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Original Article

UPEC strain characterization isolated from Mexican patients with recurrent urinary infections

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Abstract

Introduction: Any microorganism is capable of causing urinary tract infections (UTI). However, Uropathogenic *Escherichia coli* (UPEC) is responsible for the majority of UTI. A variety of virulence genes have been identified in UPEC. Diverse epidemiological studies support that specific subsets of genes are characteristic of each *E. coli* uropathogenic subtype involved in the development of cystitis, pyelonephritis, and urosepsis.

Methodology: Twenty-three *E. coli* strains isolated from women with cystitis, 25 from men with prostatitis were characterized according to serotype, virulence genes, PFGE profile and susceptibility antimicrobials.

Results: *E. coli* O25:H4–ST131 was more frequently isolated from cystitis than prostatitis. The majority of cystitis strains presented almost all the virulence genes, contrary to 60% of prostatitis strains. The strains characterized by serology, PFGE and MLST and virulence profile showed that cystitis strains shared a lineage with their own genomic fingerprint, indicating that these strains derived from a common ancestor. The prostatitis strains showed serotypes diverse and much higher degree of genetic diversity, indicating that they are an unrelated group. More than 50% of isolates were resistant to at least 7 of the antimicrobials tested. Antibiotic-tolerant cells were observed, presenting with more frequency in response chloramphenicol, tetracycline, trimethoprim-sulfometoxazole and dicloxicillin.

Conclusions: Persister cells can be detected from the beginning of the infection. The importance of these persister cells that exhibit multidrug tolerance is that a single surviving bacterium can initiate again an infection making it clinical relevant in the chronic urinary tract infection.

Key words: UPEC; persister cells; *O25:H4*

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Introduction

Urinary tract infections (UTIs) are among the most common bacterial infectious diseases in the general population. Women and girls over 2 years of age are the most affected, with at least 10-25% of females developing symptomatic UTI during their lifetime [1-4]. Many host factors increase UTI susceptibility, the most common being the obstruction of the normal urinary flow leading to stasis. Other factors include vesicoureteral reflux, incomplete bladder emptying, urinary tract instrumentation, an active sex life, and metabolic diseases, such as diabetes [5]. Adult women with acute cystitis-urethritis may refer suprapubic tenderness upon physical examination, with the typical signs and symptoms being acute onset of dysuria, urgency and increased frequency of urination. Fever is usually not present in uncomplicated cystitis-urethritis. Men with acute prostatitis complain of fever, chills, and both perineal and low-back pain together with dysuria and increased frequency of urination. Physical examination of the prostate in acute prostatitis patients should be avoided because of severe pain, and because manipulation of the prostate increases the risk of bacteremia [6].

Although virtually any microorganism is capable of causing UTI, Uropathogenic *Escherichia coli* (UPEC) is responsible for the majority of urinary tract infections (70-90%) [1,2,7-9]. A variety of virulence genes have been identified in UPEC encoding virulence factors, which allow these microorganisms to colonize vaginal and periurethral cells. This colonization produces infection of a specific target site through the attaching

of UPEC to the uroepithelium and invasion of the tissue. UPEC is also able to resist serum bactericide activity and phagocytosis. UPEC major virulence factors include fimbrial adhesins (type 1, P and S/F1C), toxins (cytotoxic necrotizing factor type 1 [CnF1]), hemolysins, surface structures that contribute to the evasion of host defenses (capsule and O antigen), and multiple iron acquisition systems (aerobactin, enterobactin, and those mechanisms similar to enterobactin, including iron and yersiniabactin) [10,11].

Various groups of O and H antigens have been associated with specific *E. coli* pathotypes of which the serogroups O1, O2, O4, O6, O7, O8, O16, O18, O25 and O75 have been associated with UTI [12,13].

Over the last decade, the emergence of multidrug resistance UPEC strains has made UTI treatment more problematic, this phenomenon increase rapidly due to the wide dissemination of UPEC strains particularly from ST131 clone, which harboring determinants for extended-spectrum β-lactamase (ESBLs) and resistance to trimethoprim-sulfamethoxazole and fluoroquinolones [13-15]. The antibiotic resistance is acquired through genetic changes and may be an important factor to antibiotic therapy failure, however, the presence of a subpopulation of multi-drug tolerance (MDT) bacteria could be other important cause of infections fail to clear despite of the absence of resistance organisms encouraging the recurrent urinary infections (recurrence is used for repeatedly occurring UTI without knowledge of strain similarity), such bacteria (persister cells) are typically thought to represent nongrowing, isogenic, physiological variants that gain MDT via the inactivation of antibiotic-targeted cellular processes through presumed metabolic dormancy [16,17]. In this current study, genetic and phenotypic characterization of a group of UPEC strains isolated from Mexican patients with cystitis and prostatitis was carried out. This is the first study to show that it is possible the early detection of persister cell utilizing the appropriate method of antimicrobial susceptibility, with base on results the physician would install the treatment scheme that would avoid the persistence or relapse of UTI.

Methodology

Strains

A collection of 49 clinical strains of *E. coli* were used in this study: 24 *E. coli* strains were isolated from unrelated women with cystitis, and 25 from unrelated men with prostatitis. The clinical strains were isolated from midstream urine, from February 2008 to November 2009. The background of the patients showed that they had been repeatedly treated for recurring urinary tract infections; unfortunately, the medical records were unavailable for all patients. All patients were being treated in the Urology department of a General Hospital in Mexico City (SSA, Ministry of Health). The project was approved by the Ethics Committee of the Hospital General (2008-87586, SSA, Ministry of Health). In all cases, patients or relatives were informed about the nature of the study and were asked to sign a consent form. Each strain was isolated and identified in the bacteriology laboratory of the same hospital using a MicroScan 96 WalkAway (Dade Behring, West Sacramento, USA). All strains were transported to the Bacterial Genomic laboratory (Faculty of Medicine, Universidad Nacional Autónoma de Mexico [UNAM]) where they were confirmed as *E. coli* pure culture and biochemically typed using conventional biochemical tests and the API20E system. All of the isolated bacterial strains were labelled and stored on sealed Dorset egg slants in a temperature-controlled room that houses the culture collection of the Faculty of Medicine UNAM (FMU).

Serotyping

The strains were serotyped by agglutination assay using 96-well microtiter plates and rabbit serum (SERUNAM), which was obtained against 185 somatic antigens (O) and 53 flagellar antigens (H) for *E. coli* and against 45 somatic antigens for *Shigella* species [18]. A highly sensitive and specific agglutination system involving only one antigen and its homologous antibody was used.

Virulence gene detection by PCR

Chromosomal DNA was isolated from overnight cultures of each *E. coli* strain, as well as from three *E. coli* control strains, (28E, 57E and CFT073). DNA was purified from the bacteria using miniprep (DNeasy Blood & Tissue Kit QIAGEN, Hilden, Germany) as previously described. Thirteen virulence genes of UPEC pathotype were detected: *cfn1* (cytotoxic necrotizing factor), *fimH* (mannose-specific adhesion of type I fimbriae), hly (α-haemolysin), *sfa* (S fimbrial adhesin central region), *uspA* (universal stress protein A), *papD* (P fimbriae element), *sat* (secreted auto transporter toxin), *ipaH* (invasion plasmid antigen H), *yad* (Yad fimbria), *foc* (FIC fimbria), *hofB-hofC* (encode type IV prepilin) and aerobactin. Each of these UPEC genes was amplified by PCR using specific primers (Table 1). Thermal cycling conditions for each gene were as follows: an initial denaturation cycle at

94°C for 2 minutes, followed by 35 cycles at 94°C for 1 minute, annealing temperature (according to each specific primer set, Table 1) for 1 minute and 72°C for 1 minute with a final cycle of 72°C for 2 minutes.

Pulse Field Gel Electrophoresis

Genomic DNA in agarose blocks was prepared using the method previously described by PulseNet standardized PFGE protocol, USA (National Molecular Subtyping Network for Foodborne Disease Surveillance, CDC) [19]. *XbaI* and *NotI* fragments were separated with a CHEF-MAPPER device (Bio-Rad, Hercules, USA) and electrophoresis was performed on 1% agarose gels and 0.5X TBE buffer at 14°C with pulse time ramped from 2.2 to 54.2 seconds over 20 hours and 6.0 V/cm. The sizes of XbaI and NotI fragments were estimated using *XbaI* fragments of *Salmonella* ser. braenderup global standard H9812. The images were digitized by Gel Logic 112 imaging system (Kodak, Rochester, USA). The fingerprinting profile in the PFGE gel was analyzed using the software package BioNumerics v.7.1 (Applied Maths, Sint Martens-Latem, Belgium). After background subtraction and gel normalization, the fingerprint profiles were subjected to typing, based on banding similarity and dissimilarity, using the Dice similarity coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) according to average linkage clustering methods.

Multilocus Sequence Typing (MLST)

Multilocus Sequence Typing characterization (MLST) (ST131) was performed as described previously by gene amplification and sequencing of the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) by use of the protocol and primers specified on the *E. coli* MLST website (htpp://mlst.ucc.ie/mlst/dbs/Ecoli) [20]. The allelic profiles (STs) of the seven gene sequences were obtained via the electronic database from the *E. coli* MLST website.

Statistical analysis

The study is an observational report. It is not necessary a statistical analysis. All data were reported according to frequency.

Table 1. Primers sequence used in the genetic characterization of UPEC strains

Gene	Primer sequence (5'-3')	Size of product	Tm	Description	Reference
cnf1	F - AAGATGGAGTTTCCTATGCAGGAG R - CATTCAGAGTCCTGCCCTCATTATT	498 pb	63°C	Citotoxic necrotizing factor	13
fimH	F - TGCAGAACGGATAAGCCGTGG R - GCAGTCACCTGCCCTCCGGTA	508 pb	63°C	Fimbrial subunit	13
hlyA	F - AACAAGGATAAGCACTGTTCTGGCT R - ACCATATAAGCGGTCATTCCCGTCA	1177pb	63°C	Hemolysin	13
sfa	F - GTGGATACGACGATTACTGTG R - CCGCCAGCATTCCCTGTATTC	440pb	55°C	Cold shock gene	13
uspA	F - CTACTGTTCCCGAGTAGTGTG R - GGTGCCGTCCGGAATCGGCGT	501 pb	56.5°C	Universal stress protein A	This study
papD	F - CCCTCCGGTTCAGCGCCTTG R - ACGACAGTACCGGGCGACCA	455 pb	57.4°C	Chaperon protein	This study
sat	F - GGTGAGTCCGGTGCATGGGC R - CAAGTTCCGCCTGCGGCTCA	412 pb	60.5°C	Secreted autotransporter toxin	This study
aerobactin	F- AACGGCCATCTTCCCCTGACAA R- CCACCGGAAGCTGCCAACT	490 pb	58.9ºC	Siderophore	This study
foc	F- TTACCGCTGCAGCAAGACACAAAG R- ACACCGCCGGCCCCTGATG	425 pb	58.1ºC	F1C fimbriae	This study
*hof*B	F- TCCGGTTGAGATCCCCATAGC R- ATAAGGCCGTGCGACCATAAAAAC	440 pb	59.2ºC	Type IV pilus 1	This study
*hof*C	F- AGGTATTTCCGCCGCTCTATCAGG R- AGTACGGGCAGCGCATTGTTTCT	531 pb	58.9ºC	Type IV pilus 1	This study
yad	F- CAACGGGCACAGCTATTTCCTTA R- TTCCTCCGGTAGTGTCATCTTCTG	416 pb	54.4ºC	Fimbriae	This study
ipaH	F - CATTGCCCGGGATAAAGTCAGAAC R- GGGCCAGTACCTCGTCAGTCA	400 PB	55°C	Invasion plasmid antigen H	29

Antimicrobial susceptibility

Antimicrobial susceptibility was assessed using both the agar dilution and disk diffusion method. Both methods were performed according to CLSI (Clinical Laboratory Standards Institute) guidelines [21] using Mueller-Hinton agar plates (BD Difco, Franklin Lakes, USA). The antibiotics used for both methods were as follows: Azithromycin (disks 15µg, BD BBL Sensi-Disc USA), Chloramphenicol (30µg), Ciprofloxacin (5µg), Doxycycline (30µg), Gentamycin (10µg), Imipenem (10µg), Nalidixic Acid (30µg), Tetracycline (30µg), Ticarcillin (75µg), Trimethoprim/Sulfamethoxazole (1.25µg/23.75µg), amoxicillin/clavulanic acid (20µg/10µg), ceftazidime (30µg) and nitrofurantoin (300µg). *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as controls in the susceptibility tests. Agar dilution was performed using two-fold increments (across a range of 0.125 to 512 µg/mL) of each antimicrobial agent incorporated into Muller-Hinton agar. Resistance was interpreted on the basis of the recommended breakpoints of the CLSI. A MIC value lower than the cut-off was used to indicate susceptibility and two-fold dilutions above the cut-off to determine resistance. The criterion for intermediate susceptibility was based on isolates growing within a one-fold dilution higher than the MIC value.

Bacteriological methods used in the study included the repeated streaking of single colonies and their cultivation to avoid the possibility of both contamination and mixing of the microorganisms, all of which led to the selection of pure colonies. Single colonies from each culture were selected using a stereoscopic microscope and were cultured three times to repeat the antimicrobial susceptibility test.

To determine the genotypes of the ESBLs, PCR was performed using the TEM-SHV and CTX-M specific primers, as reported previously [22].

Results

Virulence genes frequency

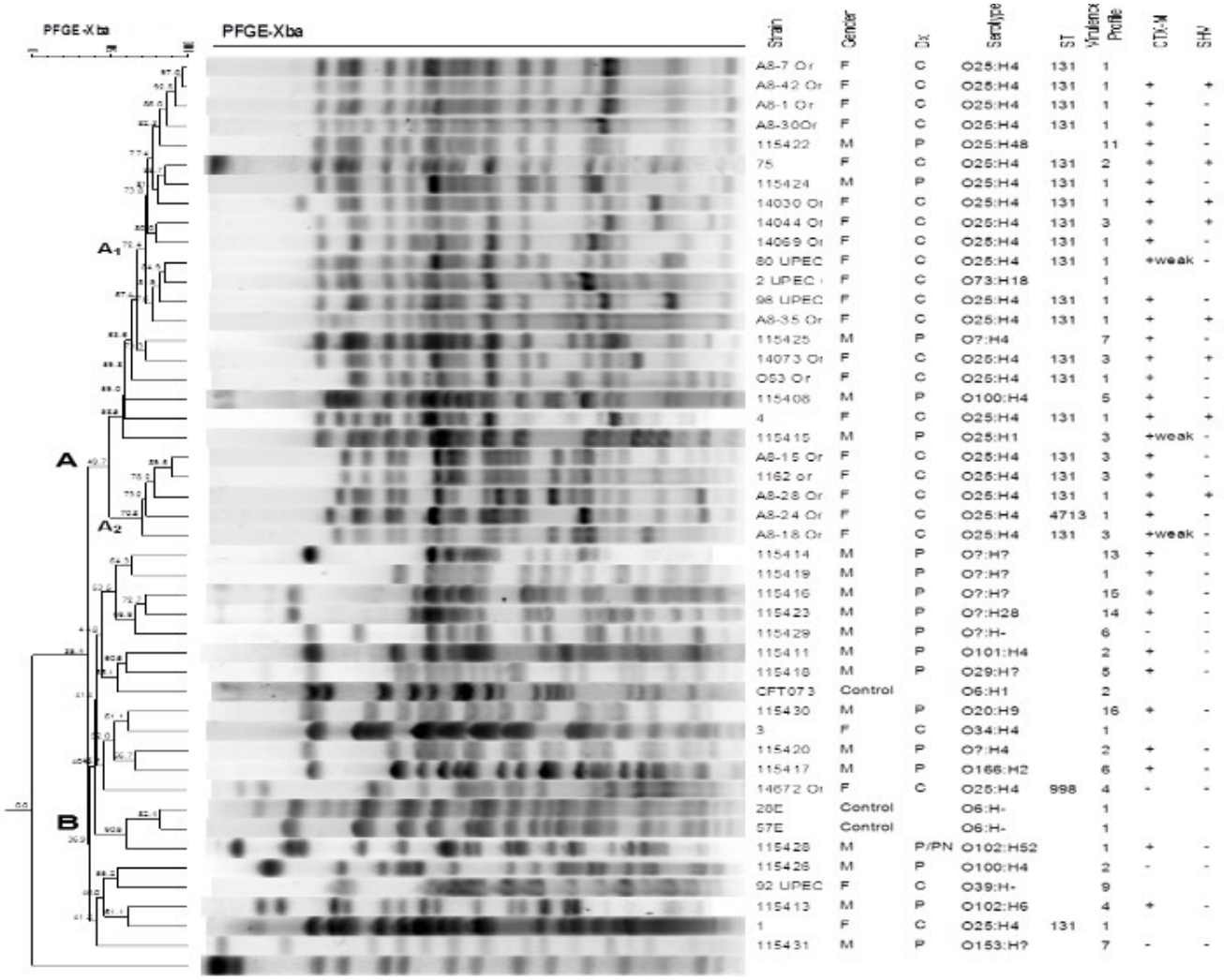
The frequency of 13 virulence genes associated with adherence, toxicity, and response to environmental conditions was identified in both strains groups. In general, the strains presented 17 different types of virulence profiles (Table 2). Of the cystitis strains, the genes *fimH*, *papD*, *sfa*, *uspA*, *ipaH*, *hofB* and *hofC* were detected in 100% of the strains; while *cnf1* and *sat* were detected in 95.6%; the aerobactin was in 91.3%; *hly* was detected in 73.9%; and the *yad* and *foc* genes only were detected in 8.6%, that correspond to 2 strains. With respect to prostatitis strains, *hofB* and *hofC* were detected in 100%; *papD* and *sfa* in 96%; *fimH* in 84%; *ipaH* in 72%; *cnf1*, *sat* and *uspA* in 64%; aerobactin in 52%; *hly* in 44%; and the *yad* and *foc* genes were not detected in some strain. Suddenly we amplify a product of 365bp by PCR using specific primers for *ipaH* gene. To confirm or discard the presence of *ipaH* gene in our

Table 2. Virulence profiles present in Uropathogenic *E. coli* strains isolated from cystitis and prostatitis.

Virulence profile	Genes												
	*fim*H	*cnf*1	*pap*D	*sat*	*sfa*	*usp*A	*hly*	*ipa*H	*yad*	*foc*	*hof*B	*hof*C	aerobactin
1	+	+	+	+	+	+	+	+	-	-	+	+	+
2	+	-	+	+	+	+	+	+	-	-	+	+	+
3	+	+	+	+	+	+	-	+	-	-	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	-
5	+	-	+	+	+	+	-	+	-	-	+	+	-
6	+	+	+	+	+	+	+	-	-	-	+	+	+
7	+	+	+	+	+	+	+	-	-	-	+	+	-
8	+	+	+	-	+	+	+	+	-	-	+	+	-
9	+	+	+	+	+	+	+	+	-	-	+	+	-
10	+	+	+	-	+	-	-	+	-	-	+	+	-
11	-	-	-	-	+	-	-	-	-	-	+	+	+
12	+	+	+	+	+	+	-	-	-	-	+	+	-
13	+	+	+	-	+	+	+	-	-	-	+	+	-
14	-	+	+	-	-	+	+	+	-	-	+	+	+
15	-	+	+	-	+	-	-	+	-	-	+	+	-
16	-	-	+	-	+	-	-	-	-	-	+	+	-
17	+	+	+	-	+	+	-	-	-	-	+	+	-

* The virulence profiles were assigned randomly based on presence or absence of each virulence gene studied. The virulence genes were detected by PCR using specific primers.

Figure 1. Pulse-Field gel electrophoresis (PFGE) profile dendrogram and genetic and phenotypic characteristics of uropathogenic *E. coli* (UPEC) isolated from patients with cystitis and prostatitis.
The dendrogram was generated by Dice similarity coefficient and UPGMA clustering methods by using PFGE images of *Xba*I digested genomic DNA. The scale bar shows the correlation coefficient (%). Upper case letters (A-B) represent the two generated clades. A1 and A2 letters represent strain subgroups. Isolate designation, patient ´s gender, diagnosis (Dx [c: cystitis; p: prostatitis]), serotype, ST, virulence profile and beta lactamase type are shown on the right.

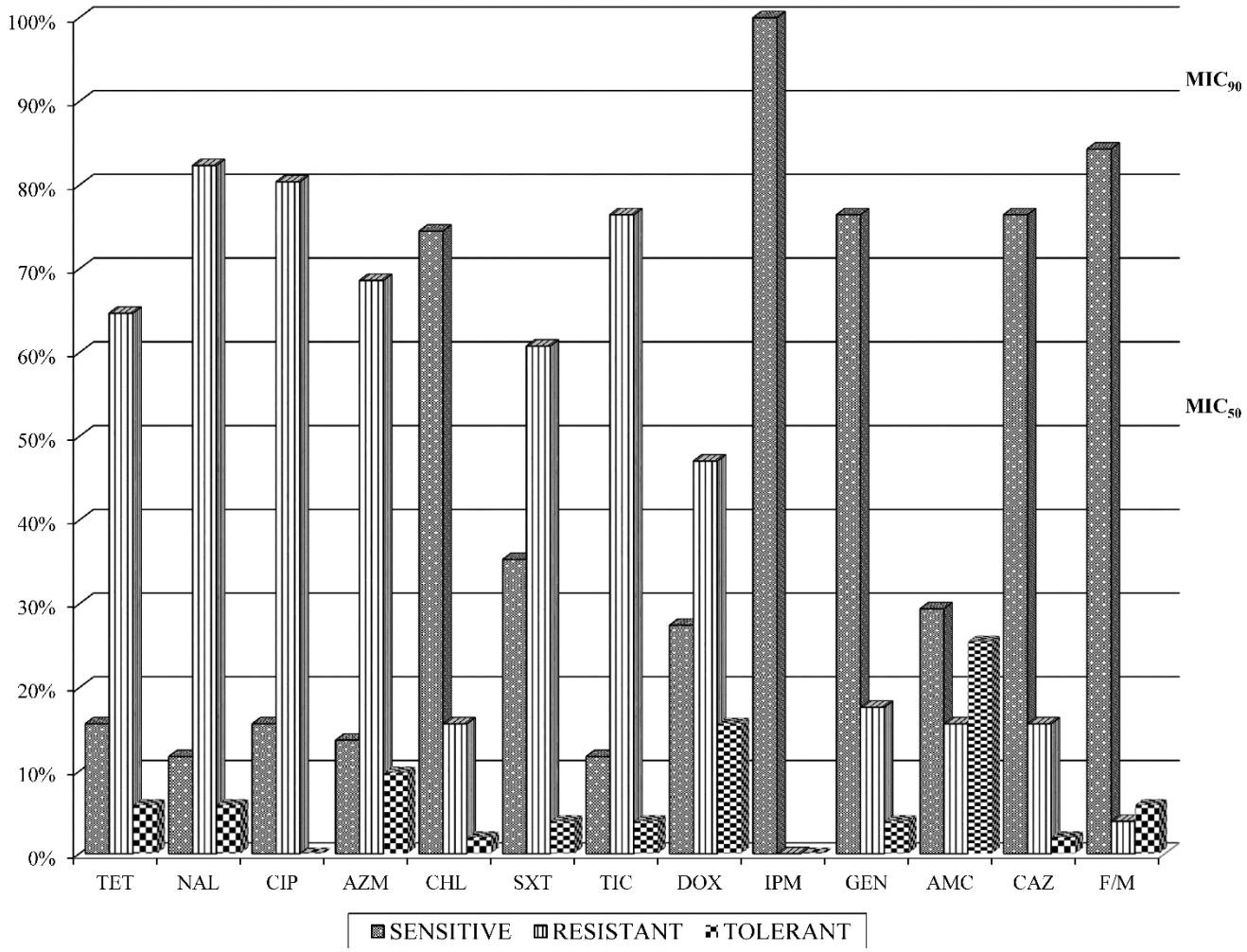


UPEC strains, the product was sequenced. Sequence analysis showed amplification of *eutE* gene (GeneBank ID submission: 1845067, accession number KT823699 and KT823700), which form part of *eut* operon for ethanolamine utilization [23].

Serotyping

Of the 24 strains isolated from women with cystitis, 21 strains were O25 serogroup in combination with H4 flagella antigen; one strain presented the O34:H4 serotype, while the remaining two strains were O39:H- and O73:H18. The strains isolated from men with prostatitis presented more serotypes. Although, we used the most complete serotyping scheme so far available, three strains could not be characterized in both the O and H antigens, other 4 strains could not be characterized in the O antigen and 2 more in the H antigen. Three strains were of O25 serogroup, 2 strains O100 and another 2 strains of O102 serogroup. Each of the O20, O29, O101, O153 and O166 serogroups was present in one strain. The H4 flagella antigen was detected in 6 strains and one of these presented the O25:H4 combination, 3 were in combination with the O100 or O101 serogroup, and the remaining 2 could not be characterized in the O antigen. Other flagella antigens present in the prostatitis strains were H1, H2, H6, H9, H28, H48 and H52 (Figure 1). Additionally, one strain was not mobile.

Figure 2. Histogram showing antimicrobial susceptibility and resistance profile of Urophatogenic *E. coli* strains isolated from cystitis and prostatitis. Antimicrobial susceptibility was assessed by the disk diffusion and agar dilution methods. Both were performed according to CLSI. Each bar represents the percent of resistance, susceptibility and tolerance of all UPEC strains for each antibiotic.



Multilocus Sequence Typing (MLST)

To assess the phylogenetic relationship more rigorously within the clonal group O25:H4, which presented non-identical PFGE profiles and to determine the ST type, we decided to carry out multilocus sequence typing analysis. The majority of the strains were ST131, except two, which one was ST998 and the other was ST 4713. The latter was an ST that had not been reported previously and, which present a change of nucleotide (g-t) at the position 430 of the *mdh* gene.

Macrorrestriction analysis

Results using *Not* and *Xba* indicated that each strain tested has a single fragment pattern. There were 6 strains (115406, 115409, 115410, 115412, 115422.2 and 115423.2) isolated from prostatitis patients that were not cut by either of these two enzymes. The *Xba* results were distributed in two major clades (A and B) (Figure 1). Clade A grouped 25 strains, with the majority of them (21) of serotype O25:H4 -ST131 (Figure 1). The clade A was subdivided into two compact subgroups A1 and A2 with 14 and 5 strains respectively. Subgroup A1 presented a Dice similarity index >72% among the strains. This subgroup was formed by 11 strains of serotype O25:H4 -ST131 and one strain serotype O73:H18, all isolated from women with cystitis and two strains of serotypes O25:H4 -ST131 and O25:H48 isolated from patients with prostatitis. Subgroup A2 presented a Dice similarity index >70% among the strains, corresponding to 5 strains serotype O25:H4 (all except one were ST131,

with the other being a novel ST4713). These strains were isolated from women with cystitis. Of them, 3 of the 5 strains presented a virulence profile type 3 and the other 2 a virulence profile type 1 (Figure 1). This ~~sub~~group also included a group of 6 strains belonging to a wider group of serotypes (O25:H1, O100:H4, O25:H4, O?:H4) and virulence profiles (type 3, 5, 1, 7). Clade B comprised 18 strains. Of these, 14 were isolated from men suffering from prostatitis, 4 from women with cystitis, and three control strains. This cluster was also more diverse with a Dice similarity index <45% among the strains (Figure 1). Of the strains isolated from women with cystitis, two belonged serotypes O34:H4 and O39:H- and two to serotype O25:H4 with one of them belonging to ST998. The strains isolated from men with prostatitis belonged to a greater diversity of serotypes and virulence profiles. Six strains could not be typed for an O antigen and 5 strains had an unidentified H antigen. One other strain was non motile (H-).

In conclusion, the genetic characterization of the strains isolated from cases of cystitis were shown to share a defined number of bands in the chromosomal profiles, apart from the serotype, the ST 131 and their 1 and 3 virulence profiles. This indicates that the strains are highly related and shared a lineage with its own genomic fingerprint. Meanwhile, the prostatitis strains presented a much higher degree of genetic diversity suggesting that this infection type is produce by unrelated strains of diverse serotypes.

Antimicrobial susceptibility

In terms of resistance, approximately 68% of the cystitis strains were resistant to more than 4 antimicrobials and 57% isolates from prostatitis were resistant at least 7 of the antimicrobials tested. The majority of strains (94%) were resistant to nalidixic acid independent of the source of isolation. On the other hand, all strains of both groups were sensitive to imipenem (Figure 2). In addition, more than 81% of the strains isolated from cystitis were sensitive to chloramphenicol, gentamycin, ceftazidime, and nitrofurantoin, while the isolates from prostatitis showed a sensitivity of 50%, 61.4%, 50% and 65% to chloramphenicol, gentamycin, ceftazidime, and nitrofurantoin respectively. From 68% to 90% of the strains from cystitis were resistant to trimethoprim-sulfamethoxazole, ticarcillin and ciprofloxacin. Regarding prostatitis strains, from 57% to 96% were resistant to tetracycline, azithromycin, doxycycline, ticarcillin and ciprofloxacin (Figure 2).

Meanwhile, 18 and 12 strains isolated from cystitis and prostatitis respectively, showed clonal subpopulations with a heterogeneous response (drug susceptible or tolerant phenotype) to one or multiple antibiotics. Initially, in both susceptibility assays, several isolates showed susceptibility to a specific drug with complete inhibition of their growth and apparently death, but several hours (12-24 hours) after, we observed the regrowth of individual colonies within the zone of inhibition produced by the antibiotic disk in the diffusion assay (Figure 3) and on agar plates with the antibiotic concentration of cut-off recommended by CLSI (which had been previously read as sensitive). These observations showed the presence of persister cells, organisms that survive exposure to a given cidal antibiotic or drug and have the capacity to revive (regrow or resuscitate) and grow under conditions without stress (antibiotic) [16].

To confirm the presence of subpopulations of persistent cells with drug-tolerant phenotype to

Table 3. List of Uropathogenic *E. coli* strains that presented clonal subpopulations (persister cells) with heterogeneous response to one or multiple antibiotics.

n	CHL	F/M	TE	CAZ	DOX	GM	STX	NA	TIC	IMP
A8-1	+	+								
A8-7	+									
A8-18	+		+	+						
A8-24	+				+					
A8-28	+				+					
A8-30		+								
A8-35					+					
A8-42	+					+	+			
2			+		+					
4			+		+					
80			+		+					
1162		+			+					
14030	+									
14069	+		+				+			
14073			+							
14672								+		
053									+	
115424							+			
115422		+					+			
115426				+						
115408							+			+
115417						+				
115413							+			
115423			+		+		+			
406			+							
412	+		+							
115414	+		+		+					
115416	+									
115430							+			
92	+	+	+	+	+	+				+

Abbrevations: CHL (Chloramphenicol), F/M(nitrofurantoin), TE (Tetracycline), CAZ (ceftazidime), DOX (Doxycycline), GM (Gentamycin), STX (Trimethoprim/Sulfamethoxazole), NA (Nalidixic Acid), TIC (Ticarcillin) and IMP (Imipenem).

different antimicrobials, three single colonies of each subpopulation derived from the first assay for both methods were selected with the aid of a stereoscopic microscope. Each colony was streaked on Mueller-Hinton agar plates (BD Difco, USA) to recheck the purity of the colony (each colony was worked again and confirmed as *E. coli* pure culture according to biochemical tests and each was newly characterized according to its serotype, virulence genes and PFGE profile) and reassess the antimicrobial susceptibility assays again, repeating the assays for three continuous. First, each surviving cell was recovered and grown in the absence of antibiotic, observing that each colony grew in number during the growth phase. Second, the antimicrobial susceptibility results showed that the subclones derived from a single colony (persister cell) maintained their growth behavior to the same antimicrobial, as was previously characterized (Table 3). In addition, approximately the half of the strains (16) with persistent phenotype showed multidrug tolerance (chloramphenicol, doxycycline, gentamycin, imipenem, tetracycline, trimethoprim/sulfamethoxazole, ceftazidime and nitrofurantoin) with a high degree of phenotypic variability, switching phenotype from sensitive to tolerant and from tolerant to sensitive to the same antibiotic in the different assays (Table 3).

The PFGE profile of each strain (persister cell) showed identical fingerprints to parent cell of which it was derivate (Figure 3), consequently the virulence profile and serotype was the same for each group of strains.

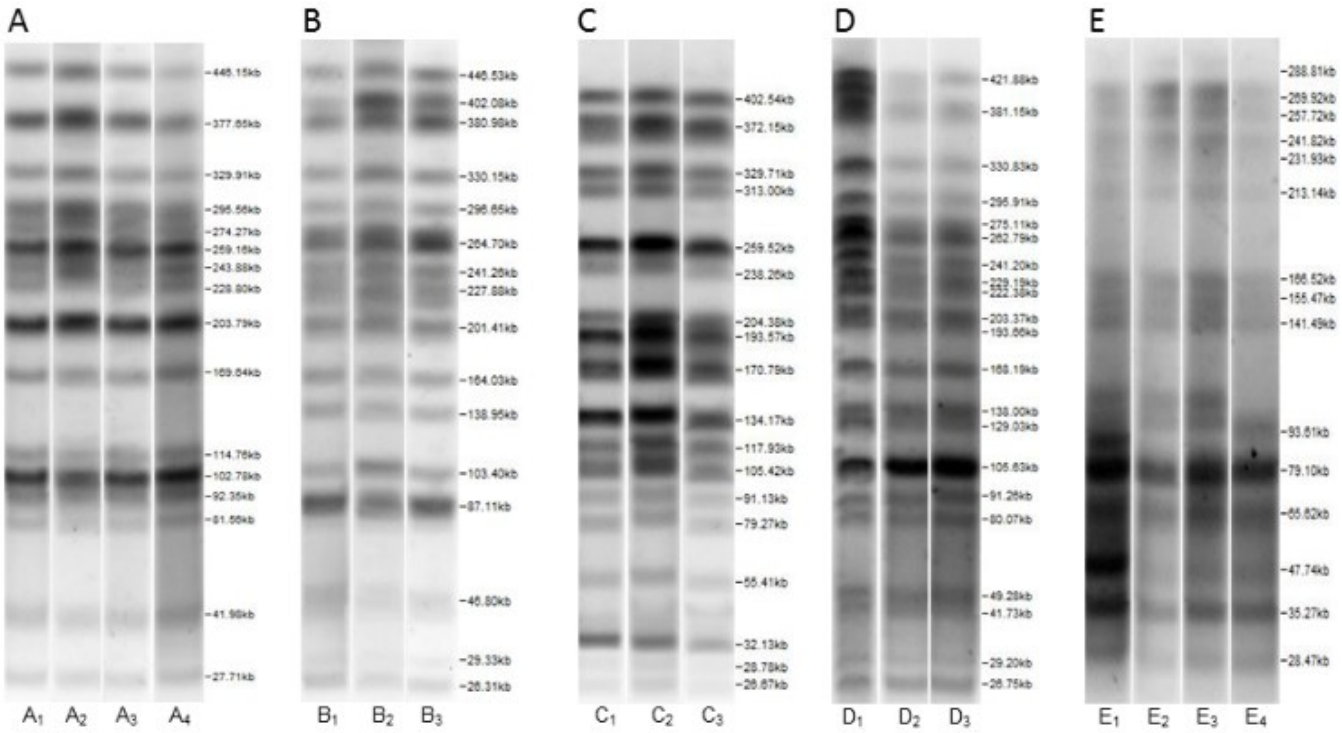
ESBLs detection

The *ctx*-M gene was presented by 100% of *E. coli* O25:H4 ST131 but only 33% presented SHV. In this study, the *E. coli* O25:H4 ST998 strain did not produce amplification of the PCR product with specific primers for both genes, indicating that this strain is not a β-lactamase producer.

Discussion

In the current study, a group of strains isolated from women with cystitis and from men with prostatitis was characterized phenotypically and genetically. The majority of the cystitis and prostatitis strains presented

Figure 3. The groups A, B, C, D and E shown PFGE profiles from parents Urophatogenic *E. coli* strains and their persister cells subclones obtained under antibiotic stress. Each group (A, B, C, D and E) is formed by the profile of the original cell (strains: 14069, A8-1, A8-28, 115408 and 115423 respectively) and by the profiles of two or three persister cell subpopulations derived of each original strain. A1, B1 and C1 show the chromosomal profiles of the original strains (parental strains) isolated of three patients with cystitis; D1 and E1 show the chromosomal profiles of the original strains (parental strains) isolated of two patients with prostatitis. A2, A3 and A4 show the restriction profiles of the persister cell subclones derived from the strain 14069; B2 and B3 show the restriction profiles of the persister cell subclones derived from the strain A8-1; C2 and C3 show the restriction profiles of the persister cell subclones derived from the strain A8-28; D2 and D3 show the restriction profiles of the persister cell subclones derived from the strain 115408; and E2 and E4 show the restriction profiles of the persister cell subclones derived from the strain 115423. In all cases, the parent cell and their persister cells showed identical chromosomal pattern.



virulence genes related to colonization of uroephitelium. In both disorders, the infection was confined to bladder and prostate epithelium, where the bacterial adherence factors like-mannose-specific adhesion of type I fimbriae, P fimbriae, and other fimbriae (such as S, and type IV pilin) play an important role for the colonization of the urinary epithelium and stimulation of a local inflammatory response.

However, *yad* and *foc* genes were not detected in our strains as expected, since they have been documented involved in the virulence of the disease and are specific to uropathogens. The *foc* operon encoding F1C fimbria and *yad* for Yad fimbriae are common to CFT073, EDL933, and MG1655 strains, both genes play an essential role in the pathogenesis of urinary tract infection [14,25]. The presence of the genes associated with toxicity (*cnf1*, *sat*, *uspA*, *hly*) in the cystitis strains showed difference in frequency of them between those strains isolated from prostatitis. Various epidemiological studies support the notion that specific subsets of genes are characteristic of each of the *E. coli* uropathogenic subtypes involved in the development of cystitis, pyelonephritis, and urosepsis [10,11,24,25]. There are reports showing that cystitis isolates differ specifically from prostatitis isolates due to the lower prevalence of *hlyA* and *cnf1* [26,27,28] and that prostatitis isolates have significantly higher aggregate virulence factors scores than cystitis isolates. In the current study, these differences were found, but contrary to those former reports, the cystitis strains of our population presented higher frequency of *hly*, *pap*, *sat* and *cnf1* than prostatitis isolates. The results of current study show to O25:H4–ST131 strains as a homogeneous population with a genetic content uniformly present. In contrast to previously reported, our O25:H4 –ST131 strains show specifically a higher frequency in the genes encoding for HlyA and Cnf1 toxins [26].

The ability to inhabit different niches during an ascending urinary tract infection and to cause particular pathologies at each site resides largely in the island genes specific to Uropathogenic *E. coli* [24,25]. However, the importance of other virulence factors should also be regarded. We know that apart from *Shigella* spp., the only other organism that is known to have *ipaH* are enteroinvasive strains of E. coli (EIEC) [29]. Interestingly, the specific primers for *ipaH* gene amplified *eutE* gene in our UTEC strains; this finding, to our knowledge, has not been formerly reported in uropathogenic *E. coli* before. The sequencing of PCR products showed that both forward and reverse primers present in their 5' and 3' ends have a homology with the *eutE* gene sequence, allowing the alignment of both primers and the product amplification. The *eutE* gene forms part of the *eut* long operon, which participates in the utilization of ethanolamine [23]. We do not know the role of this gene in the pathogenesis of urinary tract infections by UPEC strains. However, the upregulation of *eut* genes during a variety of host-pathogen interactions provides additional evidence that suggests a role for ethanolamine utilization in bacterial pathogenesis. It is unknown whether ethanolamine is simply a valuable nutritional source of carbon and /or nitrogen in a range of host environments or whether the ability to metabolize this compound contributes to host invasion or immune evasion by a more specific mechanism [23]. Further studies need to be done to determine the role *eut* operon in UPEC related urinary tract infections.

In the present study we found that the most of cystitis strains were *E. coli* O25:H4 -ST131 and the great majority are producing beta lactamases. Although, the majority of *E. coli* ST131 exhibits serotype O25:H4, a small subset exhibits serotype O16, in the current study we did not detect this last serotype among our strains. Nevertheless, the multilocus sequence typing analysis showed the presence of a novel ST (ST4713) not reported previously, which presents a change of nucleotide (g-t) at the position 430 of the *mdh* gene.

E. coli O25:H4-ST131 has been described as an important human extraintestinal pathogen responsible for community- and hospital-acquired urinary tract and bloodstream infections [14,15,30,31,32,33]. This strain is found globally and produces extended spectrum β-lactamase. It has also shown fluoroquinolone antimicrobial resistance, as well as co-resistance to aminoglycosides and trimethoprim-sulfamethoxazole [34]. There are some reports of *E. coli* ST131 strains resistant to carbapenems [35]. In the present study, our strains showed high resistance to ciprofloxacin, medium resistance to trimethoprim/sulfamethoxazole and little resistance to Gentamycin. But all the strains were sensitive to imipenem, although, was detected a subpopulation of persister cells of a culture from cystitis and one from prostatitis that showed tolerance to imipenem.

The analysis of chromosomal profiles by PFGE, serology, ST and virulence profiles of our strains suggests that cystitis isolates are closely related between them and were derived from a common ancestor. Further studies will be necessary to confirm or discard that the *E. coli* O25:H4 ST 131 strains of this study correspond to same clone circulating in different countries in the world [13], but what is striking is that

E. coli O25:4 ST131 has been isolated almost exclusively from samples of cystitis [34] from women. It is important to study a large number of cystitis strains from women as also from men in Mexico to determine if this *E. coli* type with the genetic characteristic found in this study, occurs only in cystitis isolates in both genders or exclusively in females, or is associated to recurrent urinary infection.

The antimicrobial susceptibility assays showed the emergence of clonal subpopulations with heterogeneous phenotypes to antibiotic stress. This phenomenon was observed when single colonies regrew a few hours after having declared an antibiotic sensitive strain tested. A single colony was selected and the susceptibility assay using the same antibiotic was repeated. This time, we observed at the beginning that the majority of cells no grow, but a small proportion of cells survived up to form a new colony. The key signature of persistence is the biphasic kill curve obtained when bacteria in batch culture are exposed to a bactericidal antibiotic: the killing rate is initially very high but then slows and may even level off to zero [17]. This phenomenon was first described in the 1940s, when Staphylococcal infections were seen to recur, even after extensive treatment with high doses of penicillin [36]. These types of bacteria were termed "persisters" and they have strong clinical implications in a number of infectious diseases, such as tuberculosis, syphilis and typhoid fever, where the immune system proves ineffective and a single surviving bacterium is capable of initiating infection [37]. Approximately 30% of urinary tract infections become recurrent in women [7]. The ST131 has been associated with having recurrent or persistent symptoms [38]; which is likely attributable to receiving inappropriate empirical therapy as FQs, which are largely ineffective against this clonal group. The results of present study show that this recurrence could be due to the existence of persister cells. Once the antibiotic is withdrawn, the persisters cells grow and repopulate the urinary niche leading to a recurrent infection. Recurrent infections, in turn, generate antibiotic-sensitive cells, as well as antibiotic-tolerant persisters. The infection is perpetuated in this manner in spite of prolonged therapy. Therefore, the treatment with one antimicrobial will be successful during the initial stages of the infection, killing rapidly growing bacteria (exponential growth), but it will be less efficient with slowly growing bacteria, and non-growing bacteria (stationary phase) will not be affected. The kill efficacy of antibiotics depends on the physiological state of the bacterium. Unlike resistance, which is genetically acquired and passed on to subsequent generations, persistence is a transient phenotype, which seems to be in an arrested growth state, which could spontaneously switch to fast growth and generate a population that is sensitive to the antibiotic with a small proportion of tolerant antibiotic cells. Numerous factors have been proposed as being responsible for the persistence phenotype occurring independently of stable genetic changes [39]. Recent research has shown that persistence of the model organism *Escherichia coli* depends on toxin-antitoxin (TA) loci [17,39,40,41] where the overproduction of toxin encoded by TA loci induces a persistence-like state from which the cells could be resuscitated by the induction of antitoxin gene transcription. A better understanding of the mechanisms behind bacterial persistence may lead to the design of drugs to combat chronic and recurrent infections, such as recurrent urinary infections.

Conclusions

Uncomplicated urinary tract infections are among the most prevalent infectious diseases that affect women. The results of this study show that *E. coli* O25:H4 ST131 is frequent in women with cystitis in Mexico. Being able to identify the most common type in a specific population makes it more attractive to both vaccine producers and health authorities to develop a vaccine and introduce large-scale vaccination programs as a preventive strategy. It is important to determine at the beginning whether the UTI is caused by sensitive, persister, or resistant strains, since the study of persistence has revealed the inherent non-genetic heterogeneity of bacterial populations, which might constitute a general adaptation to variable environments. The presence of persistent cells in an infection has clinical relevance, since this type of cell perpetuates the infection. This is the first study that show multidrug-tolerance profiles of different strains, which can be detect from primary isolation using the appropriate antimicrobial susceptibility method. Effective therapy of bacterial infections should not only aim at eradicating antibiotic-sensitive pathogens but also at eliminating persisters cells or preventing their emergence.

Bacteriology laboratories should return to the routine practice of carrying out antimicrobial susceptibility tests that allow persistent cells to be detected and should not just rely on automated methods, since these do not detect different bacterial subpopulations within an apparently pure culture.

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