Int.J.Curr.Microbiol.App.Sci (2024) *13*(09): 1-8

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 13 Number 9 (2024)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2024.1309.001

Adherence Patterns and Invasion of Uropathogenic *Escherichia coli* Carrying *fim*H Gene

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Keywords

Uropathogenic *Escherichia coli*, fimH gene, adherence, invasion, Vero cells

Article Info

Received:
xx July 2024
Accepted:
xx August 2024
Available Online:
xx September 2024

A B S T R A C T

The present study aims to investigate two of the Uropathogenic *Escherichia coli* (UPEC) pathogenesis mechanisms, as adherence and invasion in primate kidney epithelial (Vero) cells. Methods*:* 20 *E. coli* isolates from outpatients with UTI in one public clinic were evaluated for adherence and invasive abilities in Vero cells. Results: All 18 positive isolates for *fim*H gene adhered to the Vero cells (100%). The two UPEC isolates that did not carry the *fim*H gene did not adhere. Two different UPEC adherence patterns were observed: aggregative adherence pattern (AA) and a pattern called "no typical adherence pattern" (NTA). Among 18 adherent UPEC isolates, 11 presented AA adherence pattern (61%) and 7 showed NTA pattern (39%). The high rate of adherence of the UPEC *fim*H positive isolates (≅90%) to Vero cells suggests the expression and participation of the *Fim*H adhesin in UPEC adherence behavior. Invasive capacity of UPEC isolates ranged of about 6.5% to 15.33%. Results corroborate the fact that UPEC can persist in the urinary tract through its adhesion, as well as invade cells establishing the bacterial UTI process.

Introduction

The ability of Uropathogenic *Escherichia coli* (UPEC) for causing urinary tract infection (UTI) is associated with the production of some virulence factors directly involved with the infection process, such as Type-1 fimbria (Kucheria *et al.,* 2005; Terlizzi *et al.,* 2017; Wurpel *et al.,* 2016).

Adherence is the start stage to the urinary tract colonization by UPEC (Wurpel *et al.,* 2016; Wright *et al.,* 2006). Fimbriae help in adhesion to host cell surface and tissue invasion (which is important in pathogenesis of UPEC causing UTIs), biofilm formation and cytokine induction (Dias *et al.,* 2010; Basu *et al.,* 2013; Tabasi *et al.,* 2016). FimH is an adhesin located at the tip of Type-1 fimbriae and responsible for increasing the inflammatory responses associated with bacterial adherence (Stamm, 2006). In cystitis cases, FimH is continuously expressed and produced in the bladder environment (Firoozeh *et al.,* 2014).

Some studies have been shown that some UPEC strains can present adherence patterns like those of EPEC, as for example the aggregative adherence pattern, in urinary tract cells (Nascimento *et al.,* 2021; Schüroff *et al.,* 2021) and that the Type-1 fimbriae is responsible for aggregative adherence pattern establishment (Moreira *et al.,* 2003; Abe *et al.,* 2008).

However, there are few studies showing that UPEC can adhere and invade Vero cells (Andrade *et al.,* 2021).

The present study mainly aimed to observe the adherence behavior of UPEC strains carrying *fim*H gene in Vero cells. The ability of some isolates to invade Vero cells when exposed to the media with amikacin was also investigated.

Materials and Methods

Study design and ethical considerations

Twenty *E. coli* isolates recovered from urine samples of outpatients (16 female and four male) at a public hospital in Rio de Janeiro, from May to September 2019, were analyzed in the study. Only one representative bacterial isolate per episode of urinary tract infection was selected. Bacterial identification was determined by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) technique. The project was approved by the research ethics committee (2.920.186/CAAE number 95984018.6.0000.5243, Medical College, Fluminense Federal University).

PCR to *fimH* gene

The presence of *fimH* gene was investigated by polymerase chain reaction (PCR) and sequencing using primers (forward 5'-TGCAGAACGGATAAGCCGT GG-3') reverse 5'-GCAGTCACCTGCCCTCCGGTA-3') and conditions already reported (Basu *et al.,* 2013). Electrophoresis was performed on 1.5% agarose gel developed in GelRed® (0.5g/L) under UV light.

Cells culture conditions

The Vero cell line American Type Culture Collection (ATCC) CCL-81, derived from African green monkey kidney (*Cercopithecus aethiops*), was used to mimic the normal human renal epithelial cells (Ammerman *et al.,* 2008).

Vero cells were cultured in Minimum Essential medium (MEM, GibcoBRL) supplemented with fetal calf serum (5% v/v; GibcoBRL) gentamicin (50 µg/mL) and fungizona (2.5 µg/mL). The cells were cultured on 13 mm diameter glass coverslips placed in 24-wells tissue culture plates (Costar) to obtain the sub-confluent cell monolayer. Cultures were kept at 37ºC and 5% CO_2 for 2 to 3 days to obtain the cell confluence. Next, the monolayer was washed twice with Dulbecco's phosphate buffered saline (PBS-D; pH 7.2) and incubated with 1 mL of fresh MEM without antibiotics.

Adherence assays

Adherence was evaluated in all 20 UPEC isolates (18 *fimH*-positive and 2 *fimH*-negative) by a qualitative assay of adhesion in Vero cells (ATCC CCL-81) (Ammerman *et al.,* 2008).

Bacterial cultures were prepared in 5 mL Luria Bertani broth medium (LB; pH 7, 4), overnight, at 37 °C. Later, cell monolayers were incubated with 35 µL of each bacterial suspension in LB and incubated for 3 and 6 hours, in 5% CO_2 atmosphere at 37ºC. For the 6 hours assays, the cells were washed with PBS-D and fresh medium was added after 3h of incubation. The cells were washed twice with PBS-D to remove the non-adherent

bacteria, fixed with methanol, stained with 5% Giemsa stain for 30 minutes, and washed in distilled water and then optical microscopy slides are produced.

Enteroaggregative *Escherichia coli* 042 (EAEC 042) and Uropathogenic *Escherichia coli* (UPEC I64) strains (Figure 1) were used as positive control of adherence assays; non-inoculated culture was used as negative control (Rosa *et al.,* 1998). Experiments were performed at least three times.

Invasion assay

Invasive assays were performed according reported previously (Pereira *et al.,* 2008).

Salmonella enterica serovar Typhimurium C20 and *E. coli* DH5α bacterial strains were used as controls positive and negative, respectively.

Vero cells non-polarized monolayers were cultivated on abiotic surface in 24 well-tissue culture plate containing Minimum Essential medium (MEM, GibcoBRL) supplemented with 5% v/v fetal calf serum (GibcoBRL), 50 µg/mL gentamicin, and 2.5 µg/mL fungizona in 5% CO_2 atmosphere at 37ºC, until reach confluence.

UPEC isolates and control strains were cultured in 5 µl of Tryptic Soy Broth medium (TSB) overnight at 37ºC. Bacterial concentrations of 0.14 Optical Density (O.D.) were determined by densitometry. Aliquots of 35 µl of the bacterial suspensions (approximately 10^8 CFU ml^{-1}) were added to Vero monolayers in wells containing 1 ml of MEM usage medium without antibiotics. The infected monolayers were incubated for 3 hours at 37º C in an atmosphere with 5 % CO_2. After 3 hours of incubation, the cells were washed with PBS-D twice and MEM supplemented with 25 µg /ml of amikacin was added to half of the wells and incubated for 1 hour at 37º C in 5 % CO_2 atmosphere. After incubation, the cells were washed twice with PBS-D and lysed 1 ml of 1 % Triton X-100 (BioRad) for 30 minutes.

Aliquots of cell lysates were diluted in PBS-D (10^{-1} – 10^{-5}) and plated in Tryptic Soy Agar medium (TSA) and incubated at 37°C for 18 to 24 hours, to quantify the CFU of viable intracellular bacteria.

The invasion index was expressed as the percentage of intracellular bacteria divided by the number of inoculated bacteria (10^7) x 100 (Tang *et al.,* 1993). Experiments were performed at least three times. A percentual ≥1% was considered positive to cell invasion, which is possible to confirm the invasion result by transmission electron microscopy (the gold-standard rate); this parameter was defined based on previous studies (Santos *et al.,* 2015).

Results and Discussion

All *E. coli* isolates have had identification confirmed by MALDI-TOF MS.

Eighteen (90%) of the 20 *E. coli* isolates included in the study were *fimH* positive (14 [87.5%] from female and four [100%] from male outpatients).

All *E. coli* isolates carrying the *fim*H gene were able to adhere to Vero cells (18; 100%) and the two *fim*H negative isolates (365 e 421) were not able (Table 1).

Two different adherence patterns were observed among the bacterial isolates: aggregative adherence (AA) (11 [55%] isolates) and not-typical adherence (NTA) (7 [39%] isolates]. Two (10%) isolates did not show adherence (Table 1). The “stacked-brick” aggregative adherence (*E. coli* isolate 368) and NTA (*E. coli* isolate 424) patterns are shown in Figures 2 and 3, respectively.

Ten of 20 *E. coli* isolates (four showing AA pattern [353, 354, 368 and 397], four NTA [355, 366, 422 and 424] and 2 non-adherents [365 and 421]) were evaluated for invasive ability (Table 1). Four (50%) adherent isolates (two AA and two NTA) and the two non-adherent (100%) were able to invade Vero cells (Table 1).

Of the eight strains tested, 50% were able to invade Vero cells with high capacity of internalization. Two of 20 UPEC strains (353 and 368), that presented an AA pattern, showed an invasive rate of 14,15% and 6,50%, respectively. However, the strains 354 and 397, that showed the same adherence pattern were not able to invade the urinary cells (0,20% and 0,16% invasion rates, respectively). Among UPEC strains which have adherence pattern classified as NTA, 355 and 366 strains also revealed an invasive ability to the Vero cell, showing an elevated rate of 15,33% and 12,40%, respectively.

Table.1 Characteristics of 20 UPEC strains of the present study.

Clinical strain	Isolation data	Patient gender	Patient age	PCR to *fim*H gene	Adherence pattern in Vero cells	
					3h	6h
353	20/05/2019	F	15	+	AA	AA
354	20/05/2019	F	23	+	AA	AA
355	21/05/2019	F	79	+	NTA	NTA
356	21/05/2019	F	61	+	AA	AA
358	22/05/2019	F	75	+	AA	AA
359	04/06/2019	F	26	+	AA	AA
363	04/06/2019	F	41	+	AA	AA
364	04/06/2019	F	92	+	AA	AA
365	04/06/2019	F	76	-	NA	NA
366	04/06/2019	F	53	+	NTA	NTA
367	04/06/2019	F	62	+	NTA	NTA
368	04/06/2019	F	67	+	NTA	AA
397	10/06/2019	M	69	+	AA	AA
398	10/06/2019	M	73	+	AA	AA
399	10/06/2019	M	87	+	AA	AA
421	17/06/2019	F	81	-	NA	NA
422	17/06/2019	M	57	+	NTA	NTA
423	17/06/2019	F	70	+	NTA	NTA
424	17/06/2019	F	33	+	NTA	NTA
425	17/06/2019	F	38	+	NTA	NTA
UPEC I64					AA	AA
EAEC O42					AA	AA
C20					N	N
DH5a					N	N

Legend: UPEC I64 and EAEC O42: positive control to adhesions tests; C20: positive control to invasion; DH5a: negative control to invasion; Aggregative adherence (AA); Nonadherence (NA); Not typical adherence (NTA); N: Not tested; +: positive; -: negative.

Figure.1 Image of microscopic observation of the UPEC I64 positive-control strain of AA patter after 6h of infection in Vero cells (1000x).

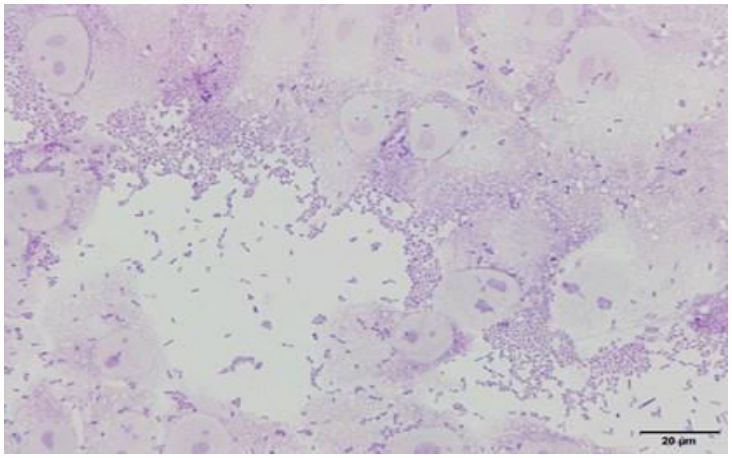


Table.2 Percentual of invasion of Aggregative adherence and Not-typical adherence UPEC strains in Vero cells.

Clinical strain	Adherence pattern in Vero cells	Percentual of invasion to Vero cells/(Result)
353	AA	14.15%/(+)
354	AA	0.20%/(-)
355	NTA	15.33% (+)
366	NTA	12.40%/(+)
368	AA	6.50%/(+)
397	AA	0.16%/(-)
422	NTA	0.42%/(-)
424	NTA	0.04%/(-)
353	AA	14.15%/(+)
354	AA	0.20%/(-)
C20		100% /(+)
DH5alfa		0.55%/(-)

Legend: C20: positive control to invasion tests; DH5a: negative control to invasion; Aggregative adherence (AA); Not typical adherence (NTA); +: positive; -: negative.

Figure.2 Image of microscopic observation of the AA pattern of UPEC isolate 368, after 6h of infection in Vero cells (1000x).

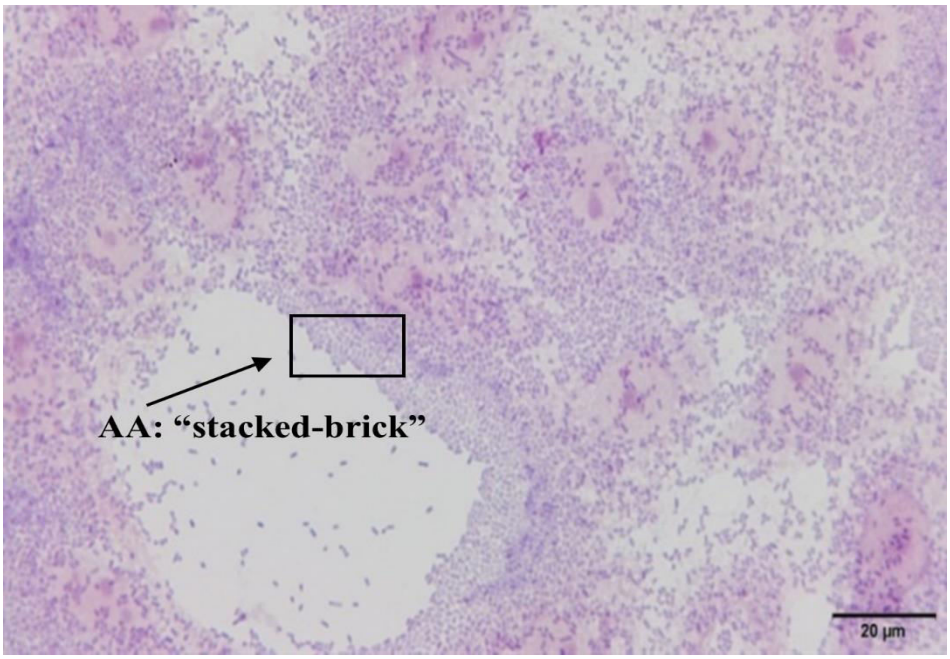
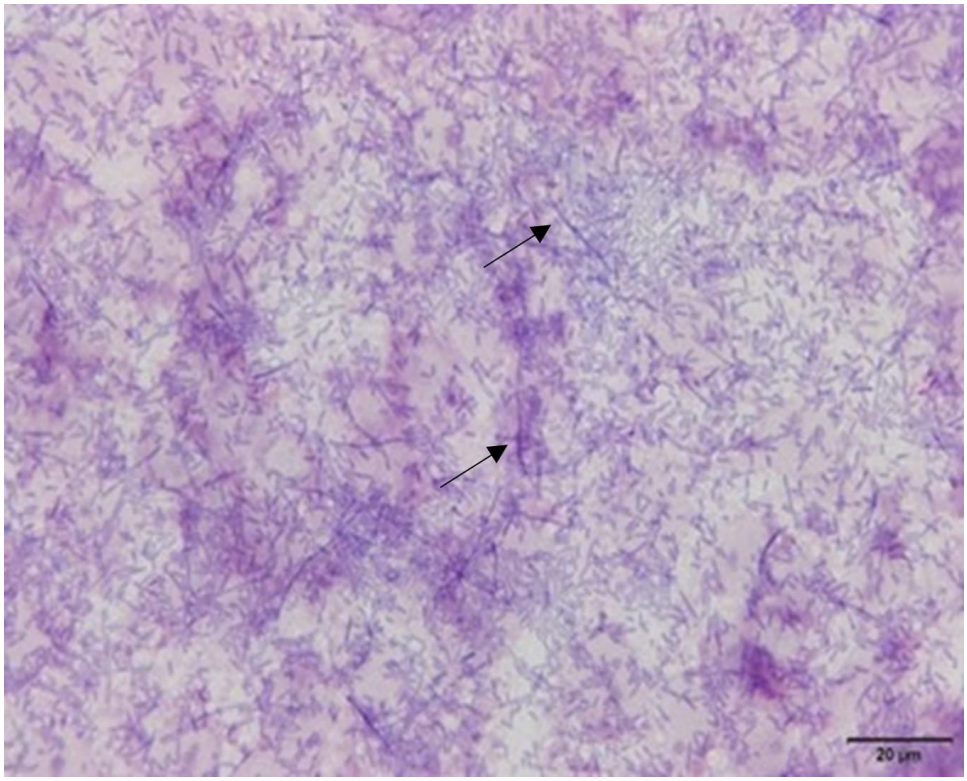


Figure.3 Image of microscopic observation of the NTA pattern of UPEC isolate 424 with filaments occurrence (arrows), after 6h of infection in Vero cells (1000x).



On the other hand, the strain 422 did not present an invasive feature, representing only 0,42% of invasion (Table 2).

All UPEC strains carrying the *fim*H gene showed to be adherent to Vero cells (18; 100%). It is evidence of the expression and participation of the FimH adhesin in UPEC adherence behavior.

Some studies have shown the Type-1 fimbriae role in the UPEC adherence, but also, in the UPEC invasion to bladder cells (Mulvey *et al.,* 2001; Martinez *et al.,* 2000; Staerk *et al.,* 2016).

A study associated the lack of type-1 fimbriae, during planktonic growth in both mouse and human urine specimens, with the reduction of bladder-cell adhesive and invasive potential of UPEC) (Staerk *et al.,* 2016).

In general invasion occurs after adhesion, through Type-1 fimbriae mediation, which adheres to the receptors on host urothelial cells, integrins α3, β1, and uroplakins, which allows UPEC to invade and colonize the bladder, forming intracellular bacterial communities (IBC) (Wiles *et al.,* 2008; Chen *et al.,* 2009; Flores-Meireles *et al.,* 2015).

After the invasion, the intracellular bacterial community's escape of host immune responses, contributing to the occurrence of persistent or chronic urinary infection (Hannan *et al.,* 2012).

In the present study, 8 adherent UPEC strains to Vero cells were also tested to their ability to invade this cell line. Invasion ability seemed not to be directly related with adherent pattern, since UPEC strains belonging to two different patterns found (AA or NTA) showed to invade Vero cells in different levels; UPEC invasion mechanisms can help bacteria to escape immune system defenses.

In summary, the adherence to Vero cells observed in all UPEC strains carrying *fim*H gene confirms the role of FimH adhesin in the colonization and infection of UPEC of the urinary tract.

Taken together, these data reinforce the participation of the FimH in the UTI pathogenesis and can reflect better

adaptation and capacity for causing infection as compared with non-adherent UPEC isolates.

We could observe that part of these strains was able to invade Vero cell lines, indicating that they possess ability of internalization as a strategy of pathogenicity.

Acknowledgements

We would like to thank to FAPERJ and to team of laboratories BMM, LAPAC and LAPESF as well as researches who contributed to the production of this article.

Author Contributions

Flávia Lúcia Piffano Costa Pellegrino: Conceived the original idea, designed the model, Writing - Original Draft Preparation, Writing - Review & Editing, Visualization, Supervision, Project Administration, and Funding Acquisition.; Sandra Maria Cordeiro da Silva Gonçalves: Writing - Original Draft Preparation, Methodology and Validation; Nathália Lucas da Silva Andrade: Writing - Original Draft Preparation, Methodology and Validation; Mariana Barros Lopes: Writing - Original Draft Preparation, Methodology and Validation; Gabriel Gomes do Rosario: Methodology and Validation; Thiago Pavoni Gomes Chagas: Methodology; Cláudia Rezende Vieira Mendonça e Souza: Methodology; Daniel Escorsim Machado: Methodology; Jamila Alessandra Perini: Methodology; Marinella Silva Laport: Methodology; Rubens Clayton da Silva Dias: Writing - Original Draft Preparation, Writing - Review & Editing, Visualization, Supervision.; Ana Cláudia de Paula Rosa Ignacio: Writing - Original Draft Preparation, Writing - Review & Editing, Visualization, Supervision.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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How to cite this article:

Sandra Maria Cordeiro da Silva Gonçalves, Nathália Lucas da Silva Andrade, Mariana Barros Lopes, Gabriel Gomes do Rosario, Thiago Pavoni Gomes Chagas, Cláudia Rezende Vieira Mendonça e Souza, Daniel Escorsim Machado, Jamila Alessandra Perini, Marinella Silva Laport, Rubens Clayton da Silva Dias, Ana Cláudia de Paula Rosa Ignacio and Flávia Lúcia Piffano Costa Pellegrino. 2024. Adherence Patterns and Invasion of Uropathogenic *Escherichia coli* Carrying *fim*H Gene. *Int.J.Curr.Microbiol.App.Sci.* 13(9): 1-8. **doi:** https://doi.org/10.20546/ijcmas.2024.1309.001