll et al. 2013) and in murine models of opportunistic wild-type *P. mirabilis* strains (Pearson and Mobley 2007; Sosa et al. 2006).

Unlike the experimental settings of infection using EAEC mutant strains in OF-1 outbred mice (Boll et al. 2013) or *P. mirabilis* in inbred CBA (Armbruster et al. 2017; Jansen et al. 2004; Massad et al. 1994) or outbred CD-1 mouse strains (Umpiérrez et al. 2013; Zunino et al. 2003), the present study was conducted in BALB/c inbred mice and low bacterial loads were observed in the organs from mice infected with each of the bacterial strains tested. The low extent of bacterial colonization may result from various factors, such as the (i) modalities of infection via the transurethral or intravesicular route, (ii) volume and CFU of the bacterial inoculum (Hagberg et al. 1983; Moayeri et al. 1991), and (iii) the genetic background that partially accounts for the low persistence of UTIs in BALB/c mice (Hagberg et al. 1983; Hannan et al. 2010).

The overall analysis revealed a divergent response between the cytokine mRNA expression at each time point and the bacterial loads in target organs. These findings may result from the molecular mechanisms that chronologically control the constitutive or induced expression of mRNA transcripts encoding bacterial products; the latter underlie the activation of signaling pathways leading to the expression of cytokine transcripts at different time points, as documented in experimental models of infection with EAEC and *P. mirabilis* (Pearson et al. 2011; Sanchez-Villamil et al. 2016; Yáñez et al. 2016). Synchronization of the molecular mechanisms that sense the host milieu is critical for bacterial survival and multiplication, as described in a murine model of UTIs with *P. mirabilis* (Pearson et al. 2011).

With the exception of IL-8 levels on day 7 p.i., concordance between the relative mRNA levels of cytokines and their immunohistochemical detection was not found. A lack of concordance between mRNA expression and the tissue localization of cytokines such as TNF-α has been observed in other experimental settings (Rugo et al. 1992). Apparent discrepancies may reflect independent signaling pathways that induce the expression of each cytokine but may also result from technical issues. Quantitative RT-PCR is a highly sensitive method for detecting transcripts in a wide array of cells;

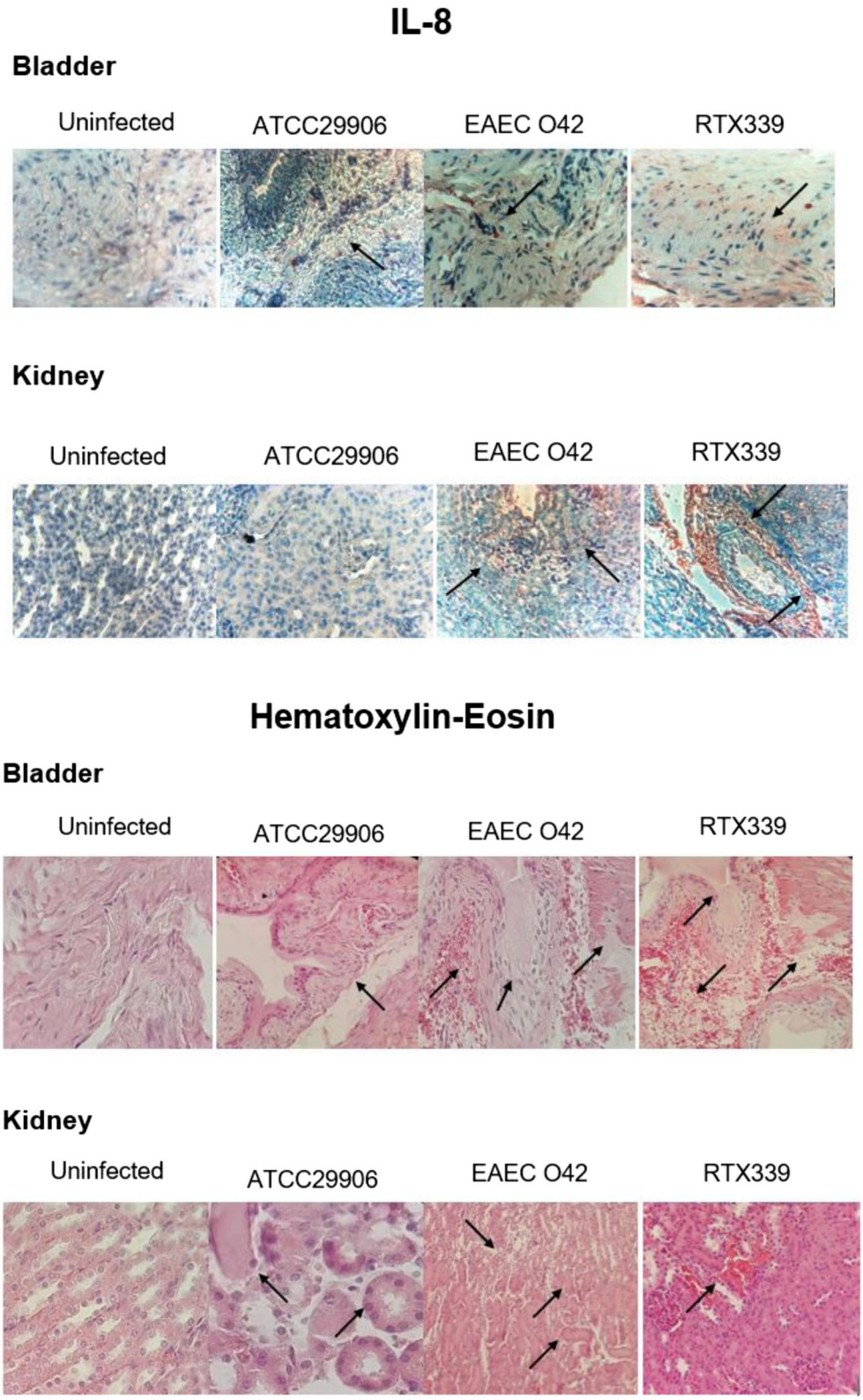

Fig. 6 Image of the immunohistochemical staining for IL-8 (upper panel) and histological examination using hematoxylin and eosin staining (bottom panel) in bladder and kidney samples from female BALB/c mice that were uninfected or infected with *P. mirabilis* ATCC29906, EAEC O42, or *P. mirabilis* RTX339 and sacrificed on day 7 p.i. In the upper panel, arrows indicate the in situ location of the IL-8 protein. In the bottom panel, arrows in the bladder depict tissue deformation and the presence of erythrocytes; in the kidney, arrows highlight renal tubule swelling (*P. mirabilis* ATCC 29906), tissue deformation (EAEC O42), and the presence of damaged erythrocytes (*P. mirabilis* RTX339). Microscopic images were captured at 40× magnification

however, the protein products are not detected when very few cells are available (Amsen et al. 2009).

In general terms, the expression of the IL-8 mRNA on day 7 p.i. matched the bacterial loads of EAEC O42 and *P. mirabilis* RTX339 in both target organs. Moreover, IL-8 expression was concordant with the visualization of the IL-8 protein in the bladders and kidneys of mice infected with *P. mirabilis* RTX339 and EAEC O42. Similar to the results

of this assay, bacterial loads and/or inflammatory responses in the bladder and/or kidney have previously been observed at 7 days p.i (Umpiérrez et al. 2013).

Upregulation of the IL-8 mRNA/protein has been confirmed in vitro and is associated with some enteroaggregative EAEC components, such as, fimbria, flagellin, and plasmid-encoded toxin (Pet) (Harrington et al. 2005; Rocha-Ramírez et al. 2016; Steiner et al. 2000). IL-8 plays a key role in the pathogenicity of EAEC by promoting the recruitment of phagocytic cells and, ultimately, epithelial damage (Rocha-Ramírez et al. 2016). *P. mirabilis* has been shown to induce the production of proinflammatory biomarkers, including cytokines and chemokines (CCL20, CXCL20, and CCL2), as well as bladder leukocyte recruitment in vivo (Armbruster et al. 2017; Umpiérrez et al. 2013). Furthermore, the increased production of IL-8 induced by *P. mirabilis* is associated with a lack of mobility and a retarded capacity to generate biofilms in vitro (Fusco et al. 2017).

Histological examinations with hematoxylin and eosin staining indicated that the *P. mirabilis* RTX339 strain displayed a greater ability than *P. mirabilis* ATCC29906 to cause substantial morphological changes and damage in the bladder and kidney. These phenotypic traits may result from virulence factors acquired from different isolates and may reveal the opportunistic nature of *P. mirabilis* isolates (Sosa et al. 2006). Like *P. mirabilis* RTX339, EAEC O42 caused substantial morphological alterations in both target organs. These findings suggest a potential role for EAEC O42 as a causal agent of UTIs, although its preferred niche is the intestinal tract (Hebbelstrup Jensen et al. 2014).

In conclusion, *P. mirabilis* ATCC29906, EAEC O42, and *P. mirabilis* RTX339 activated IL-8 signaling and induced damage in the bladder and kidney on day 7 p.i. The differences in the responses of all of the parameters may result from phenotypic virulence traits and from their interactions with the host inflammatory response. Although BALB/c mice are a useful model for assessing the pathogenicity of enteropathogenic strains (Saha et al. 2013), the current model of ascending UTIs in BALB/c mice was valuable for examining the uropathogenicity of the three bacterial strains. These findings may be an experimental reference for clinical studies intended to ascertain the potential diagnostic applications of urinary biomarkers of inflammation for bacterial strains commonly associated with enteric diarrhea (Nunes et al. 2017).

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Authors' contributions Melendez-Avalos Araceli: performed the experiments, analyzed the data, and designed the figures.

Sainz Espuñes Teresita: conceived the study.

Castrillón-Rivera Laura Estela: contributed to the bacterial count assays.

Mendoza-Pérez Felipe: provided advice on the experiments and processed the data.

Palma-Ramos Alejandro: provided advice on the immunohistochemistry assay.

Castañeda-Sánchez Jorge Ismael: primer design and provided advice on the RT-PCR assay.

Drago-Serrano Maria Elisa: wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ambite I, Nagy K, Godaly G, Puthia M, Wullt B, Svanborg C (2016) Susceptibility to urinary tract infection: benefits and hazards of the antibacterial host response. Microbiol Spectr 4(3). https://doi.org/10.1128/microbiolspec.UTI-0019-2014

Amsen D, de Visser KE, Town T (2009) Approaches to determine expression of inflammatory cytokines. Methods Mol Biol 511:107–142. https://doi.org/10.1007/978-1-59745-447-6_5

Armbruster CE, Smith SN, Johnson AO, DeOrnellas V, Eaton KA, Yep A, Mody L, Wu W, Mobley HL (2017) The pathogenic potential of Proteus mirabilis is enhanced by other Uropathogens during Polymicrobial urinary tract infection. Infect Immun 85(2):e00808–e00816. https://doi.org/10.1128/IAI.00808-16

Boll EJ, Struve C, Sander A, Demma Z, Nataro JP, McCormick BA, Krogfelt KA (2012) The fimbriae of enteroaggregative Escherichia coli induce epithelial inflammation in vitro and in a human intestinal xenograft model. J Infect Dis 206(5):714–722. https://doi.org/10.1093/infdis/jis417

Boll EJ, Struve C, Boisen N, Olesen B, Stahlhut SG, Krogfelt KA (2013) Role of enteroaggregative Escherichia coli virulence factors in uropathogenesis. Infect Immun 81(4):1164–1171. https://doi.org/10.1128/IAI.01376-12

Boll EJ, Ayala-Lujan J, Szabady RL, Louissaint C, Smith RZ, Krogfelt KA, Nataro JP, Ruiz-Perez F, McCormick BA (2017) Enteroaggregative Escherichia coli adherence fimbriae drive inflammatory cell recruitment via interactions with epithelial MUC1. MBio 8(3):e00717–e00717. https://doi.org/10.1128/mBio.00717-17

Fusco A, Coretti L, Savio V, Buommino E, Lembo F, Donnarumma G (2017) Biofilm formation and immunomodulatory activity of *Proteus mirabilis* clinically isolated strains. Int J Mol Sci 18(2): E414. https://doi.org/10.3390/ijms18020414

Goyal A, Konar M, Setia A, Narang A, Ghosh S (2010) Galactose specific adhesin of enteroaggregative *E. coli* induces IL-8 secretion via activation of MAPK and STAT-3 in INT-407 cells. Biochim Biophys Acta 1800(6):574–579. https://doi.org/10.1016/j.bbagen.2010.03.009

Gutiérrez-Lucas LR, Mendoza-Hernández G, González-Pedrajo B, Eslava-Campos C, Bustos-Martínez JA, Sainz-Espuñes T (2012) Identification of the autotransporter pet toxin in *Proteus mirabilis* strain isolated from patients with urinary tract infections. Adv Biol Chem 2:283–290. https://doi.org/10.4236/abc.2012.23036

Hagberg L, Engberg I, Freter R, Lam J, Olling S, Svanborg Edén C (1983) Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin. Infect Immun 40(1):273–283

Hannan TJ, Mysorekar IU, Hung CS, Isaacson-Schmid ML, Hultgren SJ (2010) Early severe inflammatory responses to uropathogenic E. coli predispose to chronic and recurrent urinary tract infection. PLoS Pathog 6(8):e1001042. https://doi.org/10.1371/journal.ppat.1001042

Harrington SM, Strauman MC, Abe CM, Nataro JP (2005) Aggregative adherence fimbriae contribute to the inflammatory response of epithelial cells infected with enteroaggregative *Escherichia coli*. Cell Microbiol 7(11):1565–1578

Hebbelstrup Jensen B, Olsen KE, Struve C, Krogfelt KA, Petersen AM (2014) Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev 27(3):614–630. https://doi.org/10.1128/CMR.00112-13

Jansen AM, Lockatell V, Johnson DE, Mobley HL (2004) Mannose-resistant Proteus-like fimbriae are produced by most *Proteus mirabilis* strains infecting the urinary tract, dictate the in vivo localization of bacteria, and contribute to biofilm formation. Infect Immun 72(12):7294–7305

Khan MA, Kang J, Steiner TS (2004) Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires toll-like receptor 5-dependent p38 MAP kinase activation. Immunology 112(4):651–660

Kumar S, Dave A, Wolf B, Lerma EV (2015) Urinary tract infections. Dis Mon 61(2):45–59

Massad G, Lockatell CV, Johnson DE, Mobley HL (1994) *Proteus mirabilis* fimbriae: construction of an isogenic pmfA mutant and analysis of virulence in a CBA mouse model of ascending urinary tract infection. Infect Immun 62(2):536–542

Moayeri N, Collins CM, O'Hanley P (1991) Efficacy of a Proteus mirabilis outer membrane protein vaccine in preventing experimental Proteus pyelonephritis in a BALB/c mouse model. Infect Immun 59(10):3778–3786

Nunes KO, Santos ACP, Bando SY, Silva RM, Gomes TAT, Elias WP (2017) Enteroaggregative *Escherichia coli* with uropathogenic characteristics are present in feces of diarrheic and healthy children. Pathog Dis 75(8). https://doi.org/10.1093/femspd/ftx106

Pearson MM, Mobley HL (2007) The type III secretion system of *Proteus mirabilis* HI4320 does not contribute to virulence in the mouse model of ascending urinary tract infection. J Med Microbiol 56(Pt 10):1277–1283

Pearson MM, Yep A, Smith SN, Mobley HL (2011) Transcriptome of Proteus mirabilis in the murine urinary tract: virulence and nitrogen assimilation gene expression. Infect Immun 79(7):2619–2631. https://doi.org/10.1128/IAI.05152-11

Pellegrino R, Scavone P, Umpiérrez A, Maskell DJ, Zunino P (2013) *Proteus mirabilis* uroepithelial cell adhesin (UCA) fimbria plays a role in the colonization of the urinary tract. Pathog Dis 67(2):104–107. https://doi.org/10.1111/2049-632X.12027

Rocha-Ramírez LM, Hernández-Chiñas U, Baños-Rojas D, Xicohtencatl-Cortés J, Chávez-Berrocal ME, Rico-Rosillo G, Kretschmer R, Eslava CA (2016) Pet serine protease from enteroaggregative *Escherichia coli* stimulates the inflammatory response activating human macrophages. BMC Microbiol 16(1):158. https://doi.org/10.1186/s12866-016-0775-7

Rugo HS, O'Hanley P, Bishop AG, Pearce MK, Abrams JS, Howard M, O'Garra A (1992) Local cytokine production in a murine model of Escherichia coli pyelonephritis. J Clin Invest 89(3):1032–1039

Saha DR, Guin S, Krishnan R, Nag D, Koley H, Shinoda S, Ramamurthy T (2013) Inflammatory diarrhea due to enteroaggregative Escherichia coli: evidence from clinical and mice model studies. Gut Pathog 5(1):36. https://doi.org/10.1186/1757-4749-5-36

Sanchez-Villamil J, Tapia-Pastrana G, Navarro-Garcia F (2016) Pathogenic Lifestyles of *E. coli* Pathotypes in a Standardized Epithelial Cell Model Influence Inflammatory Signaling Pathways and Cytokines Secretion. Front Cell Infect Microbiol 6:120

Schilling JD, Lorenz RG, Hultgren SJ (2002) Effect of trimethoprim-sulfamethoxazole on recurrent bacteriuria and bacterial persistence in mice infected with uropathogenic Escherichia coli. Infect Immun 70(12):7042–7049

Sosa V, Schlapp G, Zunino P (2006) *Proteus mirabilis* isolates of different origins do not show correlation with virulence attributes and can colonize the urinary tract of mice. Microbiology 152(Pt 7):2149–2157

Steiner TS, Nataro JP, Poteet-Smith CE, Smith JA, Guerrant RL (2000) Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J Clin Invest 105(12):1769–1777

Tandogdu Z, Wagenlehner FM (2016) Global epidemiology of urinary tract infections. Curr Opin Infect Dis 29(1):73–79. https://doi.org/10.1097/QCO.0000000000000228

Umpiérrez A, Scavone P, Romanin D, Marqués JM, Chabalgoity JA, Rumbo M, Zunino P (2013) Innate immune responses to Proteus mirabilis flagellin in the urinary tract. Microbes Infect 15(10–11):688–696. https://doi.org/10.1016/j.micinf.2013.06.007

Yáñez D, Izquierdo M, Ruiz-Perez F, Nataro JP, Girón JA, Vidal RM, Farfan MJ (2016) The role of fibronectin in the adherence and inflammatory response induced by Enteroaggregative *Escherichia coli* on epithelial cells. Front Cell Infect Microbiol 6:166. https://doi.org/10.3389/fcimb.2016.00166

Zunino P, Sosa V, Allen AG, Preston A, Schlapp G, Maskell DJ (2003) *Proteus mirabilis* fimbriae (PMF) are important for both bladder and kidney colonization in mice. Microbiology 149(Pt 11):3231–3237