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Microbiologic profile of *Escherichia coli* strains isolated from urinary tract infections in aged nephrology clinic patients.

Perfil microbiológico de cepas de *Escherichia coli* isoladas de infecções do trato urinário em pacientes idosos de ambulatório de nefrologia.

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RESUMO

A invasão de bactérias no sistema urinário em humanos caracteriza a infecção do trato urinário (ITU), com capacidade de causar cistite e/ou pielonefrite. A *Escherichia coli* é o principal microrganismo associado à ITU e por isso foi chamada de UPEC (*Escherichia coli* uropatogênica). A prevalência de ITU entre os sexos diminui após 65 anos em infecções comunitárias e em mulheres na pós-menopausa. As infecções urinárias podem ocorrer em pacientes ambulatoriais ou internados na presença de diversos fatores, como perfil diferenciado de resistência antimicrobiana, adesão e invasão às células uroepiteliais, presença de fatores de virulência, formação de biofilme e grupo filogenético prevalente. O presente estudo tem como objetivo relacionar a ITU por UPEC em pacientes ambulatoriais maiores de 55 anos atendidos na nefrologia. Foi detectado que mecanismos de virulência como, adesão e invasão nas células renais podem estar associados ao processo de infecção do trato urinário causado pela UPEC nesses pacientes. A formação de biofilme e a associação com o grupo filogenético D podem ser importantes determinantes da adesão precoce e da infecção.

Palavras-chave: UPEC; Infecção urinária; Virulência; Aderência; Invasão..

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ABSTRACT

The invasion of bacteria in the urinary system in humans characterizes the urinary tract infection (UTI) with the capacity to cause cystitis and/or pyelonephritis. *Escherichia coli* is the main microorganism associated with UTI and was therefore called UPEC (Uropathogenic *Escherichia coli*). UTI prevalence between genders decreases after 65 years in community infections and in post-menopausal women. Urinary infections can occur in outpatients or inpatients in the presence of several factors such as differentiated antimicrobial resistance profile, attachment, and invasion to uroepithelial cells, presence of virulence factors, biofilm formation, and prevalent phylogenetic group. The present study aims to relate the UTI by UPEC in outpatients older than 55 years attended in nephrology. It was detected that virulence mechanisms such as adhesion and invasion in renal cells may be associated with the urinary tract infection process caused by UPEC in these patients. Biofilm formation and association with phylogenetic group D can be important determinants of early adhesion and infection.

Keywords: UPEC; Urinary infection; Virulence; Adhesion; invasion.

INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) is the most common cause of Urinary Tract Infections (UTI) (WANG et al, 2024). The incidence of UTI is four times higher in women compared with men. It is estimated that 50% of women experience at least one UTI during their lifetime, usually an uncomplicated UTI (SUBASHCHANDRABOSE; MOBLEY, 2015).

UTIs caused by *E. coli* typically occur in elderly patients and are one of the most important risk factors in this age group, since it encompasses changes in the genitourinary tract, in addition to hormonal and immunological factors related to age comorbidities. Due to urinary and fecal incontinence, the use of geriatric diapers facilitates the contamination of the urinary tract by microorganisms. The lower urinary tract inflammation, such as cystitis, originates when gastrointestinal microorganisms enter the urinary tract (NICOLLE, 2001; OMLI et al, 2010; PALMS et al, 2018).

The difference in prevalence between genders decreases after 65 years in community infections. In post-menopausal women, recurring urinary tract infections are common (OLIVEIRA; SANTOS, 2018). Urinary infections can occur in outpatients or inpatients, but the risk factors and antimicrobial resistance profile are different between community urinary tract infections and nosocomial urinary tract infections (SALTOGLU et al, 2015).

The UPEC pathogenicity process involves many steps, but the most important is the attachment and invasion of uroepithelial cells (BARBER et al, 2013). UPEC strains

that cause infections present harbor virulence factors to enhance the capacity to survive and colonize the urinary tract. In addition, biofilm formation is important to the UPEC infection process, and multiple genes are involved. Biofilm formation is also implicated in the colonization of urinary bladder in animal models (SUBASHCHANDRABOSE; MOBLEY, 2015). Previous studies have investigated the genotype and phylogenetic background of *E. coli.* The most prevalent phylogenetic group in extraintestinal pathogenic *E. coli* strains is B2, followed by group D. Groups A and B1 are commensal strains phylogroups causing extraintestinal infection, but their distribution is subject to geographical variations (CHAKRABORTY et al, 2015; MARTÍNEZ et al, 2006).

The present study aims to relate UTIs, older than 55 years, with the presence of virulence and phylogenetic groups.

MATERIAL AND METHODS

Escherichia coli strains were isolated from urinary samples of eleven patients with urinary tract infection (UTI), aged 55 years and older during outpatient treatment in the nephrology department of a tertiary hospital in Rio de Janeiro, Brazil, during the periods from January to March 2014.

The strains were reisolated and confirmed by morphotintoral identification through Gram staining and subsequent identification through manual and automated biochemical tests. Manually, tube tests containing the media urea agar (Merck®) were used for urease production tests; SIM agar, LIA agar and TSI agar (all from Merck®) - H_2S production; SIM agar - for indole production and evaluation of motility; Simmons citrate agar (Merck®) - carbon source growth; LIA agar - for decarboxylation of lysine; and TSI agar - for the use of carbohydrates (glucose, sucrose and lactose). All strains were also confirmed by automated identification using the Vitek System.

The study was licensed by the Ethics Committee according to Brazilian legislation (register number: CAAE: 45780215.8.0000.5259).

The isolated strains followed the following inclusion criteria: Isolated urine samples from January to March 2014; *Escherichia coli* infection; repeat *Escherichia coli* infection; isolated strains of patients from the nephrology outpatient clinic and isolated strains of patients over 55 years of age. The exclusion criteria were as the following: infection with other bacteria; isolated strains of patients from other outpatient clinics, and isolated strains of patients under 55 years of age.

Molecular detection of virulence genes and phylogenetic groups

The primers utilized to detect *pap* (encoding pilus associated with pyelonephritis), *sfa* (adhesin-encoding operons: the central region of the *sfa*/f*oc* operon, encoding S fimbriae, and F1C fimbriae), *afa* (S fimbriae), *hly* (encoding α-hemolysin), *aer* (aerobaction) and *PAI* markers. PCR conditions were the same as that described by Yamamoto et al (1995), and Johnson & Stell (2000)**.** The phylogenetic typing was performed by multiplex PCR (CLERMONT et al, 2012; GIRARDINI et al, 2012).

Adherence assays with E. coli using VERO cell line

Initially, VERO cells were grown in flasks containing MEM (Minimum Essential Medium, Difco - BRL) supplemented with 2% FBS, gentamicin 50 µg/mL, and fungizone 2.5 g/mL, at 37°C in a humidified atmosphere of 5 % CO_2. Then, they were subcultured in 96-well plates. Each well was washed twice with PBS and D-MEM with 2% FBS, then inoculated with 35 µL of bacterial culture (grown for 18 hours at 37°C for each bacterial strain). Plates were incubated for 3 hours at 37°C, and thereafter, the cell cultures were washed, fixed, and stained with Giemsa (1:20), and the slides for viewing under a light microscope were prepared (ROSA et al, 1998), with modification. The controls used were *E. coli* DH5α to negative tack; and the EAEC 042 and UPEC I64 as positive control in all tests.

Invasion assays with E. coli using VERO cell line

The quantitative invasion assay was performed according to Santos *et al* (2015). The VERO monolayers were cultivated in 24 well-tissue culture plates containing MEM supplemented with 2% FBS, a maintenance medium at 37°C in a humidified atmosphere of 5 % CO_2 until reached semi-confluence. Bacterial strains were inoculated in TSB overnight at 37°C. Bacterial concentrations were determined by densitometry. Aliquots of bacterial suspensions (approximated 10^8 CFU/mL) were added to VERO monolayers in wells containing 1 mL of MEM medium without antibiotics. The infected monolayers were incubated for 3 hours at 37°C in a humidified atmosphere of 5 % CO_2. After 3h cell monolayers were washed with PBS-D and supplemented with MEM or 100 µg/mL of amikacin and incubated for 1h at 37°C in a humidified atmosphere of 5 % CO_2. After incubation, the cells were washed twice with PBS-D and lysed 1 mL of 1% TritonX-100

(BioRad) in PBS-D for 30 min. Aliquots of cell lysates were diluted in PBS-D (10^{-1}–10^{-5}), plated in TSA, and incubated at 37°C for 18–24h, to quantify the CFU of viable intracellular bacteria. The invasive ability of *E. coli* strains was compared with positive and negative controls, respectively, *Salmonella enterica* serovar Typhimurium (C20) and non-invasive *E. coli* DH5α (TANG et al, 1993; SANTOS et al, 2015; ROSA et al, 2001). All strains were amikacin sensitive and unable to grow in media containing (100 µg/mL amikacin). Experiments were performed at least three times.

Biofilm formation assay

To assess semi-quantitative biofilm formation, 200µL of DMEM containing 0.45% glucose in 96-well flat-bottom polystyrene microtiter plates (TPK) was inoculated with 5µL (approximately 10^5 bacterial strains) of a culture of LB grown overnight at 37ºC. The plate was incubated for 18h at 37ºC. Then, the planktonic cells were removed by rinsing three times with water and the substratum was stained with 0.5% crystal violet for 5 min. After washing with water and blotting with paper towels, the biofilm formation was quantified spectrophotometrically by the addition of 200µL of 95% ethanol to each well of a crystal violet-stained microtiter plate. After solubilization for 30 min at room temperature, 150µL was transferred to a new microtiter plate, and the absorbance was determined with an enzyme-linked immunosorbent assay plate reader at 570 nm (MOHAMED et al, 2007)**.**

RESULTS AND DISCUSSION

Urinary tract infections are one of the most common infections and previous reports showed uropathogenic *E. coli* (UPEC) as the main etiologic agent in these infections [20]. The biofilm formation, adhesion, and invasion abilities are important virulence factors to uropathogenic *E. coli*. In this study, biological, assays were employed to investigate the virulence-associated characteristics and biofilm production of *E. coli* from the urine of nephrology patients with UTI. A total of 11 UPEC isolates were analyzed for the presence of virulence factors, biofilm formation, and adhesion. These isolates were from elderly (age average for over 55 years) patients majority female (81.8%) patients attempted in nephrology ambulatory in 2014.

The phylogenetic group followed the gene inclusions in Group A, including isolates with *chuA*-/TSPE4.C2; group B1, *chuA*-/TSPE4.C2+; group B2 *chuA*+/*yjaA*+;

and group D, *chuA+/yjaA-*. Among these strains 72.2% (n=8) were positive to *chuA* gene 63,6% (n=7) were positive to *tspE4.c2* gene, while 45.4% (n=5) were positive for *yjaA.* In the phylogenetic analysis, the isolates were classified as A 27.23% (n=3), B1 19.09% (n=1), B2 18.18% (n=2), and D 45.45% (n=5). Standard strain I64 belongs to the phylogenetic group D. The results presented inversion since the prevalent group was D followed by A, and lastly, we had two strains of group B2.

In urinary tract infections, the association with the phylogenetic groups B2 and D has been reported as the most prevalent (CHAKRABORTY et al, 2015). This study presented divergence according to the literature, showing the D and A phylogroups as the most prevalent in our study. Infections caused by groups A and B1 have a better prognosis than those caused by groups B2 and D, where they present reinfection until the death of the individual (CLERMONT et al, 2012; DERAKHSHANDEH et al, 2013; CHAKRABORTY et al, 2015).

According to Nowrouzian *et al*. (2006), the phylogenetic groups B2 and D generally have virulence factors, and cause extraintestinal infections, in addition to persisting in the colonic microbiota. Our study showed a predominance of phylogenetic group D, followed by groups A and B2. Regarding the association of the virulence factors with the phylogenetic group, we can highlight that some strains of the filogroup D did not present any of the virulence factors investigated here. The small sample size examined in the present study does not allow any conclusions regarding the virulence factors associated with the phylogenetic group, and other studies are necessary to confirm this relationship. Stoppe et al. (2017) and Lee *et al*. (2016) determined that the relationship between the phylogeny and its hosts, and virulence factors is not understood, and other host-related factors must be involved (climate host, or food and habits), and other virulence factors. Further research is required to ensure the existence of patterns relating to the distribution of the phylogenetic group with different factors associated with the host and virulence genetic factors. This could facilitate the prediction of the type of *E. coli* strain that would be most prevalent in different climates or geographic locations.

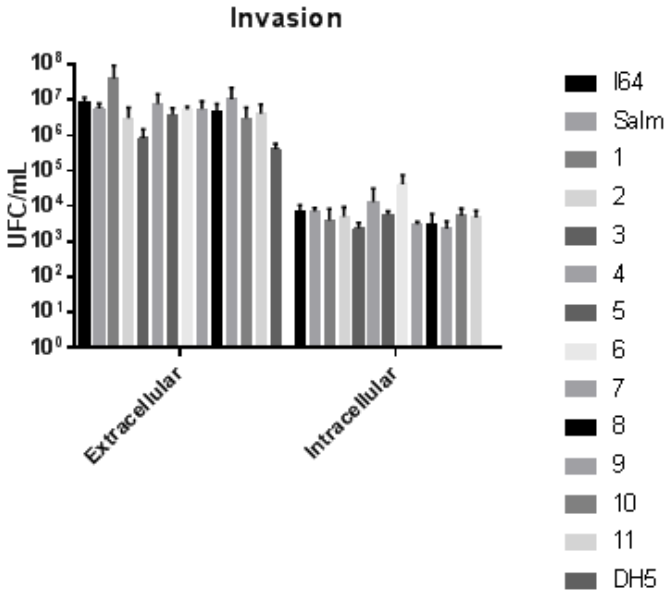
The virulence genes investigated are related to the presence of fimbriae P (*pap*); the formation of adhesins (*afa* and *sfa*); the presence of aerobactin siderophore in iron uptake (*aer*); detection of alpha-hemolysin (*hly*). In this study, the presence of pathogenicity island (*PAI*) was investigated. In these islands are located chromosomal genes that can code some virulence factors.

The results showed 36% of strains (3, 4, 6, and 11) with only one virulence factor present, eighteen percent of strains (5 and 7) with two factors, one strain (1) with four factors, and 36 % of the strains (2, 8, 9, 10) with absence of the virulence factors studied. This study emphasizes the prevalence of the *aer* gene, supporting the research of Johnson (1991) in the prevalence of this gene. Also, the association of this gene with the *pap* and *sfa* (strain 1) genes, and with pathogenicity island using *PAI* (strains 5 and 7) was reported in this study. Strain 11 presented only the *aer* gene. The presence of the gene *PAI* in strains 3 and 6 were also described. Strains that present virulence factors do not necessarily present one factor per strain, and one strain may present more than one factor or none.

Four strains (36%) did not present any of the six virulence genes investigated in this study, corroborating with Oliveira *et al*. (2011) who reported that 10% of the analyzed strains did not present positivity for the same markers used in this study. And Blanco et al. (1997) observed the absence of the *hly*, *pap*, and *sfa* genes in the strains of UPECs investigated by them. Yun (2014) presented all these virulence factors in about 64 samples of UPECs analyzed. Our study showed five isolates that were positive for *PAI* markers, and of these, three are associated with some of the virulence factors investigated.

Therefore, the capacity for adhesion and invasion of urinary tract cells was investigated. Five isolates (2, 3, 4, 7, and 10 – 46%) presented aggregative adhesion (AA) pattern on the surfaces of renal Vero cells, while the other strains (1, 5, 6, 8, 9 and 11 – 54%) presented a moderate or discrete pattern of adhesion in this cell line. However, it was not possible to associate the phylogenetic type with the adhesion pattern. Besides, the adhesion pattern was not also associated with the presence of virulence genes. Probably because the adhesion process is associated with other virulence genes not investigated in this study as adhesins and fimbriae. The same result was found when the invasion was investigated (Figure 1). The isolates were able to enter renal Vero cells but it was not associated with specific virulence genes analyzed in this study. Interestingly, the invasion percentage was higher for isolates belonging to phylogenetic groups A and D than for B2 isolates.

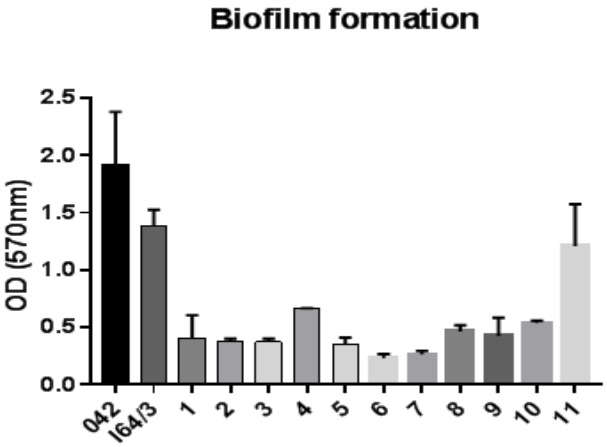
Figure 1: Extracellular and intracellular comparison in the invasion of Vero cells by *Escherichia coli* isolated from urinary infection.



Fonte: SOUZA, 2018

Biofilms are microbial communities that are adherent to each other and surfaces. The biofilms confer protection against hydrodynamic flow conditions, to the immune system of the host such as phagocytes, and antibiotic activity (HANNA *et al*, 2003). Many UTI cases are associated with biofilm formation, mainly catheter-associated urinary infections (COSTERTON *et al*, 1999). To assess whether the ability to form biofilms is associated with the adhesion, invasion, or the presence of specific virulence genes, the adherence to the abiotic surface using 96-well microtiter plates was evaluated. All UPEC strains were tested and compared with the control strains 042 and I64 and classified as non-biofilm producer OD < 0.500; weak biofilm producer 0.500 ≥ OD ≤ 1.000, or strong biofilm OD > 1.001. Almost all isolates were classified as non-biofilm formers compared with strains 042 and I64. The exceptions were strains 4 and 10 which were classified as weak biofilm producers and strain 11 which was considered strong biofilm former (Figure 2). When the biofilm results, we compared concerning the adhesion and invasion, isolate 4 presented an aggregative pattern and was able to invade Vero cells, whereas strains 10 (weak biofilm former) and 11 (strong biofilm former) presented a typical pattern and were able to invade. Therefore, the ability to form biofilm could be associated with adhesion and invasion. In addition, the presence of virulence genes investigated in this study could not be associated with biofilm formation.

Figure 2: Formation of biofilm in strains of *Escherichia coli* isolated from urinary infection.



Fonte: SOUZA, 2018

Reisner et al. (2006) described a variation of biofilm formation in strains of *E. coli*, failing to identify a biofilm association with pathogenic strains. Therefore, biofilm formation depends on several factors, such as growth medium composition, and biofilm formation surface. *E. coli* can respond in different ways to changes in environmental conditions.

CONCLUSION

Despite studies considering each urinary infection case as unique, previous UTIs can be connected. A possible explanation is that quiescent reservoirs inside the urothelium can be formed before infection. Thus, each infection can increase the risk of recurring urinary infections, corroborating with Barber et al. (2013).

Age is an important factor in increasing the prevalence of UTI in the elderly. Tavares et al. (2011), Melo et al. (2017), Oliveira and Santos (2018), and Yan et al. (2024), confirmed that with the increasing population aging, the occurrence of geriatric syndromes, among them urinary incontinence, would lead to a UTI in this age group of the population. Due to functional and structural changes in the urinary system and impairment of functional independence of the urinary system.

There is no need for the presence of all virulence factors for adhesion and bacterial invasion in host cells to cause urinary tract infection. The invasion percentage in Vero cells was higher in isolates belonging to first by phylogenetic group D (45,45%)

followed by phylogenetic group A (27,27%), with phylogenetic group B2 being the third most incident. According to Clermont et al. (2012), Derakhshandeh et al. (2013), Chakraborty et al. (2015) and Munkhdelger et al. (2017), the outcome was far better when caused by A and B1 groups than B2 and D. This study presented somewhat divergent results from the above-mentioned authors.

Uropathogenic *Escherichia coli* (UPEC) can lead to an innocuous existence until they gain access to the urinary tract, where they can cause a urinary infection (BARBER et al, 2013; NOZARIAN; ABDOLLAHI, 2015). The number of virulence factors investigated in this study was few, considering that *E. coli* has a large arsenal, which allows it to successfully colonize the urinary tract of the host. Thus, the identification of these other virulence factors becomes an advantageous tool to better understand the pathogenesis of UTIs caused by this type of bacteria, corroborating with Rozwadowski and Gawel (2022). Furthermore, national studies to evaluate the incidence and risk factors associated with UTI in the elderly population are still insufficient and further research about this topic as suggested by Melo et al. (2017) is still necessary.

Biofilm formation can be an important determinant of early adhesion and infection. Although not all virulence factors are investigated. Biofilm may function as an important attribute in the establishment of infection. However, we could not identify a biofilm association with pathogenic strains due to variations of factors involved in the biofilm formation test (REISNER et al, 2006).

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