Veterinary Microbiology 239 (2019) 108445

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Infectious potential of human derived uropathogenic *Escherichia coli* UTI89 in the reproductive tract of laying hens

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ARTICLE INFO

Keywords:
Anthrozoonosis
Uropathogenic *E. coli*
Avian pathogenic *E. coli*
Salpingitis
Urinary tract infection

ABSTRACT

Avian pathogenic *E. coli* (APEC) and human uropathogenic *E. coli* (UPEC) harbour common virulence factors in spite of being associated with disease in different hosts. APEC strains have been shown to have zoonotic potential. In contrast, it is not known whether UPEC strains can cause infection in immunologically competent hens. The objective of the current study was to compare the ability of the well-characterized UPEC strain, UTI89, and the APEC strain, F149H1S2, to infect human and avian cells in culture and to cause salpingitis in an infection model in adult laying hens. *In vitro* characterization showed that the strains grew equally well in human urine, and both were able to infect human intestinal (Int407) and bladder (J82) epithelial cell lines, and they survived in avian macrophages (HD11) to the same extent. Groups of adult birds were inoculated with 10^8 bacteria directly into the oviduct using a surgical procedure. After an infection period of 48 h, bacterial load in the oviduct was determined by dilution series, and pathology was determined based on gross lesions and histological observations. Similar counts of UPEC UTI89 (ST95) and the APEC strain F149H1S2 (ST117) were obtained from tissues of infected birds, and salpingitis as evaluated by clinical score and histopathology was observed to a similar extent after infection with the two strains. Together, the results showed that UPEC UTI89 and APEC F149H1S2 have a similar potential for causing salpingitis in laying hens in the model used. No infection differences were observed between the UPEC UTI89 wild type and a mutant strain with knock-out of the well-known virulence gene, *fimH*, (UPEC UTI89ΔfimH), showing that the salpingitis model is not suitable for the detection of all UPEC virulence factors.

1. Introduction

Some *Escherichia coli* types have acquired virulence factors which allow survival and growth outside of the intestine. These are collectively referred to as Extraintestinal Pathogenic *E. coli* (ExPEC) and can be subdivided into four pathotypes: Uropathogenic *E. coli* (UPEC), Neonatal meningitis *E. coli* (NMEC), Avian pathogenic *E. coli* (APEC), and Sepsis-Associated *E. coli* (SePEC) based on the clinical symptoms of the host. UPEC and APEC, which are the focus of the current study, show genomic similarity (Johnson et al., 2007; Moulin-Schouleur et al., 2007), but the typical infection site and host differ. Knowledge obtained from genomic studies of UPEC and APEC strains do not indicate a clear separation (Jorgensen et al., 2019), and a potential zoonotic risk may exist (Ron, 2006).

APEC infections in poultry affect the respiratory tract and the reproductive tract with airways and the cloacae as the entry ports, respectively. Once the respiratory tract is infected with APEC, the infection can progress and cause air sacculitis, polyserositis and subsequently septicaemia. Colonization of the reproductive tract results in salpingitis or a salpingitis-peritonitis syndrome (SPS), which also can lead to septicaemia (Guabiraba and Schouler, 2015).

In humans, UPEC can cause urinary tract infection (UTI) by entering the urinary tract system *via* the urethra, *i.e.*, an ascending infection. Dependent on the UPEC strain and the host status, this can lead to infection of the bladder (cystitis), infection of the kidneys (pyelonephritis), and in rare cases blood stream infections (septicaemia/bacteraemia) (Wiles et al., 2008).

UPEC and APEC share virulence genes which cannot be linked directly to the host, nor to the pathotype (Ewers et al., 2007). Such genetic similarities have led to speculation of a zoonotic potential of APEC

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https://doi.org/10.1016/j.vetmic.2019.108445
Received 11 June 2019; Received in revised form 24 September 2019; Accepted 3 October 2019

strains (Manges and Johnson, 2012). Several studies have documented the ability of APEC strains to infect in *in vivo* models for human UTI (Jakobsen et al., 2012; Zhao et al., 2009; Stromberg et al., 2017; Jakobsen et al., 2010). In contrast, studies of the anthrozoonotic potential of UPEC using *in vivo* models for APEC infections have only been performed with young animals without a fully developed immune response. In these studies, Moulin-Schouleur et al. (2007) found that UPEC strains, belonging to phylogenetic group B2, were lethal to 1-day-old specific pathogen free (SPF) chicks, especially the ones of serotype O18:K1 or O2:K1, belonging to the phylogenetic subgroup B2-1. They also inoculated 9 strains into the air sacs of 3.5-week-old SPF chickens and again found that strains of serotype O18:K1 or O2:K1 caused a typical avian colibacillosis. Zhao et al. (2009) confirmed that a UPEC strain was lethal when inoculated subcutaneously in 1-day-old chickens. However, so far, no studies have investigated the pathogenesis of UPEC stains in infections in adult birds. This is of relevance from a veterinary point of view, since it would imply a putative risk that poultry can be infected with human derived strain. This study aimed to compare the ability of the well characterized UPEC UTI89 strain and a likewise well characterized APEC strain F149H1S2 to infect cell cultures derived from humans and hens, and to investigate whether a typical UPEC strain can cause salpingitis in adult hens.

2. Materials and methods

2.1. Bacterial strains and media

The UPEC isolate UTI89, used in the current study, was isolated from a patient with an acute bladder infection (Mulvey et al., 2001) and later sequenced (Chen et al., 2006). The sequence type of UPEC UTI89 is ST95, its phylogroup is B2 and it is of serotype O18:K1:H1 (Mulvey et al., 2001). The strain harbours a plasmid, pUTI89 (Chen et al., 2006), which has characteristics of both F plasmids and other known virulence plasmids (Cusumano et al., 2010). Also an UTI89Δ*fimH* mutant was used (Andersen et al., 2012). The mutant lacks the *fimH* gene encoding the tip of the major adhesion factor type 1 fimbria, and is less pathogenic in UTI in mice (Connell et al., 1996).

The APEC isolate F149H1S2 represented the typical APEC strain. This strain was isolated from the oviduct of a broiler breeder, which had been affected with salpingitis-peritonitis according to post-mortem examinations (Pires-dos-Santos et al., 2014). The sequence type of APEC F149H1S2 was determined to be ST117, the serotype to be O83:H4, and the phylogroup to be D (Guerra et al., 2018a). No studies have reported the plasmid content of this strain, but according to a search in the sequencing data (Supplementary Material I, performed by Guerra et al. (Guerra et al. (2018b)) using PlasmidFinder (Carattoli et al., 2014) three plasmids of the IncF family (IncFIB(AP001918), IncFIB(pLF82) and IncFII) are present with more than 96% identity. APEC plasmids are typical of the IncF family, a family of plasmids that is capable of carrying transfer, multidrug resistance and virulence functions (Olsen et al., 2012; Johnson and Nolan, 2009).

Strains were propagated on plates of LB agar (MP Biomedicals, Taastrup, Denmark). For overnight cultures, one colony on an LB plate was inoculated in 10 mL LB medium (MP Biomedicals, Taastrup, Denmark) and incubated overnight at 37 °C and 125 rpm. Blood agar plates were blood agar base (Oxoid, Roskilde, Denmark) supplemented with 5% blood from cattle. Prior to use, the blood agar plates were incubated at 37 °C overnight to ensure sterile plates, no growth observed.

2.2. Comparison of virulence genes

Virulence genes reported to be present in APEC and/or UPEC strains (Johnson et al., 2006; Johnson et al., 2008; Rodriguez-Siek et al., 2005; Jahandeh et al., 2015) were examined for in UPEC UTI89 and APEC F149H1S2 using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) (Boratyn et al., 2013) and the published genome sequences of the two strains (Chen et al., 2006; Guerra et al., 2018a).

2.3. Growth assays in LB and urine, ex vivo

The LB medium was prepared according to the manufacturer's standards (MP Biomedicals, Taastrup, Denmark). The growth study in LB was carried out with three biological and technical replicates by a BioScreen C™ for 24 h at 37 °C as previously described (Moller et al., 2016).

Fresh human morning urine was collected from a 32-year-old male. The urine was filtrated using two coffee filters, followed by filtration through a 0.45 µm pore filter (Minisart filters, Sigma-Aldrich, Copenhagen, Denmark) and finally a 0.2 µm pore filter (Minisart filters, Sigma-Aldrich, Copenhagen, Denmark). Overnight cultures of bacteria were prepared by inoculating one colony of each strain into urine and letting the cultures grow over-night (ON) at 37 °C with shaking at 225 rpm. Next day, growth assays with biological triplicates of each strain were set up in 100 mL conical flasks by inoculating the overnight culture to OD_{600} 0.05 with a final volume of 10 mL filtrated urine. Samples for OD_{600} measurements were collected until stationary phase was reached.

Growth rates were calculated from lag phase to stationary phase. The growth rates were calculated as the change in OD_{600}-unit per hour, as previously described (Hall et al., 2014). Growth rates were determined in biological triplicates for UPEC and APEC grown in LB and urine.

2.4. Cell culture assays

Two carcinoma human cell lines, intestinal cells, Int407 and bladder cells, J82, were used for cell invasion experiments. Both types of cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Slangerup, Denmark) supplemented with 100 µg/mL penicillin/streptomycin (P/S) (Thermo Fisher Scientific, Slangerup, Denmark) and 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Slangerup, Denmark) in cell culture flasks incubated at 37 °C with 5% CO_2 with high humidity. Prior to infection studies, passages were made to multiply the number of cells by detaching the cells from the surface of the cell culture flasks by trypsinization, as previously described (Staerk et al., 2016). Bacteria, from an overnight culture, were prepared and dissolved in an appropriate amount in DMEM with 10% FBS, resulting in a MOI of 100:1. The bacteria were added to the cells and the infection took place for 1 h with incubation at 37 °C with 5% CO_2. The non-infectious/extracellular bacteria were washed away three times with PBS followed by a bactericidal procedure with DMEM with 10% FBS and 100 µg/mL gentamicin (Thermo Fisher Scientific, Slangerup, Denmark) for 1 h. Next, the cells were washed with PBS to remove the antibiotic-containing medium. The infectious/intracellular bacteria were released by lysing the cells with 1% Triton X-100 (Merck, Copenhagen, Denmark) followed by serial dilution in PBS to count the bacterial colony-forming-units (CFUs) on LB plates.

The avian macrophages HD11 were used for survival studies of strains in macrophages. The macrophages were grown in Roswell Park Memorial Institute (RPMI) 1640 medium, GlutaMAX™ Supplement, HEPES by Thermo Fisher Scientific supplemented with 25 µg/mL gentamicin and 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher Scientific) in cell culture flasks incubated at 37 °C with 5% CO_2, high humidity, as previously described (Herrero-Fresno et al., 2014). Briefly, bacteria from an overnight culture were inoculated into fresh media resulting in a start OD_{600} value of 0.05 and the bacteria were grown until OD_{600} 1.00. After reaching OD_{600} 1.00 the bacteria were precipitated and dissolved in an appropriate amount in RPMI 1640 medium with 10% FBS. The bacteria were added to the macrophages (MOI = 100) and cells were incubated at 37 °C with 5% CO_2 for 1 h.

Next, the macrophages were washed twice with preheated (37 °C) PBS and subsequently, RPMI 1640 medium with 10% FBS and 50 μg/mL gentamicin were added (T = 0). At 1, 3, and 19.5 h post this point, dilution series of intracellular bacteria were performed by removing the media, washing with PBS and releasing bacteria by 0.1% Triton X-100 treatment. 10 μL of each bacterial dilution was spotted in triplicates onto LB plates, incubated at 37 °C and counted the next day.

2.5. Induction of salpingitis in birds

Infection experiments were conducted with permission from the Food and Veterinary Administration, licence number 2013-15-2934-00923 (experiment 1) and 2019-15-0201-01611 (experiment 2). The hens were acclimatized one week prior to the study start for each experiment.

In the first experiment, 42 Bovans Brown layers (Hendrix-ISA LLC ©), 26 weeks of age, were used. The birds were separated into five groups in isolated pens within the same room. Three groups (group 1–3) consisted of ten birds each, whereas a last group (group 4) consisted of 12 birds. Each group a rooster was placed to calm the hens in time prior to inoculation. Group 1 remained un-infected and was used to ensure the absence of infection prior to the study (n = 5, killed before the study) and to rule out transmission from other groups, since the pens were within the same room (n = 5, killed at the end of the study). Swabs from oviduct, spleen and liver were spread onto blood agar plates to verify that no bacteria could be cultivated from these sites in un-infected birds. Groups 2, 3, and 4 were surgically inoculated with UPEC UTI89, APEC F149H1S2 and sterile PBS, respectively, using the methods previously published (Pors et al., 2014) (see below).

In the second experiment, 30 commercial Lohmann Brown Layers, 23 weeks of age, were used. The birds were separated into three groups of ten birds each in isolated pens in separate barrier rooms. Groups were surgically inoculated with UPEC UTI89, UPEC UTI89Δ*fimH* and sterile PBS, respectively.

Infection of the oviduct was performed by injection of surgically freed oviducts of fully anaesthetized birds as described previously (Pors et al., 2014). Briefly, overnight cultures of the respective bacterial strain were prepared by inoculating a single colony into 10 mL of LB liquid media followed by incubation overnight at 37 °C with 125 rpm shaking. The next day, bacterial cultures were precipitated by centrifugation (4000 rpm for 10 min), followed by removal of the liquid media and resuspension of each bacterial pellet in 10 mL sterile PBS. The OD_{600} was diluted to 1.25, which corresponded to approximately 1×10^9 CFU/mL. Syringes were prepared with an injective volume of 0.1 mL consisting of the inoculum and PBS and stored on ice prior to injection. The injected CFUs of both bacterial inoculum and PBS inoculum were determined by CFU dilution series before and after the surgical inoculation procedure. The average inoculums (mean ± SD) of the first experiment were $9.1 \times 10^8 \pm 7.5 \times 10^7$ CFUs for UPEC UTI89 and $7.1 \times 10^8 \pm 2.2 \times 10^8$ CFUs for APEC F149H1S2, and $6.2 \times 10^8 \pm 2.3 \times 10^8$ CFUs for UPEC UTI89 and $8.2 \times 10^8 \pm 3{,}1 \times 10^8$ CFUs for UPEC UTI89Δ*fimH* in the second experiment. As expected, no colonies were obtained from plating the PBS solution.

0.1 mL bacterial suspension or PBS was injected into a pre-determined spot of the oviduct of each bird. The surgical wound was closed, and the hens were given pain killer (Buprenorfin, 0.05 mg/kg) and after recovery from anaesthesia moved back to their pens. Birds were observed continuously for pain-related behaviour, discomfort and clinical symptoms until the end of the infection period. Furthermore, analgesic (Buprenorfin, 0.05 mg/kg) treatment 8 and 16 h were administered post infection.

2.6. Post mortem examination and sampling

Birds were euthanized by cervical dislocation 48 h post challenge. They were aseptically opened and subjected to post mortem inspection, including recording of gross lesions within the peritoneum, oviduct, ovary, lungs, spleen and liver. Care was taken to prevent potential contamination from the surroundings within the peritoneum. For each bird, swab samples of the spleen and liver were taken by gently pushing the sterile swab into the organ, rotating it, pulling it out and immediately spreading the swab content onto blood agar plates for testing whether the infection had spread with the blood. The oviduct was placed onto a disinfected plastic bag prior to disentanglement. Swab samples from infundibulum, magnum and istmus were spread onto blood agar plates to determine if the bacteria were uniformly spread inside the oviduct. For the first experiment, tissue from infundibulum, magnum and istmus was collected for histology. Next, the tubular shaped oviduct was opened, and a sterile object glass was used to scrape the upper mucosa layer of the inside of the oviduct. The scrape was weighed and collected in a falcon tube and stored on ice prior to bacterial enumeration by plating.

2.7. CFU counts in oviduct scrape

To enable pipetting, the oviduct scrape was mixed (1:1 W/W) with sterile PBS and disintegrated by vortexing. This solution was used for dilution series. Each solution 10^{-1} to 10^{-8} was spotted in 10 μL spots onto LB agar plates in two dilution series and three technical replicates of each dilution, incubated at 37 °C overnight and the CFUs were counted the following day.

2.8. Histology and immunohistochemistry

The collected tissue samples were placed into histology cassettes and immediately soaked into 4% neutral buffered formaldehyde (Merck, Copenhagen, Denmark) for 24 h for tissue fixation. Standard procedures for histology were applied by graded concentrations of ethanol and xylene followed by embedding into paraffin wax, from which tissue sections of 3–5 μm were sliced. Haematoxylin and eosin (H &E) staining was performed according to a standard protocol (Stevens and Wilson, 1996). Evaluation of histology slides by light microscopy was based on a scoring scheme from 0 to 4, where 0 was normal, 1 was mild cell infiltration, 2 was moderate cell infiltration, 3 was widespread cells infiltration and/or scarce necrosis, and 4 was widespread necrosis. In this study cell infiltration is considered by presence of heterophil granulocytes, macrophages and lymphocytes together with oedema and exudation. For each of the three parts of the oviduct, the individual results were summarized to a total lesion score (Supplementary material I).

Sections for immunohistochemical detection of *E. coli*, cut from the paraffin-embedded tissues, were rinsed twice in TBS pH 7.6 (3.64 g/L Tris hydrochloride (Sigma-Aldrich Copenhagen, Denmark), 0.86 g/l Tris base, 6 g/L sodium chloride) for 5 min. The endogenous peroxidase was blocked by 0.6% hydrogen peroxide in TBS for 15 min. The TBS rinse was repeated twice. Preprocessing of 1.8×10^{-4} g/mL protease in TBS for 5 min. The TBS rinse was repeated thrice. Blocking was performed for 5 min in Ultra V block (LabVision/AH diagnostics, Tilst, Denmark). The primary antibody used for immunohistochemistry was rabbit anti *E. coli* antibody (4 mg/mL, Bio-Rad, Kidlington, Denmark), which reacts with LPS and capsule of all *E. coli* O and K antigenic groups. It was diluted 1:1000 in TBS for 1 h at room temperature. As a nonsense control, rabbit immunoglobin fraction (Agilent DAKO, Glostrup, Denmark) was used, which was diluted 1:500 in TBS for 1 h at room temperature. The TBS rinse was repeated twice. An HRP one Polymer (AH diagnostics, Tilst, Denmark) was added for 30 min., and the TBS rinse was repeated twice. Subsequently, the AEC chromogen single solution was added (AH diagnostics, Tilst, Denmark) for 10 min. and rinsed in distilled water twice for 5 min. The corresponding section was dissolved in Mayers hematoxylin (VWR, Søborg, Denmark) for 10 s, rinsed in running water for 1 min, followed by two final rinses in distilled water for 4 min. Lastly, the section was mounted with glycerol-

gelatin and subsequently evaluated by light microscopy based on presence of *E. coli*.

2.9. Statistical analysis

Comparison of infection potentials of the two strains in the different models were performed by unpaired *t*-test in GraphPad Prism, Prism 5 for Windows (GraphPad Software, La Jolla, California USA). The detection limit for CFU was 5×10^3 bacteria per gram oviduct scrape or mL lysed cell-culture due to the 10 µL spotting method. For statistical purposes, birds where no bacteria were detected were given a value of 1/10 of the detection limit, *i.e.* 5×10^2 CFU/gram. Statistical analysis of the histology scores were carried out for each of the oviduct sections (infundibulum, magnum and istmus) by nonparametric Mann Whitney test in GraphPad Prism, Prism 5 for Windows (GraphPad Software, La Jolla, California USA). Significance levels was set to $P < 0.05$.

3. Results

3.1. Presence of virulence genes in APEC F149H1S2 and UPEC UTI89

The virulence genes in the genome sequences of APEC F149H1S2 and UPEC UTI89 were examined. All virulence genes present in one or both strains are shown in Table 1. Fourteen virulence genes were found in both strains, eight virulence genes were only present in APEC F149H1S2, and eleven virulence genes were only present in UPEC UTI89. In Supplementary Material III, an extended version of Table 1 is shown, where putative virulence genes reported in literature are included as well.

3.2. Growth studies in LB and urine, ex vivo

UPEC UTI89 and APEC F149H1S2 showed overlapping growth curves in LB and human urine (Supplementary Material I and Fig. 1, respectively). Minor differences were calculated for the growth rates (mean ± SD) in both LB and urine. In LB, the growth rates were 0.08 ± 0.01 OD_{600}/hr for UPEC UTI89 and 0.07 ± 0.00 OD_{600}/hr for APEC F149H1S2 and in urine, the growth rates were 0.37 ± 0.05 OD_{600}/hr for UPEC UTI89 and 0.29 ± 0.01 OD_{600}/hr for APEC F149H1S2. None of the growth rate differences were statistically significant ($P = 0.173$ in LB and $P = 0.066$ in urine).

3.3. Invasion into human epithelial cell lines

As a first step of comparing the infection potential of UPEC and APEC, *in vitro* infection studies with two human cell lines were conducted (Fig. 2). The average CFU numbers were 1.5×10^4 CFUs for UPEC UTI89 and 2.5×10^3 CFUs for APEC F149H1S and the infection ability of UPEC UTI89 was significantly higher than that of APEC F149H1S2 ($P < 0.01$ and $P < 0.001$ for infection of Int407 and J82, respectively).

3.4. Survival in avian HD11 macrophages

We also evaluated the ability of the strains to survive inside phagocytic cells, through infection studies using the chicken derived macrophage cell line, HD11. Both strains increased in numbers at time T = 3 compared to T = 1 and then declined again at time T = 19.5 h (Fig. 3). There was no significant difference in the ability of the two strains to survive and proliferate in the intracellular environment of the macrophages ($P = 0.078$ and $P = 0.79$ for T = 3 compared to T = 1 and T = 19.5 compared to T = 1, respectively).

3.5. Oviduct infections in hens

Finally, we performed *in vivo* infection of the oviduct of egg-laying hens to examine whether the UPEC strain could cause salpingitis in hens. 24 h post infection all infected hens (both strains) showed clinical signs due to the infections with depression, listless and reduced active compared to the hens inoculated with sterile PBS. The infected birds remained depressed throughout the experimental period (48 h) and it was not possible to distinguish between the UPEC and APEC groups.

The UPEC strain was able to cause lesions related to the oviduct

Table 1
Virulence genes in APEC F149H1S2 and UPEC UTI89.

Virulence Gene	Present (+) in UPEC UTI89	Present (+) in APEC F149H1S2	Description
auf genes	+		Adhesion: Auf fimbria
fim genes	+	+	Adhesion: Type 1 fimbriae
pap genes	+	+	Adhesion: Type P fimbria
sfa genes	+		Adhesion: Type S fimbria
csg genes	+	+	Afimbrial adhesins: Curli
kpsMT	+		Capsule: Protectins
fliC	+	+	Motility: Flagella protien H antigen
usp	+		Multi-functional factors
ompA	+	+	Outer membrane protein
ompT	+	+	Outer membrane protein
ompC	+		Outer membrane protein
ompF	+		Outer membrane protein
ompX	+		Outer membrane protein
tsh		+	Serine protease autotransporter
iss		+	Serum resistance
traT	+		Serum resistance
feoB	+	+	Siderophore
ipfA		+	Siderophore
irp1	+	+	Siderophore
irp2	+	+	Siderophore
iucD		+	Siderophore
sitA	+	+	Siderophore
vjj		+	Siderophore
aer	+		Siderophore: Aerobactin
entS	+	+	Siderophore: Enterobactin
iroN	+	+	Siderophore: Enterobactin
ireA		+	Siderophore: Hemin uptake system
chuA	+		Siderophore: Hemin uptake systems
fyuA	+	+	Siderophore: Yersiniabactin
ybtP	+		Siderophore: Yersiniabactin
astA	+	+	Toxin: Arginine succinyltransferase
cnf1	+		Toxin: Cytotoxic necrotizing factor
hlyA	+		Toxin: Haemolysin
hlyF		+	Toxin: Haemolysin
senB	+		Toxin: Enterotoxin
vat		+	Toxin: Vacuolating autotransporter
upaG		+	Type V secretion system proteins

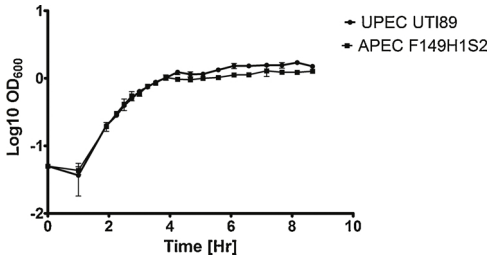

Fig. 1. Growth of UPEC UTI89 and APEC F149H1S2 in human urine, *ex vivo*. The experiment was performed in triplicates. Error bars denote standard deviations. Growth rates (mean ± SD) were calculated to be 0.37 ± 0.05 OD_{600}/hr for UPEC UTI89 and 0.29 ± 0.01 OD_{600}/hr for APEC F149H1S2. No statistical difference was found between strains.

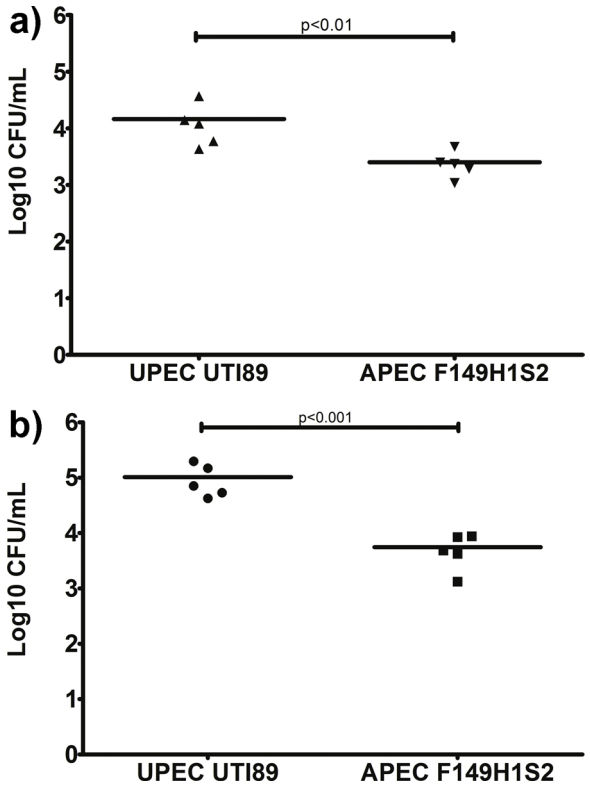

Fig. 2. Invasion of UPEC UTI89 and APEC F149H1S2 into human intestinal, Int407 (A), and bladder, J82 (B), epithelial cell lines. The bacteria were added to the cells at a MOI of 100:1 and infection progressed for 1 h. The statistical analysis showed that the difference in mean $\log_{10}$ CFU was significant.

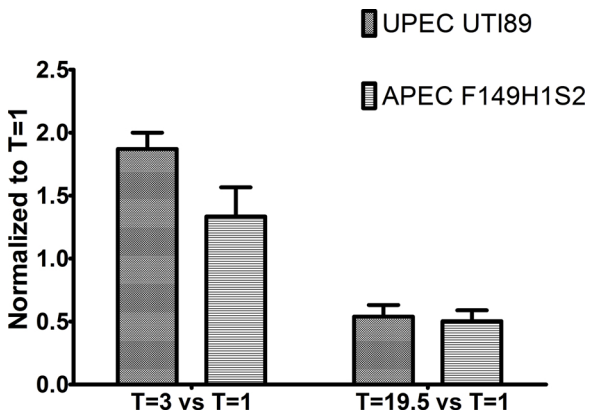

Fig. 3. Survival of UPEC UTI89 and APEC F149H1S2 in avian macrophages (HD11). The survival of bacteria in macrophages were analysed at three time points; 1, 3, and 19.5 h. All CFUs were normalized to the time point 1 h (T = 1). The statistical analysis showed that the differences between strains were non-significant.

similar to the APEC strain with presence of inflammation and fibrinous exudation in both the salpinx lumen (salpingitis) and peritonitis (data not shown). In all birds inoculated with *E. coli*, inflammation was seen in peritoneum by cloudy peritoneal membranes and presence of fibrinous exudation. Fibrinous exudation was also seen on the ovaries and inside the oviduct for all birds inoculated with the APEC and UPEC strains. Eight and nine birds inoculated with UPEC and APEC, respectively, were considered having stopped being in lay due to degeneration of the follicles. No systemic infections were observed in the birds, since no *E. coli* were cultured positive from neither liver nor spleen. All the birds receiving sterile PBS were culture negative at all sample point and

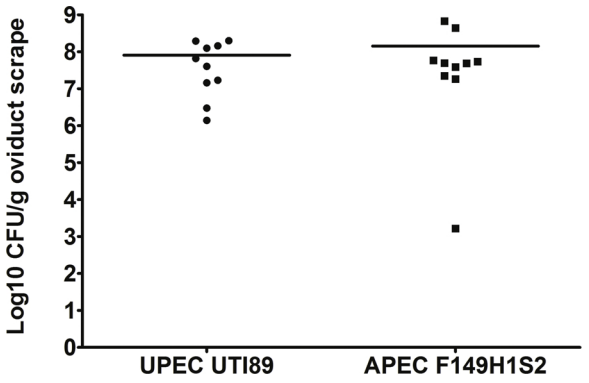

Fig. 4. Infection with UPEC UTI89 and APEC F149H1S2 in the oviduct of laying hens. Bacteria were inoculated directly into the oviduct and the infection progressed for 48 h, where the birds were euthanized. Each dot represents CFU counts from the oviduct scrape from a hen. The statistical analysis showed that the difference in mean $\log_{10}$ CFU was non-significant.

demonstrated no gross lesions as well as being in lay. Bacteria were recovered from the oviduct of all birds infected with both strains, and no significant difference ($P = 0.44$) was observed between CFU counts from the oviduct of the two groups (Fig. 4). No bacteria were obtained from the birds injected with PBS. No bacteria were recovered from birds sacrificed just prior to and after the experiments.

To examine inflammation of the infected oviduct, histology scoring was performed for three parts for each bird and compared to scoring of healthy birds inoculated with PBS (Supplementary material II). Fig. 5 shows representative examples of the most common observations that were made during the histology scoring evaluations. All birds inoculated with bacteria showed accumulation of inflammatory cells in the stroma and in the submucosa layer of the oviduct, although in varying amounts. The red arrowheads in Fig. 5a and b show loci of monocytes (lymphocytes and macrophages) from infundibulum sections of birds injected with APEC F149H1S2 and UPEC UTI89, respectively. Heterophil granulocytes are indicated by black arrowheads. Necrosis was only seen in the submucosa layer of the istmus part of the oviduct in one bird, which was injected with APEC F149H1S2 (not shown). No significant differences were seen in the histology score in the infundibulum ($P = 0.90$) and in the magnum ($P = 0.79$) sections, whereas the istmus ($P = 0.04$) section showed a significantly higher score in the APEC F149H1S2 group than in the UPEC UTI89 group (Supplementary material II).

For both APEC F149H1S2 and UPEC UTI89, evaluation of immunohistochemistry slides revealed the oviduct infection to be non-invasive (Fig. 5c and d, respectively) as none of the strains showed obvious invasion into epithelial cells or the submucosa. The epithelial lining was damaged by serous exudation and both the UPEC and APEC strains were observed embedded in the mucous layer on top of the luminal aspect of the epithelium or phagocytized by luminal macrophages or heterophils. This tissue damage and localization of bacteria was mostly prevalent in the magnum part (eight and seven birds inoculated with APEC and UPEC strains, respectively), but similar findings were found both for the infundibulum and istmus part of the oviduct.

3.6. Evaluation of the importance of fimH for the ability of UPEC to cause salpingitis in hens

The results above clearly indicated that UPEC can cause salpingitis in hens, and consequently, the surgical salpingitis model could be a potential model for studies of UTI, as well. For this to be relevant, it should be able to detect differences in pathology between a UPEC wild type strain and a mutant with reduced pathology in UTI. To investigate this, an additional salpingitis infection study was performed to compare

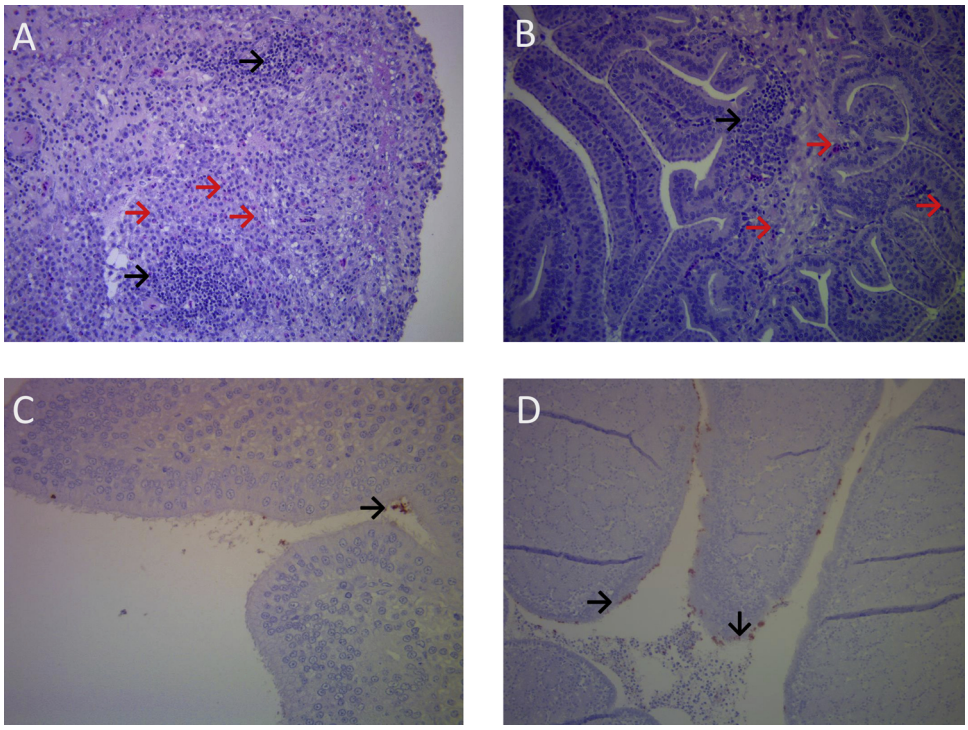

Fig. 5. Histochemistry and immunohistochemistry of oviduct sections representing examples of the most common oberservations. Histochemistry of (A) APEC F149H1S2 from an infundibulum section (20X objective) and (B) UPEC UTI89 from an infundibulum section (20× objective). Red arrowheads direct (lymphocytes and macrophages) and black arrowheads direct heterophil granulocytes. Immunohisto chemistry staining *E. coli* red of (C) APEC F149H1S2 from an istmus section (40× objective) and (D) UPEC UTI89 from a magnum section (20× objective). Small arrowheads direct to *E. coli* embedded in mucous or phagocytized (C).

the ability of UPEC UTI89 and a UPEC UTI89Δ*fimH* mutant, which according to literature is less virulent than the wild type strain (Connell et al., 1996), to cause salpingitis in the hen. 24 h post infection all infected (both strains) hens showed clinical signs (depression, listless and less activity), while hens inoculated with sterile PBS had normal active behaviour. One of the infected hens with the UPEC UTI89 wild type showed severe signs of infection (ruffled feathers, inactive and reluctance to move) and was euthanized for animal welfare reasons at this time point. Subjectively, 48 h post infection, the wild type infected hens appeared more depressed and showed lower activity in the pen than the hens infected with the deficient type 1 fimbria UPEC, although those hens were depressed as well.

The UPEC UTI89Δ*fimH* mutant was able to cause lesions related to the oviduct with a score that was not significantly different than the score of the wild type UPEC strain with presence of inflammation and fibrinous exudation in both the salpinx lumen and the abdominal cavity (data not shown). All birds inoculated with *E. coli* showed inflammation in peritoneum with cloudy peritoneal membranes and presence of fibrinous exudation. Fibrinous exudation was also seen on the ovaries and inside the oviduct for all birds inoculated with *E. coli*. In fact, the amount of exudate in hens (peritoneum, ovaries and salpinx) inoculated with UPEC UTI89Δ*fimH* was bigger than for the hens inoculated with the wild type UPEC UTI89 strain. No systemic infections were observed in the birds, since no *E. coli* were cultured positive from neither liver nor spleen. The birds receiving sterile PBS did not culture positive form any sample point and demonstrated no gross lesions. No significant difference in CFU counts from the oviducts of infected hens was seen for UPEC UTI89 and UPEC UTI89Δ*fimH* (Fig. 6).

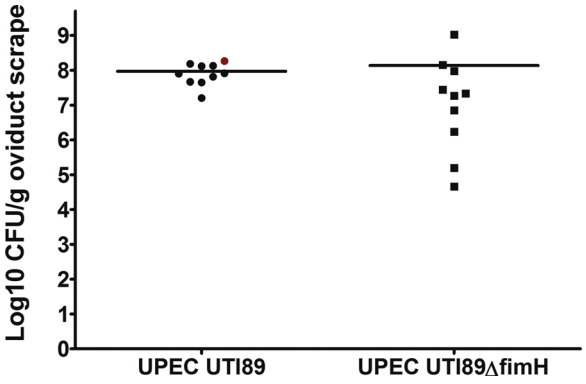

Fig. 6. Infection with UPEC UTI89 and UPEC UTI89Δ*fimH* in the oviduct of laying hens. Bacteria were inoculated directly into the oviduct and the infection progressed for 48 h, where the birds were euthanized. Each dot represents CFU counts from the oviduct scrape from a hen. The statistical analysis showed that the difference in mean Log_{10} CFU was non-significant. The red dot of the hens infected with UPEC UTI89 indicated a hen euthanized after 24 h due to severe clinical symptoms related to the infection and pronounced asocial behaviour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

Despite the differences in host association and infection sites between UPEC and APEC, they share phylogenetic and genetic relationship (Johnson et al., 2007) and they share a number of virulence genes (Manges and Johnson, 2012). Therefore, Manges and Johnson (2012) suggested that poultry products could serve as a source of ExPEC that causes sepsis in humans. Furthermore, studies have shown that UPEC are able to infect young chickens (Moulin-Schouleur et al., 2007; Zhao et al., 2009) and that APEC are able to infect human UTI model systems (Jakobsen et al., 2012; Zhao et al., 2009; Stromberg et al., 2017; Jakobsen et al., 2010). The main aim of this study was to examine whether UPEC strains are also able to cause salpingitis in adult hens in a similar manner as APEC, and thereby further supporting that the distinction between UPEC and APEC strains is arbitrary. The UPEC strain used was UPEC UTI89, which has sequence type ST95. Mora et al. (2009), hypothesized that ExPEC strains of this sequence type had a particular zoonotic potential towards humans and birds, and Jorgensen et al. (2019), showed that UPEC and APEC of this sequence type were genetically overlapping. Additionally, this sequence type is common for poultry infections (Poulsen et al., 2017; Heidemann Olsen et al., 2016).

The majority of virulence factors observed in the genomes of the two strains were shared, though differences appeared. UPEC UTI89

encodes more adhesion factors than APEC F149H1S2, and some siderophore- and toxin genes differed. A previous study (Ewers et al., 2007) examining the distribution of ExPEC virulence genes, found that *sfa* and *hlyA* were rarely found in APEC strains. Neither of these genes were present in the APEC F149H1S2 strain, but were present in UPEC UTI89. Our study is consistent with the previously reported higher prevalence of *tsh* and *iss* in APEC compared to other ExPECs (Ewers et al., 2007; Heidemann Olsen et al., 2016), since they were only present in APEC F149H1S2.

Prior to the *in vivo* infection experiments, we compared the *in vitro* infection potential of the APEC and UPEC strains. This showed nearly equivalent growth curves of APEC F149H1S2 and UPEC UTI89 in human urine. Urine has been designated a poor growth medium (Hull and Hull, 1997), and the results indicate that both strains are capable of growing under this condition, and it supports the uropathogenic potential of APEC strains as previously suggested (Skyberg et al., 2006).

The UPEC UTI89 strain showed a superior ability to invade human derived, epithelial cell cultures compared to the APEC strain, whereas one earlier study has showed that another APEC strain, APEC_01, was better at invading the human intestinal epithelial cell line (Int407) compared to UPEC UTI89 (Miquel et al., 2010). It was speculated that adhesion and invasion might not be crucial for *E. coli* when gaining access into the blood of poultry, since the oviduct has an opening directly into the peritoneum, which allows *E. coli* a chance of reaching blood capillaries within the peritoneum without adhering or invading the oviduct epithelium. In contrast, *E. coli* needs to invade the deeper epithelial cells or travel up to the kidneys in order to cause sepsis from the urinary tract in humans.

There was no significant difference between UPEC and APEC survival inside avian HD11 macrophage-like cells, which, to our knowledge, has not previously been investigated. Together, the results of our *in vitro* experiments confirmed that APEC strains have the ability to invade human epithelial cells, including bladder cells, and they showed that UPEC strains may be potentially pathogenic to birds. Prompted by this observation, we moved on to investigate whether the UPEC strain could cause pathology in a salpingitis model in adult, immunocompetent birds.

The surgical salpingitis model used (Pors et al., 2014) was developed with brown layers, since broiler breeders are more susceptible to infections than layers (Olsen et al., 2016), and despite that our APEC strain originated from an infection in layers, we chose to maintain this set-up. The salpingitis model has not been validated for its ability to detect the effect of single virulence factors, but a commensal *E. coli* strain was shown not to cause pathology (Pors et al., 2014). In our study, UPEC was confirmed by clinical signs, histopathology and immunohistochemistry to be able to cause salpingitis in hens, and only a non-significant difference was observed in CFU in the oviduct for hens infected with UPEC and APEC. Salpingitis may lead to a condition where virulent *E. coli* are present in the blood stream (colisepticemia) (Saif, 2008). In the current study, neither of the strains caused this condition, suggesting that the immune system controlled the infection at the site of inoculation. A weakness of the surgical model used was that it by-passed the normal ascendance from the cloaca, and we cannot conclude on the ability UTI89 to carry out this part of the infection.

A significant difference was seen in histology scoring of the istmus sections of the oviducts, where APEC scored higher than UPEC. This may indicate a higher virulence of the APEC strain, but no significant differences were observed in other sections of the oviduct. The study indicated that the majority of *E. coli* causing salpingitis in hens are located in the extracellular state, and this was found to be similar for UPEC and APEC. Taken all observations together, including the number of bacteria in the oviducts and the histology scores, the study showed that UPEC UTI89 can cause salpingitis in adult egg-laying hens to a similar level as the APEC strain tested. Thus, an anthrozoonotic potential for UPEC in adult hens is possible, however, to be fully able to infect hens under normal conditions, a series of barriers are to be overcome to allow transmission of UPEC from human to bird (Plowright et al., 2017), and it has to be able to perform infection by the natural route, *i.e.* ascending through the cloaca. We have not evaluated that in the current study.

The observation of UPEC-induced salpingitis in adult hens opened the question of whether the salpingitis model could also be used as an alternative to mice as *in vivo* UTI model. However, this prospect was refuted since no significant difference was observed between the virulent wild type UPEC UTI89 and UPEC UTI89Δ*fimH* whether clinical signs, scores of pathology or counting of bacteria were used for scoring. We did observe small, insignificant infection differences between the wild type and the mutant strains, a competition study might have been of relevance since it is more sensitive for detection of minor differences between strains.

fimH has been showed to be an important virulence factor in human UTI (Hagan et al., 2010) and the mutant is less virulent in murine models of UTI (Connell et al., 1996). The mutant is only deficient in the type 1 fimbria expression, and it is still capable of expressing other adhesion factors such as S and P fimbriae. Probably therefore, earlier studies have found that the mutant was not essential, but only reduced UTI in the murine model (Connell et al., 1996) and was able to cause a secondary surface colonization to bladder epithelium cells (Andersen et al., 2012). However, despite of these other adhesion factors, none of them completely compensated for the loss of type 1 fimbria, since the mutant always was less virulent (Andersen et al., 2012; Connell et al., 1996). Connell et al. (1996) hypothesized that the different fimbriae, and probably other virulence factors, act in concert to achieve the virulent phenotype. Yet, no significant difference in CFUs, found in oviduct of hens, was observed between the wild type UPEC UTI89 and UPEC UTI89Δ*fimH*, which suggests that type-1 fimbriae are not virulence factors during colonization and growth in the oviduct of hens. The viscous mucus layer covering the epithelial lining of hens is likely to trap bacteria. This may explain why type 1 fimbriae are not needed for infection in this organ.

The milieu of the human urinary tract system and the oviduct of hens differ. In the human urinary tract system, UPEC experience planktonic growth and are at constant risk of being voided during urination. Probably therefore, the adhesion factors of UPEC are of importance during UTI. Additionally, some adhesion factors also mediate host cell invasion (Pizarro-Cerda and Cossart, 2006), which possibly enhances bacterial survival by providing protection from host immune defences and allowing the pathogens greater access to deeper tissues. In the oviduct, the majority of APEC resides on the surface according to the results of immunohistochemistry in the current study. The inner surface of the oviduct is covered by a mucus layer consisting of mucins composed of glycoproteins into which sugar residues, such as *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid), are incorporated (Ariyadi et al., 2012). The immunohistochemistry revealed no intracellular *E. coli*, indicating that APEC are non-invasive or that intracellular APEC were too few to be observed. Thus, it remains unknown whether cellular invasion can contribute to recurrent salpingitis as has been suggested for UPEC in recurrent UTI in humans (Glover et al., 2014).

Interestingly, CFU variation of the UTI89Δ*fimH* mutant was remarkably higher than variation in CFU of the WT strain. The reason for this is unknown, but it may indicate that local environmental factors which influence expression of other surface antigens than type 1 fimbriae may vary between hens. Previous studies (Pors et al., 2014; Olsen et al., 2016) showed that predicting the salpingitis-virulence of APECs solely based on the genetic profile of the bacteria was difficult. This could indicate that additional strain features besides virulence factors are of importance when causing salpingitis. According to a previous study where the surgical model was used, the oviduct is not just an incubation chamber for injected bacteria, since a non-pathogenic faecal strain (D2-2) performed less good than pathogenic APEC strains (Olsen et al., 2016). The faecal strain was however isolated from 4 out of 10

layers, and the main difference was that no pathology was observed. Thus, CFU counts are probably not reliable as the only scoring in this model, but needs to be combined with observations on clinical signs and pathology, as performed in the current study.

5. Conclusion

This study demonstrated that the human cystitis isolate UPEC UTI89 can cause salpingitis in adult laying hens at a similar level as APEC F149H1S2 originally isolated from an avian oviduct with salpingitis. The two strains also grew equally in human urine and survived similarly well in avian macrophages, whereas UPEC UTI89 infected and survived better than APEC F149H1S2 in human bladder - and intestinal epithelial cells. Although UPEC UTI89 infected the oviduct of hens to the same extent as APEC F149H1S2, the *in vivo* salpingitis model is likely not a good model for human UTI, as no phenotypic difference was observed between the UPEC UTI89 wild type and the UPEC UTI89Δ*fimH* mutant strain lacking type 1 fimbria, a major virulence factor in human UTI.

Acknowledgements

Funding: This work was supported by The Danish Research Council for Technology and Production, Grant number DFF – 4184-00050.

Sisse Mortensen was supported by a PhD fellowship from University of Copenhagen.

A special thanks to the animal keepers for close observations of the hens during experimentation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108445.

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