The role of bacterial hypermutation in biofilm formation and antibiotic resistance in urinary tract infections caused by pathogens of the *Enterobacteriaceae* family

PhD Thesis

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Szeged

2014

1. List of publications concerning the subject

I. **Kovacs B**, Le Gall-David S, Vincent P, Le Bars H, Buffet-Bataillon S, Bonnaure-Mallet M, Jolivet-Gougeon A.

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FEMS MICROBIOLOGY LETTERS 347:(2) pp. 116-122. (2013) IF: 2.049

II. Jolivet-Gougeon A[¥], **Kovacs B**[¥], Le Gall-David S, Le Bars H, Bousarghin L, Bonnaure-Mallet M, Lobel B, Guille F, Soussy CJ, Tenke P.

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2. Table of contents

3. **Introduction**

Eradication of infectious diseases is constantly challenged by micro-organisms that develop new survival strategies. Previous studies suggest that mutational events play a predominant role in bacterial adaptation and confer a selective advantage [1, 2]. Early experiments detecting mutators used mutagenized laboratory strains of bacteria, which coupled with different selection strategies. LeClerc et al reported high mutation frequency among *Escherichia coli* and *Salmonella* pathogens, challenging the theory that mutators were rare among bacterial populations [3]. These findings demonstrated that natural populations could respond to environmental selection in two ways, i.e. by enhanced mutation frequencies and by recombination.

Proteins involved in the DNA mismatch repair pathway (methyl-directed mismatch repair (MMR)) help to replace nucleotides introduced erroneously into the replicated DNA and also hinder recombination between non-identical DNA sequences. Deficiencies in any of the DNA mismatch repair pathway mechanisms can lead to a hypermutator phenotype. Transient mutator status, which involves reversion or recombination within the mutator alleles or depletion of the MMR system proteins, allows the organism to temporarily benefit from the elevated mutation frequency for adaptation while reducing the risk of accumulating deleterious mutations. Using a mathematical model, Rosche & Foster (1999) showed that transient hypermutators play a role in adaptive mutation in *E. coli* [4].

The idea to investigate the association between hypermutation and biofilm forming capacity as well as antibiotic resistance in urinary tract infections (UTI) was based on several observations. The elevation of antibiotic resistance is a global problem, which affect most of the antibiotics used in UTI. *E. coli* hypermutators more frequently found in UTI than in other origin [5]. Both biofilm formation and acquisition of antibiotic resistance in urinary tract infections have an extensive literature. There is one clinical entity, in which significant relation between hypermutation and antibiotic resistance or between hypermutation and biofilm exists, namely cystic fibrosis (CF) lung infection. In CF the alveoli and bronchioli are blocked by a thick secretion, which is colonized and infected by bacteria. This mucus leads to the formation of

bacterial microenvironments known as biofilms in the long run, which protect the pathogen against the penetration of antibiotics making infection even more difficult to eradicate.

Chronic infection, bacterial adaptation, presence of persisters and transient mutators, protection against antibiotics and antibiotic pressure are the phenomena which are both present in CF lung infection and in UTI, making these two entities similar in a certain respect. This gave me the idea to investigate hypermutation, biofilm formation and antibiotic resistance in the context of urinary tract infections.

3.1 Hypermutation

3.1.1 Molecular mechanisms of hypermutation - DNA repair

Siegel & Bryson discovered the mutS gene in an azaserine-resistant derivative of *E. coli* had a mutator phenotype and carried a deletion in the mutS gene [6]. The majority of naturally occurring strong mutators have defects in the MMR system; the mutations are mainly in mutS [7], but deletions in genes encoding beta-clamp proteins [8] and in mutH, mutL and mutU (uvrD) have also been described (Figure 1.).

Inactivation of basal excision repair genes, e.g. mutY, mutM, mutT, can also cause a major increase in mutation rate. MutY, MutM and MutT reduce the level of 8-oxo-dG, and MutD supplies proofreading activity. In *Bacillus anthracis*, the mutY and mutM single knockouts are weak mutators by themselves, but the mutY mutM combination results in very high mutation rates due to G: CAT : A transversions [9]. Bacterial strains that are unable to perform any of these activities are unable to repair mismatches and, consequently, their mutation frequency increases.

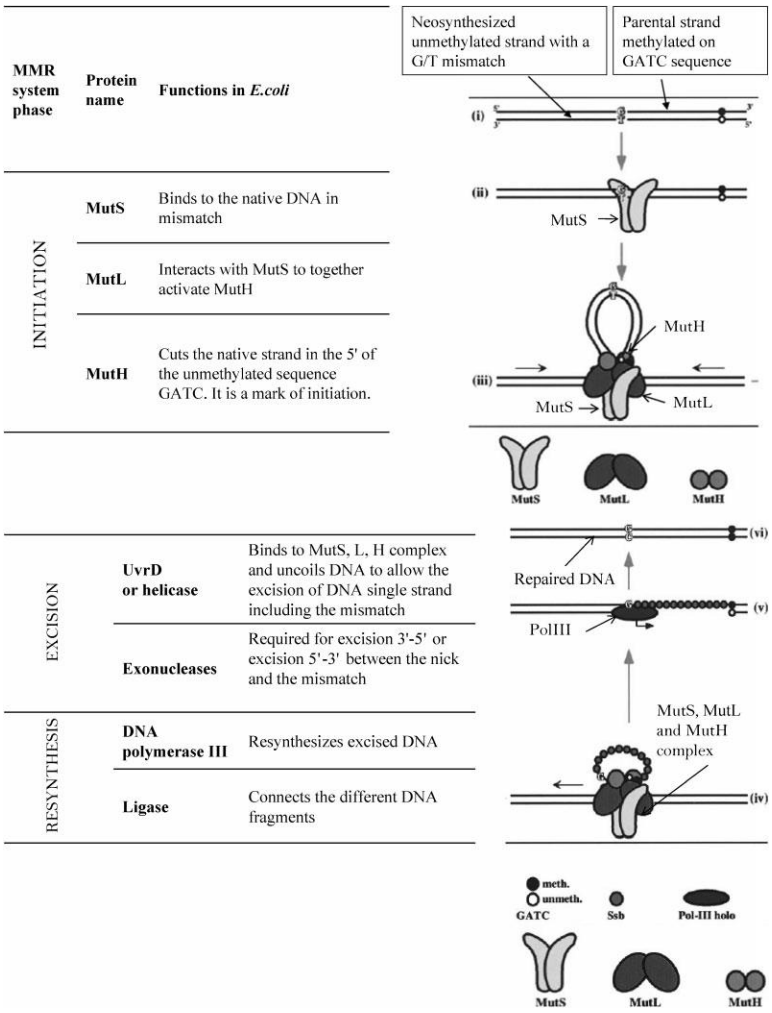


Figure. 1. MMR system DNA repair mechanism. Adapted by permission from Macmillan Publishers Ltd: EMBO J (Jiricny, 1998), copyright 1998; and by permission from Annual Reviews (Schofield & Hsieh, 2003). The initiation of mismatch repair requires MutS, MutL and MutH; the latter generates a nick in the unmethylated strand of a nearby hemimethylated d(GATC) sequence. Both MutS and MutL are dimeric ATPases: MutS recognizes and binds the mismatch and recruits MutL, which binds the MutS– mismatch complex. The endonuclease MutH is stimulated to catalyze endonucleolytic cleavage at the unmethylated d(GATC) site in the presence of MutL and MutS. DNA unwinding is then initiated at the nick by DNA helicase II (UvrD) and the repair itself is catalyzed by the DNA polymerase III holoenzyme.

Mismatch repair systems are highly conserved in bacterial populations and evolved from common ancestors, i.e. the Hex system in Gram-positive *Streptococcus pneumoniae* and the Mut system in Gram-negative *E. coli* and *Salmonella enterica serovar Typhimurium*. There is homology between HexA and MutS and between HexB and MutL in *Streptococcus pneumoniae* [10, 11] and *Saccharomyces cerevisiae* [12].

Disruptions of genes other than mut can also result in a hypermutator phenotype. For example, DNA adenine methylation by DNA adenine methyltransferase (Dam) plays an important role in DNA replication, gene expression regulation and DNA methylation for strand discrimination during mismatch repair [13]. Strains deficient in Dam are hypersensitive to DNA-damaging agents or reactive oxygen species [14]. Insertional inactivation of dam and/or drg (damreplacing genes) results in hypermutator phenotypes [15].

Other mutants with inactivated DNA repair genes have also been reported. These include isolates with inactivation of the alkylation damage repair gene ada/alkA in several *Mycobacterium tuberculosis* isolates and in one *Mycobacterium bovis* strain [16] and *E. coli* isolates with an inactivated dnaQ gene, which encodes the epsilon subunit of DNA polymerase III [17].

Recently, Rodríguez-Rojas & Blázquez looked for additional genes involved in hypermutability by screening a *Pseudomonas aeruginosa* library with random insertions. Mutational inactivation of pfpI, which putatively encodes a member of the DJ-1/ThiJ/PfpI superfamily, was found to confer a hypermutator phenotype [18].

3.1.2 Molecular mechanisms of hypermutation – Recombination

Recombination requires the genetic recombination genes recB, recC, recD and recA, which are part of the bacterial RecBCD recombination system. These proteins generally require near-perfect homology between the two complementary DNA strands, but this requirement is greatly relaxed in MMR-deficient mutants. Recombination-deficient recA and recB null mutant strains are deficient in adaptive reversion. A hyper-recombinogenic recD strain is hypermutable, and its hypermutation depends on functional recA and recB genes (Harris et al., 1994) [19]. Worth et al.

(1994) showed that MutS abolishes recA catalyzed strand transfer between the DNA of the fd and M13 bacteriophages, which vary by 3% at the nucleotide level, but has no effect on M13–M13 or fd–fd exchange [20]. Although MutL has no effect on M13–fd heteroduplex formation, the protein dramatically enhances inhibition of MutS-mediated strand transfer. Analysis of strand-transfer intermediates that accumulate in the presence of MutS and MutL indicates that the proteins block branch migration, presumably in response to mispairs within the newly formed heteroduplex. Transduction between *Salmonella typhimurium* and *Salmonella typhi* is blocked by the activity of the recipient's mismatch repair system, which senses sequence divergence and disrupts heteroduplexes in favor of recipient sequences [21].

3.1.3 Determination of mutation frequencies

All the mutations present in a given population at a chosen time can be measured by determining the mutation frequency in distinction to the "mutation rate", which is frequently used in the same manner, but refers to the rate of mutation within the genome per generation [22]. Usually the mutation frequency of a bacterial population is of the order of 10^6 to 10^7, but numerous studies have described organisms which exhibit permanent mutation frequencies 10-1000-fold or even higher than the median value of their bacterial population, including clinical pathogens and animal sources [5] [23] [24]. These clones are termed as hypermutators. In order to better define the medium value of mutation frequencies for bacteria with a hypermutator phenotype, polymorphisms in rifampicin resistance genes have been studied by Baquero et al, who defined 4 categories of *E. coli* strains according to their findings on mutation frequencies (f), and named them as hypomutable ($f \leq 8x10^{-9}$), normomutable ($8x10^{-9} < f < 4x10^{-8}$), weak mutator ($4x10^{-8} \leq f < 4x10^{-7}$), and strong mutator ($f \geq$ to $4x10^{-7}$) [25]. Denamur et al proposed that a strain should be considered a mutator strain when the frequencies of mutations that conferred resistance to rifampicin were 10-fold higher than the median value of mutagenesis observed for all the studied strains (these were termed '10-fold mutators'). Strains that displayed a >50-fold increase in mutagenesis were considered strong mutators (50-fold mutators) [5]. *E. coli* is far the most frequent pathogen in UTI, followed by other species of the *Enterobacteriaceae* family. The

former is investigated in detail, but data about the mutation frequency of the latter group was missing in the literature.

3.2 Hypermutator incidence in urinary tract infections

Hypermutators make up about 0.1% of the natural *E. coli* population [3], but this incidence is sometimes higher in clinical strains [5, 25]. Denamur et al studied a population of 603 *E. coli* strains, some commensal (i.e. fecal samples collected from healthy unrelated human subjects) and some pathogenic (from patients with bacteraemia, from patients with UTIs, enteroinvasive or enterohaemorrhagic pathogens, or from newborn meningitis) [5]. They found no significant difference in the frequency of mutators between the two groups. However, mutator strains occurred significantly more frequently among UTI strains. These results were confirmed by Baquero, who found a higher frequency of weak mutators in urinary tract isolates (25 %) and in blood isolates (38 %). Studying 696 *E. coli* strains from patients with UTIs in Sweden, Denmark and Spain he found that 23% were weak mutator and 0.7% was strong mutator [25]. The proportion of weak mutators among the Danish isolates was significantly lower than in the Spanish blood isolates, indicating geographical differences in the mutation frequency distribution profiles.

3.3 Hypermutation and biofilm formation

The initial goal of this thesis was to show a possible relationship between hypermutation and antibiotic resistance in UTI. After the first results I started to collect the literature when I realized some possible similarity between the microenvironment of CF lung infection and of urological biofilms. It is now evident, that *P. aeruginosa* isolated from respiratory samples in patients with cystic fibrosis showed a higher proportion of hypermutable strains in biofilms. So we decided to do a pilot study about the biofilm formation feature of the samples which we could compare with

the hypermutation and resistance feature. Since we observed some promising data, we have done an adhesion test as the initial step of biofilm formation for the whole series.

The ability of microorganisms to adhere to surfaces is influenced by electrostatic and hydrophobic interactions, ionic strength, osmolality and urinary pH [26, 27]. Several theories have been put forth to explain the complex interaction that occurs as a microbe approaches and then attaches to a surface. However, the precise mechanisms of attachment to biomaterials are still under investigation.

In order to react to a surface or an interface like an air-water interface, bacteria must be able to 'sense' their proximity to these surfaces. The planktonic 'free-floating' bacterial cells release both protons and signaling molecules as they move through the bulk fluid. These protons and signaling molecules must diffuse radially away from the floating cell, if not adjacent to any surface or interface. But a significantly higher concentration of either protons or signaling molecules can develop on the side of the bacterial cell close to any surface. This allows the cell to sense that it is near to a surface because diffusion is limited on this side [28]. After the planktonic bacterial cell has sensed the surface, it may commit to the active process of adhesion and biofilm formation.

There is no single process or theory, which can completely describe microbial adhesion. The initial adhesion is reversible and involves hydrophobic and electrostatic forces. It is followed by irreversible attachment provided by bacterial polysaccharides which anchor the organisms to the surface. Subsequently, colonization takes place in accordance with species' factors, such as slow migration and spreading, rolling, packing and adhesion. A developed biofilm consists of groups of microorganisms, sometimes in mushroom-like forms, separated by interstitial spaces that are filled with the surrounding fluid [29]. The growth rates of organisms on a surface as well as the strategies used by microorganisms to spread over a surface are important for colonization. These strategies are species specific and can influence the distribution of a biofilm on a surface [30].

Mutator phenotypes in P. aeruginosa can enhance microcolony initiation and growth during biofilm formation. It is showed that bacteria within microcolonies exhibit enhanced mutation frequencies and vice versa, bacteria with an elevated mutation frequency can exhibit enhanced

microcolony development. Microcolonies are foci of cell division and growth within biofilms [31], which may explain why mutations are localized within microcolonies of biofilms.

3.4 Hypermutation and antibiotic resistance

The increasing resistance of the most common uropathogens against the majority of antibiotics used in UTI is a global problem. The mechanism of *Enterobacteriaceae* acquiring resistance has been widely studied and many of the modalities have been described. If mutators have a role in the emergence of antibiotic resistance under natural conditions, such strains are expected to occur in natural populations, including clinical specimens [8]. Although the relationship between hypermutability and the acquisition of antibiotic resistance has been extensively investigated, the evidence of the association among different species remains conflicting except for cystic fibrosis [25, 32]. From the available data, it is still not clear whether high mutation frequencies are particularly important for the global evolution of pathogen populations, including antibiotic resistance acquisition [3, 33-35].

4. Hypoteses and objectives

4.1 Hypoteses

(1) Clones of the most common uropathogens belong to the *Enterobacteriaceae* family with hypermutable phenotype are more likely to be resistant to antibiotics.

(2) The higher the mutation frequency of the studied population the greater the ability to initiate biofilm formation.

4.2 Objectives

(1) To show the frequency of mutation for the first time in a highly diverse collection of *Enterobacteriaceae* strains collected from urine of UTI patients and from blood stream isolates.

(2) To investigate the capacity of strains to initiate biofilm formation.

(3) To explore the relationship between mutation frequency and biofilm formation.

(4) To determine the antibiotic resistance of the isolates against the most common antibiotics used in UTI.

(5) To show a possible link between hypermutable strains and acquisition of antibiotic resistance.

5. Material and Methods

5.1 Bacterial strains

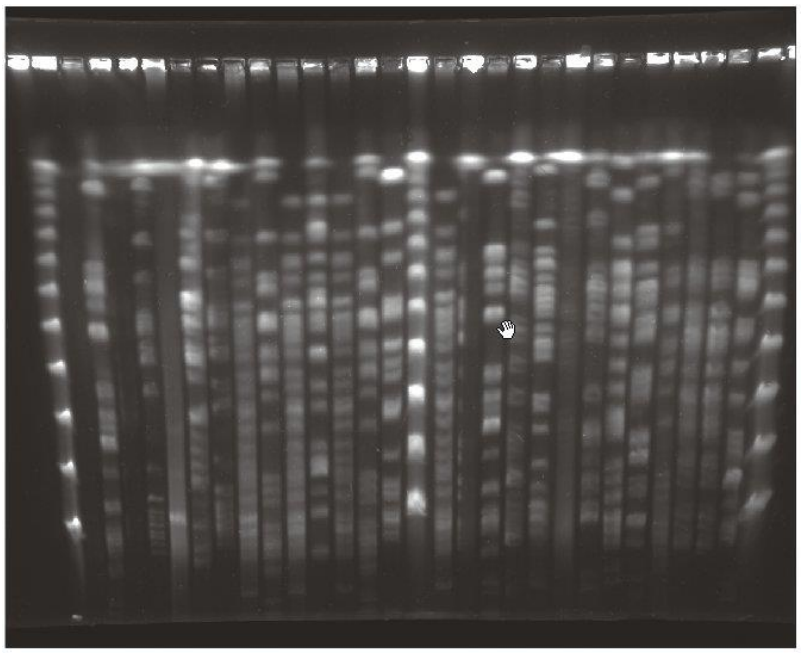
A total of 4000 clinical strains were screened for *Enterobacteriaceae* species admitted to the Urologic and Nephrologic Department of Pontchaillou University Hospital, in Rennes, France from January to December 2007. Samples with polymicrobial results were excluded.

The next step was the duplication of each strain in order to get the collection for the research. Agar medium was used to check the purity of the strain, than they were conserved on -80°C. The special deep-frozen medium contains pearls (15-20 pieces) (Cryobank TM Mixed (4x16), Mast Group Ltd, Bootle, Merseyside UK), each of which is sufficient for reproducing the baseline strain and gave the opportunity to repeat the dilution procedure three times or to repeat any case of questioned result. For the security of conserving the strains for one year and avoiding an unexpected event, deep-frozen samples was made in duplicate and held separately.

The blood samples were selected and studied by the institute followed the protocol I used for the UTI samples and the results were provided for further analysis.

5.2 Pulse-field gel electrophoresis (PFGE)

Preparation of the cellular DNA for PFGE followed the protocol of Allardet-Servent et al with minor modifications [36]. DNA contained in agarose plugs was restricted for 4 h with SfiI endonuclease (20 U) (New England BioLabs® Inc.) for *Proteus mirabilis* strains and with XbaI (40 U) (New England BioLabs® Inc.) for all other species. Electrophoresis was performed at 6 V/cm with a field angle of 120° for 22 hours with a switch time of 5 to 35 s at 14 °C and 24 hours at 30 to 70 s at 14 °C for *P. mirabilis* using a contour-clamped homogeneous electric field DR III apparatus (CHEF-DR III, BioRad, Ivry-sur-Seine, France). Bacteriophage lambda DNA ladders (Sigma) were included as size standards. Gels were then stained using SYBR® Green I (Lonza Inc.) and photographed under ultraviolet light (Picture 1.). PFGE patterns were compared by calculating the Dice correlation coefficient with the Gel Compar II software (Applied Maths, St-Martens-Latem, Belgium) and were clustered into a dendrogram using the unweighted pair group matching method (tolerance, 2.0%) (Figure 1.). Two isolates were considered genetically related if their Dice coefficient was 85% or higher. Solely the unique patterns were considered for the statistical analysis.



Picture 1. Photograph of DNA particles under ultraviolet light after PFGE.

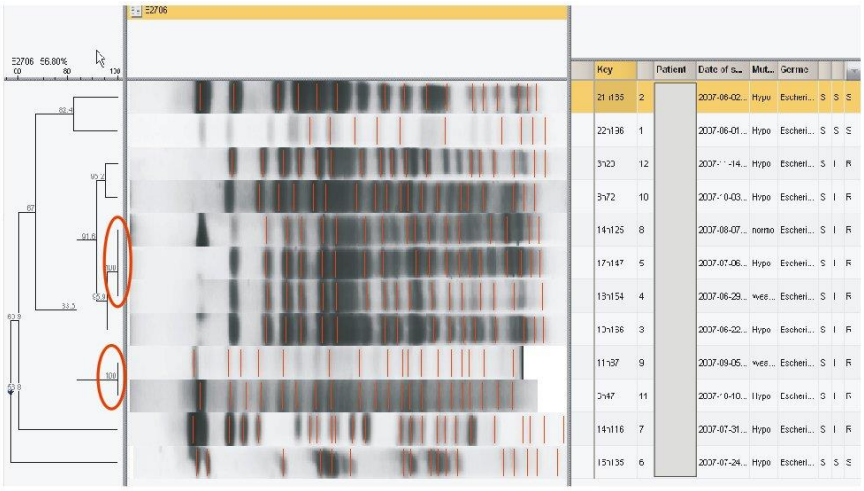


Figure 1. Comparison of PFGE patterns by dendograms. Photograph made by the Gel Compar II software (Applied Maths, St-Martens-Latem, Belgium).

5.3 Determination of mutation frequencies

Bacteria from the strain collection were spread onto agar plate (soy tryptase, TS) 18 hours (h), at 37°C to verify the purity of the strain. One isolated colony is incubated in 10ml Luria Bertani (LB) broth for 18h, at 37°C, continuously stirred at 150 rpm. Serial dilutions of the preculture made in sterile distilled water (10ml) 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} and 10^{-8} respectively. Then 100µL of dilutions 10^{0} and 10^{-1} are spread onto agar LB + rifampicin, and 10^{-6}, 10^{-7} and 10^{-8} onto agar LB without rifampicin (Figure 2.).

The mutation frequency corresponds to the number of bacteria resistant to rifampicin / the total number of bacteria:

$$= \frac{\frac{N \times 10^{-1} + N \times 10^{-2}}{2}}{\frac{N \times 10^{-7} + N \times 10^{-8}}{2}}$$

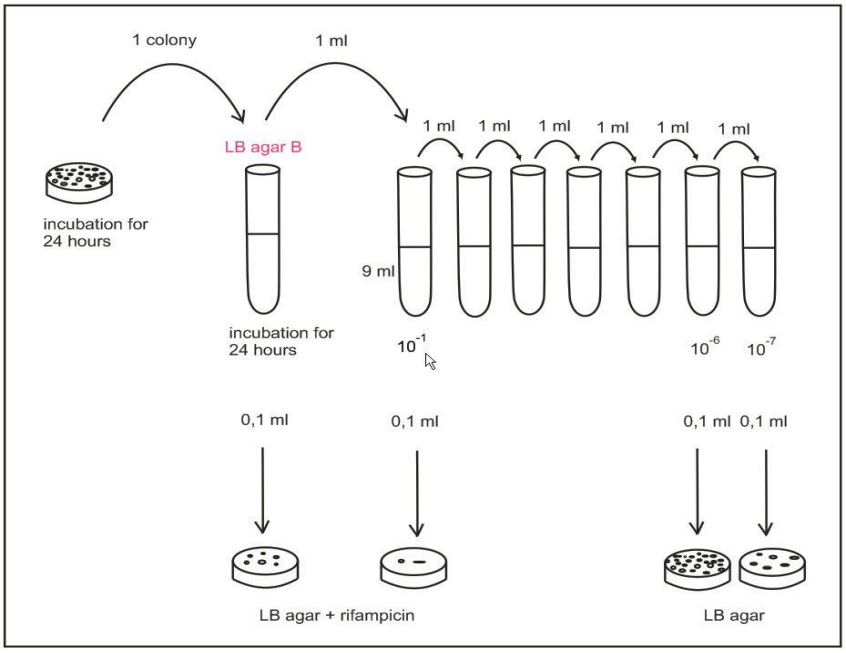


Figure 2. Serial dilution method for mutation frequency counting.

The number of colonies was counted and mutation frequencies were estimated. We used two methods for the evaluation of mutation frequencies. We considered the categorization described by Baquero, where a strain determined as normomutable when the mutation frequency (f) was equal or close to the modal point of the distribution of mutation frequencies. The results for the different species were categorized as previously described for *E. coli* strains. If the value is less than or equal to ($N \leq$) $8x10^{-9}$, it means that the bacterium is hypomutable, if the value is $8x10^{-9} < N < 4x10^{-8}$, it means that the bacterium is normomutable, when it is $4x10^{-8} \leq N < 4x10^{-7}$, it is a week mutator, and finally, if it is $N \geq$ to $4x10^{-7}$, it means that the strain is a strong mutator [25]. According to Denamur et al, strains displaying a > 50-fold increase of the median value of mutagenesis were considered strong mutators and a 10- to 50-fold increase as weak mutators [5].

The experiment was repeated in triplicate. The results were related to the mean value obtained from three independent cultures of about 10^8 CFU/mL. When mutation frequencies were $\geq 4 \times 10^{-8}$, mutation frequencies were also tested with lower (about 10^7 CFU/mL) or higher (about 10^9 CFU/mL) inocula and, if necessary, with another antibiotic (fosfomycin 30 µg/mL), to eliminate the possibility of 'jack-pot' emergence that might disturb the calculation of mutation frequencies.

5.4 Biofilm formation

The initiation of biofilm formation was assayed using polystyrene microtiter plates, as described previously, with some modifications [37]. Specifically, one fresh colony of each strain was inoculated into 10 mL tryptic soy (TS) broth and cultured for 2 h. Samples (150 µL) of exponential-growth-phase bacteria were removed and incubated overnight (18 h) at 37 °C in 96-well microtiter polystyrene plates (Falcon MicrotestTM 96; Becton Dickinson, Meylan, France). After removal of the medium, crystal violet (0.4% solution; 150 µL) was added to the emptied wells to stain the biofilm if present. The biofilm was quantified at least in triplicate for each sample. Streptococcus gordonii (strain Challis/ATCC 35105/CH1/DL1/V288) and bovine Salmonella Heidelberg B182 [24] were used as positive controls. Escherichia coli HB 101 and sterile TS culture broth were used as negative controls. The mean OD570 nm value (ODm) was calculated for three wells. A cut-off value (ODc) was established as three standard deviations

above the mean OD570 nm of the three negative controls in each plate. Biofilm production was calculated as the ODm/ODc ratio as recommended by Stepanovic et al [37]. The studied strains were classified into four categories: no biofilm producer (ratio ≤ 1), + biofilm producer (1 < ratio ≤ 2), ++ biofilm producer (2 < ratio ≤ 4) and +++ biofilm producer (ratio > 4). The raw value of the ratio was used for the correlation calculations.

5.5 Susceptibility testing

Susceptibilities of commonly used antibiotics for treatment of urinary tract infections or surgical prophylaxis (nalidixic acid, ciprofloxacin, norfloxacin, cefalotin, cefoxitin, ceftazidime, cefotaxime, fosfomycin, gentamicin, amoxicillin, amoxicillin+clavulanic acid and cotrimoxazole) were determined at admission, using agar diffusion and E tests methods, according to the current CASFM (Comité de l'Antibiogramme de la Société Française de Microbiologie (2007) and CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) guidelines 2007. AmpC overproducers were detected by their characteristic antibiotype, namely resistance to all beta-lactams except carbapenems and cefepime with no influence of clavulanic acid on MIC values of cefotaxime and cefepime. Production of extended spectrum beta-lactamases (ESBL) was detected using double-disk synergy assay and E-tests (ESBL E-test, AB Biodisk).

5.6 Statistical analysis

Continuous data were expressed as mean values ± standard deviation or as median and percentile 25 and 75, and discrete data as percentages. Comparisons between two groups were performed by two-sided Fisher's exact test for count data or the Mantel–Haenszel chi-square test for stratified data or Student's *t* and chi-square tests, *P* value of <0.05 was considered as statistically significant. For the purpose of analysis, "non-susceptible" count included both the intermediate

and resistant categories. Statistical analyses were performed with the SPSS software (SPSS Inc., Chicago, Illinois, USA).

6. Results

6.1 Pulse-field gel electrophoresis (PFGE)

Three-hundred sixty-nine clinical strains of Enterobacteriaceae were selected from urine samples of 218 patients for further analysis after purity examination and the exclusion of polymicrobial samples. The evaluation of PFGE determined 222 unique clones (isolated from 195 patients). Four, 3 and 2 different clones were isolated from the same patient in 1, 7 and 18 cases, respectively. Two patients harbored a strain with identical PFGE pattern, mutation categories and susceptibility values to another patient's. One of each of these duplicated clones was excluded from the analysis. There were 15 patients who had more than one identical clone from which we used the first one. The remaining 222 different *Enterobacteriaceae* strains from urine samples together with the 213 blood isolates were distributed as indicated in table 1:

Species	**№ of isolates with unique PFGE pattern**	
	Urine	**Blood**
E. coli	161	149
K. oxytoca	7	11
K. pneumonia	15	13
C. koseri	2	4
C. freundii	11	-
P. mirabilis	10	8
M. morganii	5	3
S. marcescens	7	6
P. rettgeri	4	-
H. alvei	-	2
E. aerogenes	-	2
S. enterica Typhi	-	1
E. cloacae	-	14
Total	**222**	**213**

6.2 Mutation frequencies

The mutation frequency distribution was calculated for the whole collection. According to Baquero's criteria, 10.3% hypomutable ($f \leq 8x10^{-9}$), 64.8% normomutable ($8x\ 10^{-9} < f < 4x10^{-8}$), 23% weak mutator ($4x10^{-8} < f < 4x10^{-7}$), and 1.8% strong mutator ($f \geq 4x10^{-7}$) strains was revealed. One-hundred weak mutators (57 UTI and 43 BSI strains) and 8 strong mutators were isolated [five UTI strains (one *C. freundii* and four *E. coli*) and 3 BSI strains (two *E. cloacae* and one *E. coli*)] (Table 2.). The distribution pattern of mutation frequencies for the *Enterobacteriaceae* collection without the *E. coli* strains showed close similarity to the distribution of the *E. coli* strains (Figure 3.). In this collection of *Enterobacteriaceae*, a submajority population whose mutation frequency was 1×10^{-8} ($1 \times 10^{-8} \leq f < 2 \times 10^{-8}$), and a smaller subpopulation with a mutation frequency 10-fold higher ($1 \times 10^{-7} \leq f < 2 \times 10^{-8}$) was already described as normomutators and weak mutators [25]. Between these two populations, strains have intermediate rates, mainly between 3×10^{-8} and 1×10^{-7}. To confirm the distinct modal mutation frequencies and the threshold proposed by Baquero et al, the analysis has been repeated and limited to strains with the characteristic rate of 1×10^{-8} and 1×10^{-7}, respectively, considered as normomutators and weak mutators. In this specific population, 115 strains were normomutators (including 84 *E. coli* and 31 other *Enterobacteriaceae*; 63 from BSI and 52 from UTI) with a mutation frequency between 1×10^{-8} and 1.98×10^{-8}. We also found 27 weak mutators (including 19 *E. coli* and 8 other *Enterobacteriaceae*: 8 from blood cultures and 19 from urine) with a mutation frequency between 1×10^{-7} and 1.91×10^{-7}. Mutation frequency calculations using the criteria defined by Denamur et al helped to highlight 10-fold and 50-fold mutator *E. coli* strains: for the whole collection, 4.1% of the UTI and 1.9% of BSI isolates were 10-fold mutators, while 0.9% of the UTI and none of the BSI isolates were 50-fold mutators [5].

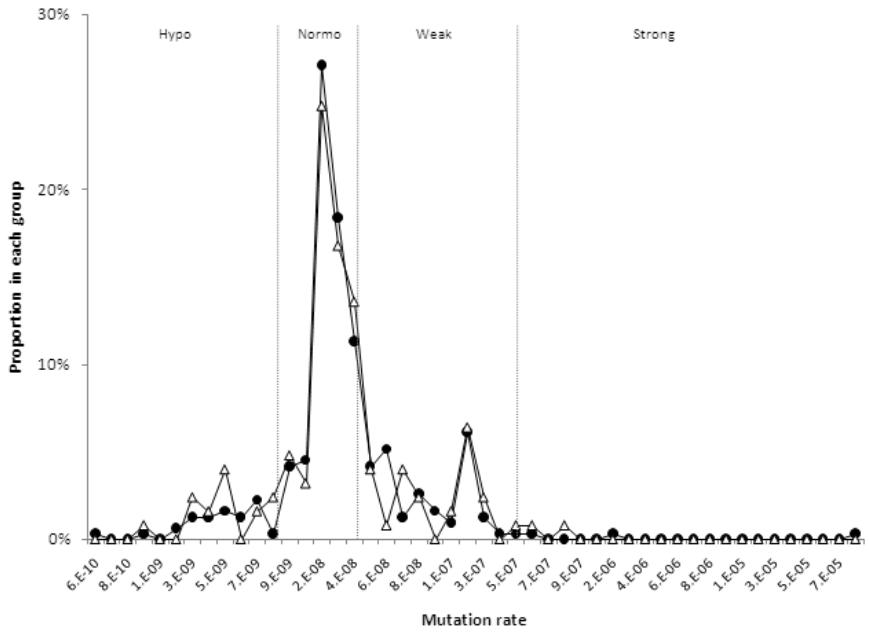


Figure 3. Distribution of the mutation frequencies to rifampicin resistance for 310 Escherichia coli (black circles) and 125 other *Enterobacteriaceae* (white triangles) clinical strains. The vertical lines indicate the thresholds proposed for *E. coli* by Baquero et al. (2004). The mutation frequencies of *E. coli* and other *Enterobacteriaceae* species showed the same distribution, which was similar to that presented in the paper by Baquero et al. (2004).

Mutation frequency (*f*) status*	*Enterobacteriaceae* species	Total (No of isolates)
Hypomutable strains $f \leq 8x10^{-9}$	*E. coli*	29
	K. oxytoca	7
	K. pneumoniae	2
	C. koseri	2
	S. marcescens	2
	C. freundii	1
	M. morganii	1
	P. mirabilis	1
	Total Hypo	45
Normomutable strains $8x10^{-9} < f < 4x10^{-8}$	*E. coli*	203
	K. pneumoniae	20
	P. mirabilis	10
	E. cloacae	9
	K. oxytoca	9
	S. marcescens	9
	M. morganii	6
	C. freundii	5
	P. rettgeri	4
	C. koseri	3
	H. alvei	2
	E. aerogenes	1
	S. enterica Typhi	1
	Total Normo	282
Weak mutator strains $4x10^{-8} < f < 4x10^{-7}$	*E. coli*	73
	P. mirabilis	7
	K. pneumoniae	6
	C. freundii	4
	E. cloacae	3
	K. oxytoca	2
	S. marcescens	2
	C. koseri	1
	E. aerogenes	1
	M. morganii	1
	Total Weak	100
Strong mutator strains $f \geq$ to $4x10^{-7}$	*E. coli*	5
	E. cloacae	2
	C. freundii	1
	Total Strong	8
Total		435

Table 2. Mutation frequency distribution according to Baquero's criteria (2004).

6.3 Hypermutation and biofilm formation

Higher biofilm-forming capacity was observed in UTI strains than in BSI strains: 140/222 (63.1%) and 90/213 (42.3%), respectively (Table 3-4.). The results were similar for the *E. coli* subpopulation in UTI strains (87/161; 54.0%) and BSI strains (71/149; 47.7%). The Mantel–Haenszel chi-squared test for stratified data (with *E. coli* in stratum 1, and other *Enterobacteriaceae* in stratum 2) gave a chi-square statistic = 18.4, a P-value < 0.001 and an Odds ratio = 2.26; 95% CI [1.55–3.28]. As expected among the urease producer *Proteus mirabilis strains*, 10/10 (100%) and 0/8 were biofilm producers in UTIs and BSIs, respectively (P < 0.001). Of the *Klebsiella pneumoniae* isolates, which are also urease positive, 14/15 (93.3%) of UTI and 3/13 (23.1%) of BSI isolates were biofilm producers (P < 0.001). No significant difference in biofilm production was demonstrated for *E coli* UTI strains (87/161; 54.0%) vs. BSI strains (71/149; 47.7%). Some biofilm-producing species (i.e. *P. mirabilis*) are particularly isolated from urine during pyelonephritis associated with bacteraemia, compared with digestive translocation or other origin. The great majority of *K. pneumoniae* UTI strains are biofilm producers; however, this is not the case for BSI strains (urinary, pulmonary or digestive origin) (Table 3A-B). In our series, only 35.2% of the BSI strains had a proven urinary tract origin.

Mutation frequency (*f*) status*	*Enterobacteriaceae* species	Urinary strains		
		No biofilm producers**	+Biofilm producers	Total
Hypomutable strains $f \leq 8x10^{-9}$	*E. coli*	6	11	17
	C. freundii	-	1	1
	Total Hypo	6	12	18
Normomutable strains $8x10^{-9} < f < 4x10^{-8}$	*E. coli*	45	54	99
	K. pneumoniae	-	13	13
	P. mirabilis	-	3	3
	K. oxytoca	1	5	6
	S. marcescens	2	4	6
	M. morganii	2	3	5
	C. freundii	1	4	5
	P. rettgeri	1	3	4
	C. koseri	-	1	1
	Total Normo	52	90	142
Weak mutator strains $4x10^{-8} < f < 4x10^{-7}$	*E. coli*	21	20	41
	P. mirabilis	-	7	7
	K. pneumoniae	1	1	2
	C. freundii	-	4	4
	K. oxytoca	-	1	1
	S. marcescens	-	1	1
	C. koseri	-	1	1
	Total Weak	22	35	57
Strong mutator strains $f \geq$ to $4x10^{-7}$	*E. coli*	2	2	4
	C. freundii	-	1	1
	Total Strong	2	3	5
Total		82	140	222

Table 3A.

Mutation frequency (*f*) status*	*Enterobacteriaceae* species	Blood strains		
		No biofilm producers**	+Biofilm producers	Total
Hypomutable strains $f \leq 8x10^{-9}$	*E. coli*	5	7	12
	K. oxytoca	3	4	7
	K. pneumoniae	1	1	2
	C. koseri	2	-	2
	S. marcescens	1	1	2
	M. morganii	-	1	1
	P. mirabilis	1	-	1
	Total Hypo	13	14	27
Normomutable strains $8x10^{-9} < f < 4x10^{-8}$	*E. coli*	59	45	104
	K. pneumoniae	5	2	7
	P. mirabilis	7	-	7
	E. cloacae	8	1	9
	K. oxytoca	2	1	3
	S. marcescens	1	2	3
	M. morganii	-	1	1
	C. koseri	1	1	2
	H. alvei	1	1	2
	E. aerogenes	1	-	1
	S. enterica Typhi	-	1	1
	Total Normo	85	55	140
Weak mutator strains $4x10^{-8} < f < 4x10^{-7}$	*E. coli*	13	19	32
	K. pneumoniae	4	-	4
	E. cloacae	3	-	3
	K. oxytoca	-	1	1
	S. marcescens	-	1	1
	E. aerogenes	1	-	1
	M. morganii	1	-	1
	Total Weak	22	21	43
Strong mutator strains $f \geq$ to $4x10^{-7}$	*E. coli*	1	-	1
	E. cloacae	2	-	2
	Total Strong	3	-	3
Total		123	90	213

Table 3B.

Table 3A-B. Mutation frequencies and ability to form a biofilm (on polystyrene plate) of a collection of clinical *Enterobacteriaceae* species (n = 435), isolated from blood and urine samples. * Categorisation according to Baquero's criteria (2004). ** Biofilm production was

calculated as the ODm/ODc ratio as recommended by Stepanovic et al.(2007). The studied strains were classified into 2 categories: no biofilm producers (ratio $\leq$ 1), +biofilm producers ratio > 1). The raw value of the ratio was used for the correlation calculations.

Higher percentage of urine sample isolates presented in every level of biofilm production. The largest difference between urine and blood strains experienced among the strongest biofilm producers (Figure 4).

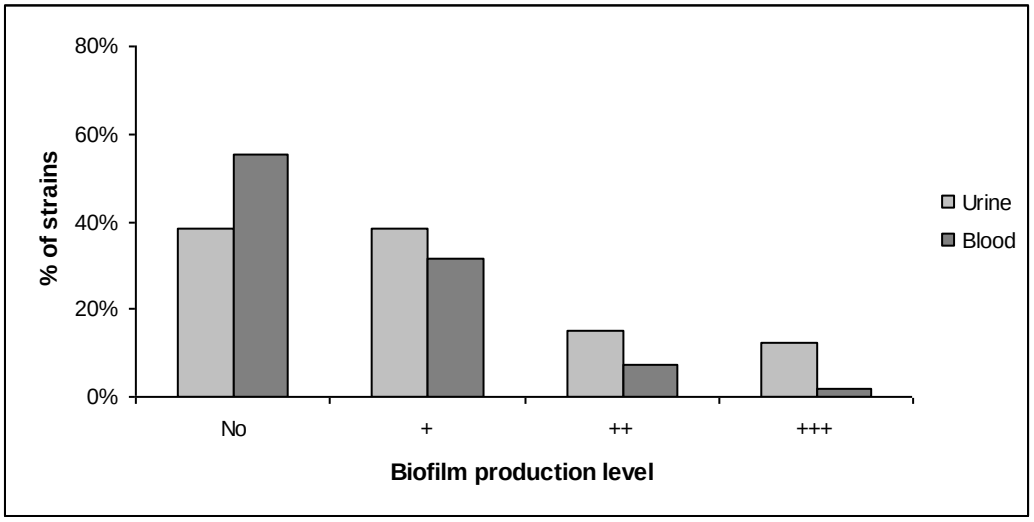


Figure 4. Biofilm production level of *Enterobacteriaceae* strains: Comparison between strains isolated from urinary tract infections (n = 222) and from bloodstream infections (n = 213). Biofilm production was classified using a 4-group classification system [37].

There was no significant correlation (Pearson's R correlation coefficient and P-values < 0.05 were considered statistically significant) between mutation frequency and the capacity to initiate a biofilm (R = 0.030, Figure 5.), irrespective of the source of the strain (urine: R = 0.022; blood: R = 0.057) or the group of species (*E. coli*: R = 0.038; other *Enterobacteriaceae*: R = 0.020).

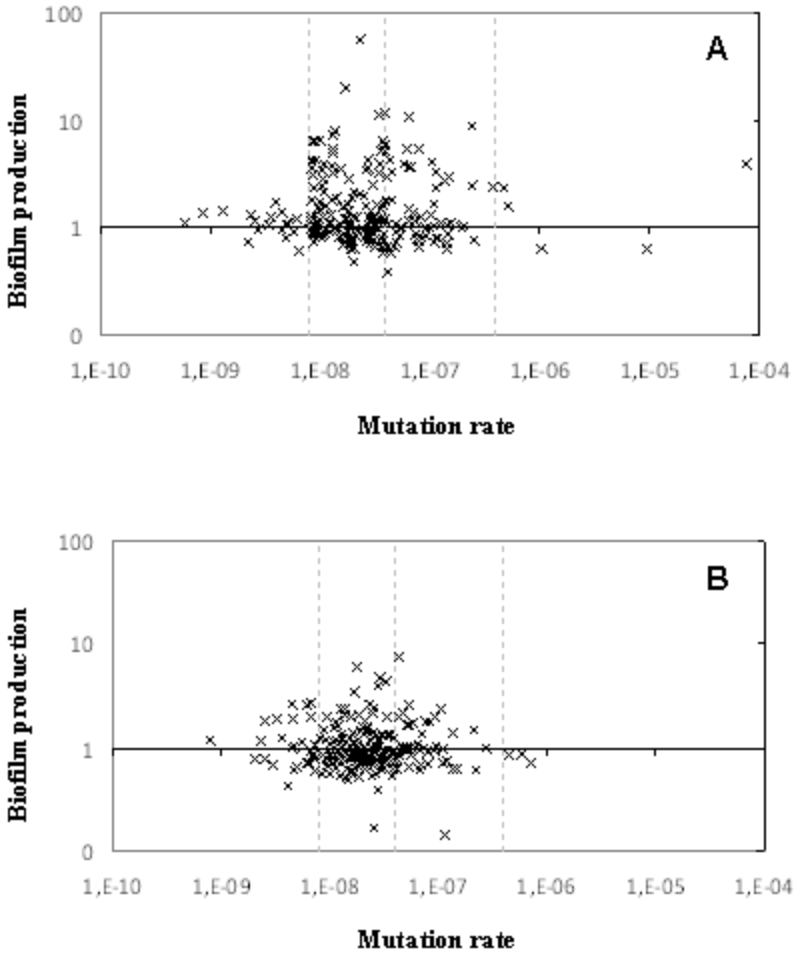


Figure 5. Relationship between rifampicin resistance mutation rates and biofilm production for 435 *Enterobacteriaceae* strains: 222 from urinary tract infections (A) and 213 from bloodstream infections (B). Biofilm production was calculated as the ODm/ODc ratio as recommended by Stepanovic et al. (2007). The dotted vertical lines indicate the thresholds of mutation according to Baquero et al. (2004).

The ability of the bacterial population to form a biofilm was then studied between the two main peaks of mutation frequencies: 1 x 10^{-8} (1 x 10-8 ≤ f < 2 x 10^{-8}) and 10-fold higher (1 x 10^{-7} ≤ f < 1 x 10^{-8}). In the first group, the biofilm production, assessed by the DOm/DOC ratio, ranged from 0.49 to 20.04, with an average of 1.64 and a standard deviation of 2.16. In the second

group, these values were 0.14, 4.13, 1.38 and 0.97, respectively. The number of strong mutators (i.e. MMR deficient type) was very low and the genetic basis was not characterized.

The highly biofilm-producing strains were normomutators except one weak mutator (*E. coli*), and 6 out of 7 had a urinary origin (three *K. pneumoniae*, two *E. coli*, one *K. oxytoca* and one *S. marcescens*). Most of the *P. mirabilis* (7 out of 10) had a weak mutator phenotype, and all of them were biofilm producers (one was +biofilm producer, five were ++biofilm producers, and two were +++biofilm producers).

6.4 Susceptibility testing

Referring to a previous study in the same hospital, from May 2003 through April 2004, activity of fosfomycin on E. coli appears unchanged, in hospitalized patients, in contrast to the increased resistance rates to other antibiotics usually prescribed for UTI [38]. Resistance rate increased by 21% for amoxicillin, 9% for amoxicillin combined with clavulanic acid, 3.8% for third generation cephalosporins, 15.5% for cotrimoxazole, 8,7% for first-generation quinolones, and 13.6% for norfloxacin, and 9,1% for ciprofloxacin (Table 4.). These results are in line with other recent works [39, 40]. Seven strains harbored an extended-spectrum beta-lactamase (ESBL) phenotype and 11 strains an overproduced cephalosporinase.

	Year	Cip	Nor	Nal	Caz	Ctx	Cxt	AC	Amx	Gen	Fos	Tsu
Overall Resistance %	2007	18.0	22.5	23.0	8.1	8.1	11.3	56.3	68.9	5.4	6.3	34.7
	2004	8.9		14.3				47.3	47.9		6.0	19.2

Table 4. Comparison of antibiotic resistance in three years' time from the same geographical area. Amx: amoxicillin; AC: amoxicillin clavulanate; Ctn: cephalotin; Cxt: cefoxitin; Ctx: cefotaxime; Caz: ceftazidime; Gen: gentamicin; Fos: fosfomycin; Tsu: cotrimoxazole; Nal: nalidixic acid; Nor: norfloxacin; Cip: ciprofloxacin

Fluoroquinolon-cotrimoxazole cross resistance in case of ciprofloxacin, norfloxacin and nalidixic acid was 60%, 67,5% and 68%, respectively.

6.5 Hypermutation and antibiotic resistance

No significant difference in the distribution of mutator (strong and weak) and non-mutator (normo and hypo) phenotypes was observed between the antibiotic resistant and susceptible isolates for any of the tested antibiotics (Table 5A-B.), neither if we examined the samples as a whole, nor as species by species. The number of strong mutators was too low to demonstrate a possible relationship between antibiotic resistance and acquisition of a strong hypermutator phenotype, although strong mutators were more frequently found in resistant strains, particularly: i) for quinolones, ii) a little for cefotaxime-ceftazidime, and iii) importantly for gentamicin. The percentage of mutators is almost the same among resistant and susceptible groups, except for fosfomycin, where we found just a few isolates (Figure 6.).

Mutation rate status	**Antimicrobial susceptibility**											
	Gen		**Fos**		**Tsu**		**Nal**		**Nor**		**Cip**	
	S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
H+N (n)	**158**	**8**	**154**	**13**	**108**	**58**	**128**	**38**	**129**	**37**	**136**	**30**
H+N (%)	**95**	**5**	**92**	**8**	**65**	**35**	**77**	**23**	**78**	**22**	**83**	**17**
W+St (n)	**52**	**4**	**54**	**1**	**37**	**19**	**43**	**13**	**43**	**13**	**46**	**10**
W+St (%)	**92**	**8**	**98**	**2**	**66**	**34**	**77**	**23**	**77**	**23**	**82**	**18**
P	**0.356**		**0.166**		**0.874**		**0.9522**		**0.977**		**0.884**	

Table 5A.

Mutation rate status	Antimicrobial susceptibility											
	Amx		AC		Ctn		Cxt		Ctx		Caz	
	S	R	S	R	S	R	S	R	S	R	S	R
H+N (n)	47	120	70	96	63	103	147	18	153	12	153	12
H+N (%)	28	72	42	58	38	62	89	11	93	7	93	7
W+St (n)	22	33	27	29	27	29	48	7	51	6	51	6
W+St (%)	40	60	48	52	47	53	88	12	90	10	90	10
P	0.08		0.36		0.237		0.88		0.512		0.415	

Table 5B.

Table 5A-B. Statistical analysis comparing the mutation rate status with antimicrobial susceptibility. Mutation frequencies (f) were defined according to Baquero et al (2004): hypomutable H ($f \leq 8x10\text{-}9$), normomutable N ($8x10\text{-}9 < f < 4x10\text{-}8$), weak mutator W ($4x10\text{-}8 \leq f < 4x10\text{-}7$), and strong mutator S ($f \geq$ to $4x10\text{-}7$). Amx: amoxicillin; AC: amoxicillin clavulanate; Ctn: cephalotin; Cxt: cefoxitin; Ctx: cefotaxime; Caz: ceftazidime; Gen: gentamicin; Fos: fosfomycin; Tsu: cotrimoxazole; Nal: nalidixic acid; Nor: norfloxacin; Cip: ciprofloxacin; S susceptible, R: resistant or intermediate; H: hypomutable strains; N: normomutable strains; W: weak mutators; St: strong mutators; n: number of strains; p:*p* value.

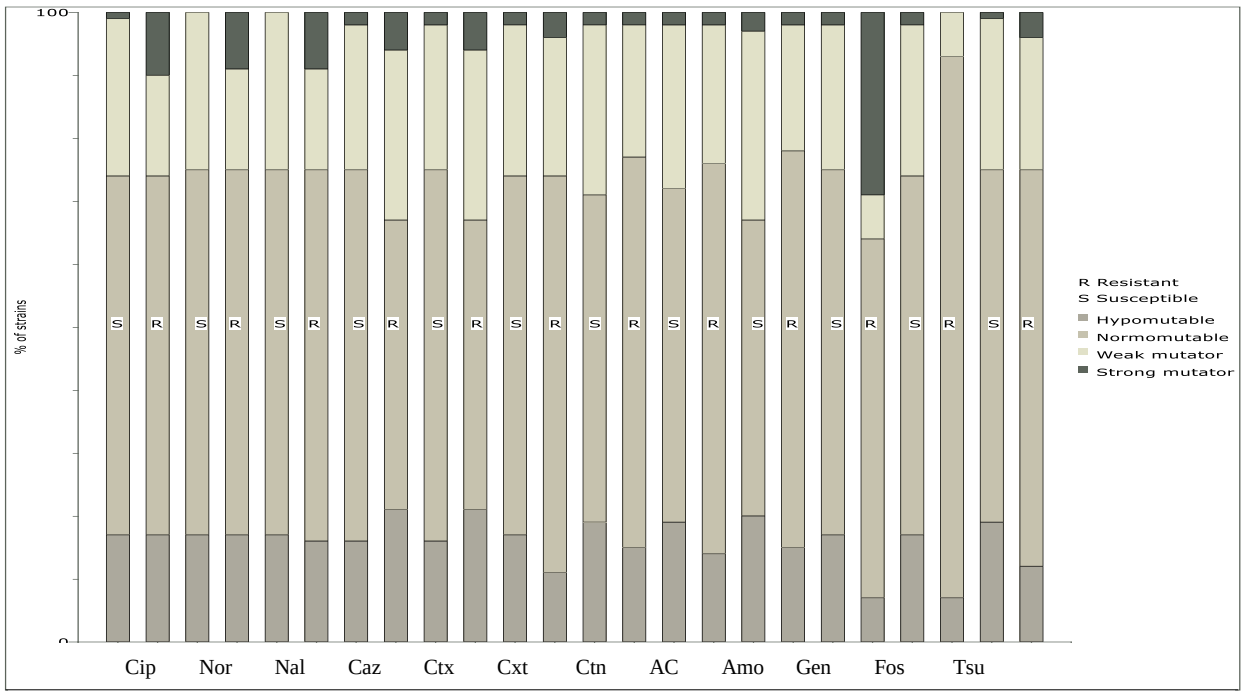


Figure 6. Distribution of mutation frequencies within the susceptible and resistant UTI strains by antibiotics. Amx: amoxicillin; AC: amoxicillin clavulanate; Ctn: cephalotin; Cxt: cefoxitin; Ctx: cefotaxime; Caz: ceftazidime; Gen: gentamicin; Fos: fosfomycin; Tsu: cotrimoxazole; Nal: nalidixic acid; Nor: norfloxacin; Cip: ciprofloxacin.

6.6 'Highlights' of the clone dendogram

There were patients with multiple isolates up to 12, but they had three PFGE patterns at most. Clones with similar PFGE pattern were excluded from the analysis, although interesting information could be gained about the possible changes of the clones during a longer period of time. Comparison of mutation frequency changes and biofilm forming capacity, or possible alteration in the antimicrobial susceptibility could also yield some exciting data. Hence, we examined patients with multiple isolates even though they had similar PFGE pattern.

One third of the patients had the same PFGE pattern with the same antibiogram through their whole series of isolates. One patient's strong mutator clone changed to hypomutable after one and a half month, with a 100% similarity of its PFGE pattern. Another clone changed to weak

mutator from strong mutator in three months' time. The third sample got hypomutable from strong mutator in one month, than consequently showed hypomutatbility along with the same antibiogram for six months. One isolate became hypomutable from strong mutator after seven months, with a 97% PFGE similarity. Our last example showed five isolates with the same PFGE pattern during four months, started with weak mutator phenotype which turned to hypomutable, than normomutable followed by a weak mutator phenotype, which altered to hypomutable in the end.

7. Discussion

7.1 Mutation frequencies

The distribution of mutation frequencies for the *E. coli* strains was in close agreement with the distribution reported by Baquero et al, who found a high rate (25%) of weak mutator and 0.7% of strong mutator strains in a collection of 696 *E. coli* isolated from urinary tract infections [25]. Polymorphisms in the rifampin resistance mutation frequency for the whole *Enterobacteriaceae* collection included the blood stream isolates showed similar pattern as well, even in case of excluded *E. coli* species. These results confirm that the mutation frequency classification developed for *E. coli* by Baquero and applied to *Stenotrophomonas* [41] can also be extended to other *Enterobacteriaceae* species.

Compared to the study by Denamur et al, we found fewer 10-fold and 50-fold mutator *E. coli* strains. Using their classification criteria for the whole collection, 4.1% of the urinary isolates and 1.9% of blood isolates were 10-fold mutators, while 0.9% urinary isolates were 50-fold mutators and we did not find any from blood cultures. The median value of mutagenesis in the Denamur report was $5x10^{-9}$, meanwhile $2.2x10^{-8}$ in this study, indicating the difficulty to compare different studies and contributing to the controversy in this topic [5]. The difference in the mutagenesis value might be due to geographical variation or differences in host or strain

characteristics. In 2004 Baquero showed considerable difference in distribution of weak mutators between the Danish (16%) and Spanish (25%) *E. coli* isolates from UTI. In the same study Spanish weak mutators tended to have higher frequencies of mutation than the Swedish ones [25]. In our experiment, there were numerous isolates with an intermediate mutation frequency between the normomutable and weak mutator frequency as well. Since incorrect assessment of the nature of these mutator strains could distort the measurement of the association with the ability to produce a biofilm, we performed a repeated analysis limited to strains with the characteristic rate of 1×10^{-8} and 1×10^{-7}, respectively, considered as normomutators and weak mutators. We found 115 normomutable strains and 27 weak mutators.

7.2 Hypermutation and biofilm formation

In the clinical context, it is estimated that 80% of acute and chronic infections are biofilm-related [42]. Uropathogenic *E. coli* (UPEC) is known to form biofilms easily [43, 44]. Previous studies are in accordance with our results, showing that biofilm was produced by about half of the strains [45, 46]. However, taking into consideration the site of isolation, this percentage only reached 42.3% in BSI strains compared with 63.1% in UTI strains in our series. Soto et al found 43% and 40% of biofilm-forming strains from patients with cystitis and pyelonephritis, respectively, while this percentage reached 63% in case of prostatitis [45].

By definition, hypermutable strains are expected to have higher capacities for adaptation, and some mutations might be linked to an increase in biofilm formation [47]. Numerous studies, especially with *P. aeruginosa* isolated from respiratory samples in patients with cystic fibrosis, showed a higher proportion of hypermutable strains in biofilms. Driffield et al found a 105-fold increase in mutability of P. aeruginosa strains (from CF patients) in biofilm, compared with planktonic cells [48]. Several authors have attempted to explain the large amount of hypermutators in a bacterial population known to readily form biofilms. Bacterial biofilm formation can be induced by DNA damaging agents (involved in mutations) triggering the SOS response, through a connection between stress-inducible biofilm formation and the RecA-LexA interplay [49]. Damaging agents have been described, including silver nanoparticles used for

their antibacterial properties [50], oxidative product created by other bacteria present in the biofilm (i.e. *S. gordonii* producing H2O2) [51], or some antibiotics such as fluoroquinolones [52].

To explain the large amount of hypermutators in biofilms, some authors have involved the formation of persister cells, in a quiescent state, rest in the biofilm or inside urothelial cells in the bladder [53] [54]. The appearance of persister cells in the biofilm promotes survival and may be related to the hypermutator phenotype. Persister cells are especially found in late cultures, and therefore in chronic infections. Considering all the above information, we could expect a relationship between the mutation frequency of a collection of clinical *Enterobacteriaceae* strains and their capacity to initiate a biofilm.

Contrary to what could be expected, there was no significant correlation (Pearson's R correlation coefficient and P-values < 0.05 were considered statistically significant) between mutation frequency and the capacity to initiate a biofilm (R = 0.030, Fig. 3), irrespective of the source of the strain (urine: R = 0.022; blood: R = 0.057) or the group of species (*E. coli*: R = 0.038; other Enterobacteriaceae: R = 0.020).

However, association was realized in a small series. As expected with the urease producer *Proteus mirabilis*, previously described as the predominant organism in biofilms from encrusted catheters [55] and urinary stones, 10/10 (100%) and 0/8 were biofilm producers in UTIs and BSIs, respectively (P < 0.001). Nevertheless, 7 out of 10 isolates of *P. mirabilis* from UTIs showed a weak mutator phenotype, all of which were biofilm producers in a different level (one was +biofilm producer, five were ++biofilm producers, and two were +++biofilm producers).

Thus, solid conclusions may be obtained for weak mutators, but not for MMR deficient strong mutators (n = 8). Moreover, among the few highly biofilm-producing strains, only one weak mutator type (*E. coli*) was detected, while seven were normomutators among other strains (three *K. pneumoniae,* two *E. coli,* one *K. oxytoca* and one *S. marcescens*). These strains were mostly isolated from urine samples (6 out of 7).

The relationship between mutation frequency and the capacity to form a biofilm is very complex and could be dependent on the state of growth. Contrary to the mentioned examples, García-Castillo et al showed decreased mutation frequencies of hypermutators in biofilms compared

with planktonic conditions [56]. The emergence of biofilm production frequently implies increased adhesion between bacterial cells, which could be responsible for errors in estimating the number of colonies counted to measure the frequency of mutation.

7.3 Hypermutation and antibiotic resistance

The relationship between the acquisition of antibiotic resistance and hypermutator phenotype is controversial. Mutation frequencies can vary among studies and often did not correlate well with antibiotic-resistant strains known to have developed mutational resistance [57]. In a large series of *S. aureus*, including many methicillin-resistant *S. aureus* (MRSA) strains, O'Neil and Chopra could not discover a mutator phenotype and concluded that mutator status is not necessary for the generation of high-level vancomycin resistance in *S. aureus* [58]. Analyzing more than 300 *E. coli* isolates, Baquero et al were unable to find any significant association between mutator phenotype and resistance against ciprofloxacin in countries with either low or high prevalence of fluoroquinolone resistance [25]. Despite the fact that mutational-antibiotic resistance development was a frequent outcome in a series of 103 acutely infected intensive care unit patients, the prevalence of hypermutable strains was found to be lower than 1%, saying there was no association between hypermutation and resistance [59].

However, significant difference between mutator and non-mutator isolates regarding the antibiotic resistance in cystic fibrosis (CF) lung infection has been previously documented [34, 60]. The same association was found within *Staphylococcus aureus* isolates in CF patients, although the authors hypothesize easier adaptation benefit prior to the hypermutable feature, because they found several mutations involved in stress response and adaptation [61]. Studying other chronic processes of the lung it was found not only that hypermutable strains were much more resistant to all the antibiotics, but also that most of the antibiotic-resistant strains were hypermutable [32]. Another series showed a more quickly evolved resistance level along with a higher resistance rate in the mutator background than in the wild type (Schaaff et al. 2002) [62]. Hypermutable strains were more frequently found (43%) in a collection of 89 extended-spectrum beta-lactamase (ESBL)-producing isolates from different patients than in non-ESBL (26%) *E.*

coli strains, although these results did not supported either the possibility that mutator strains could be better recipients of ESBL-encoding plasmids or a putative mutagenic effect on the recipient cell derived from the ESBL plasmid acquisition [23]. Komp Lindgren et al have already suggested an unequivocal coherence between elevated mutation rate and fluoroquinolone resistance, by DNA sequencing for fluoroquinolone resistance-associated mutations in *E. coli* genes [63].

In this study, no significant difference in distribution of mutation frequencies (hypo- and normomutable strains *versus* weak and strong mutators) was observed between the resistant and susceptible isolates for any of the examined antibiotics, even if the strain was ESBL or AmpC overproducer, or had fluoroquinolone resistance.

However, when statistical analysis compared strong mutators *versus* all the other strains, a statistical difference was observed only with fluoroquinolones. This data correspond to the fact that point mutation in *gyrA* gene can result quinolone resistance. Strong mutators were also found in resistant strains a little more frequently for cefotaxime-ceftazidime. Mutation in AmpR transcription regulator may induce the AmpC β-lactamase [64, 65], which resulted in clones with similar features than ESBL clones, except the fact that they are resistant against β-lactamases. The association of strong mutators and gentamicin resistance is suggested in figure 6. The number of resistant species was only 12, out of which 30% were mutators. The very low resistance rate is welcomed considering the human population, but not enough to draw a solid conclusion. Another interesting data was that pathogens kept showing low resistance rate against fosfomycin, and we found only one mutator amongst them.

7.4 'Highlights' of the clone dendogram

PFGE allowed to compare the isolates, which let us define the similarity of the bacteria isolated from different patients or from the same host. Multiple urinary tract infections affecting a patient within a year could be caused by reinfection or relapse. The latter case presumes the continuous presence of the pathogen. Chronicity is an issue in the mutagenesis and transient mutator status, which is thought to be involved in the adaptation of bacteria to the environment. Thus we

examined all the species belonging to a patient and all the species with the same PFGE pattern. Comparison of mutation frequency changes and biofilm forming capacity, or possible alteration in the antimicrobial susceptibility was determined.

Our results showed examples for mutation frequency changes along with constant PFGE pattern. Variation of shifting included all the possibilities, involving the phenomenon of transient mutator status, which may involve reversion or recombination within the mutator alleles or depletion of the MMR system proteins, allows the organism to temporarily benefit from the elevated mutation frequency for adaptation while reducing the risk of accumulating deleterious mutations. Antibiograms might alter together with changes of the PFGE pattern or mutation frequency as well, although we could not define any association.

7.5 Critical remarks, limitation of this study

A significant number of patients had recurrent infection, which presumes former antibiotic consumption. Previous antibiotic treatment possibly contributes to selection of mutators in in vitro experiments. Mutators can be favored under such conditions because they generate antibiotic resistance conferring mutations at a higher rate than what is generated by nonmutators. In addition, they also generate more mutations that compensate for the fitness reduction associated with antibiotic resistance [5]. We could not control the prescriptions made by other hospitals or the general practitioners and did not have information about the antibiotic consumption and about the course of healing, which rises several unanswerable questions like what kind of antibiotics did they receive and how long, were these infections due to resistant strains, whether the selected patients got the sufficiently prolonged therapy for that certain infection, was there a therapy failure, is there impairment between therapy failure and the development of resistance. Consequently, the effect of previous antibiotic treatment on our results is inestimable.

8. **Conclusions**

We were the first to confirm that the mutation frequency classification developed for *E. coli* by Baquero et al and applied to *Stenotrophomonas* [41] can also be extended to other *Enterobacteriaceae* species isolated from urinary tract infections.

We could not show linear dependence or significant correlation between mutation frequency and the capacity to initiate a biofilm. However, association was realized in a small series of *Proteus mirabilis*, where 7 out of 10 isolates were weak mutators, all of which were biofilm producers. This association may support our basic idea of this research, as cystic fibrosis and certain urinary tract infections are similar in a certain respect. Further investigation with larger *P. mirabilis* sample size might refine this relationship. Not finding a correlation between increased mutation frequency and initiation of biofilms in *Enterobacteriaceae* might be directly linked to the fact that strains were mainly isolated from acute infections, and does not mean that there is no relevant effect of mutation frequency in other stages or aspects of biofilm growth.

We found statistical difference between the antibiotic resistant and susceptible isolates for the tested antibiotics in case of quinolones, when the analysis compared strong mutators *versus* all the other strains, but we found no significant difference in the distribution of mutator (strong and weak) and non-mutator (normo- and hypo-) phenotypes. Although the number of strong mutators was too low to demonstrate a possible relationship between antibiotic resistance and acquisition of a strong mutator phenotype. The same conclusion has to be drawn for gentamicin, although the suggested association is an unexpected finding and would be a promising novelty, the sample size is sparse.

Alteration in mutation frequency and antibiotic resistance in isolates with identical PFGE patterns belonging to the same patients suggests the importance of chronicity and transient mutator status. Further investigation of these features might help to better understand the mechanisms of bacterial adaptation.

9. **Acknowledgements**

This work was supported by the European Urological Scholarship Program (EUSP) of the European Association of Urology (EAU) and by grants from Conseil Regional de Bretagne and from Fondation des Gueules Cassees.

I thank Marie-France Travert for technical assistance with PFGE and Anne-Marie Gouraud for technical assistance with the biofilm assays.

I express my gratitude to my mentor, Anne Jolivet-Gougeon, who introduced me to the world of clinical microbiology examinations, showed me the associations of the topic and taught me the know-how of writing the articles. Her persistent hard work gave me the model and her continuous care encouraged me to step over the difficulties during my scholarship year.

I give special thanks to my chief, Peter Tenke, who taught me to combine clinical and scientific work, showed me the road to science and provided all the circumstances to pursue this work.

I thank my colleagues in the department for undertake more work during my absence from the hospital.

Finally I thank the most to my wife and daughters to support me and to show great tolerance for my absence from family life.

10. **Magyar nyelvű összefoglaló**

Hipermutáció szerepe a biofilm képződésben és az antibiotikum rezisztencia kialakulásában *Enterobacteriaceae* családba tartozó kórokozók által okozott húgyúti fertőzésekben

10.1 Bevezetés

A fertőző betegségek gyógyítását a mikroorganizmusok újabbnál újabb túlélési mechanizmusokkal nehezítik meg. Kutatások támasztják alá, hogy a mutációnak, amely a baktériumok természetesen meglevő tulajdonsága, alapvető szerepe van a kórokozók adaptációjában, és szelekciós előnyt jelent [2, 3]. Mutáns laboratóriumi sejtvonalakban korai tanulmányok olyan mutátorokat figyeltek meg, melyek különböző szelekciós stratégiákkal rendelkeztek. LeClerc és munkatársai *E. coli* és *Salmonella* egyedeket vizsgálva magas mutációs frekvenciáról számoltak be, amely megkérdőjelezte azon elmélet létjogosultságát, mely szerint baktérium populációkban a mutátorok előfordulása ritka. Ezen eredmények azt mutatták, hogy természetes populációk a környezeti szelekciós nyomásra két módon reagáltak, megemelt mutációs frekvenciával, és rekombinációval.

Adott populációban, adott időben a mutációk száma meghatározható, mely megadja a populáció mutációs frekvenciáját (f). Ismertek olyan organizmusok, klinikai pathogéneket is beleértve, amelyek 10-1000 szeres, vagy akár még nagyobb állandó mutációs frekvenciával rendelkeznek saját populációjuk medián értékéhez képest, melyet hipermutációnak nevezünk. Kutatásom előtt az irodalomban *E. coli*-tól eltekintve nem volt adat húgyúti infekciókból származó más, *Enterobacteriaceae* családba tartózó kórokozók hipermutátor prevalenciájáról.

A hipermutáció és biofilm formáló készség, illetve hipermutáció és antibiotikum rezisztencia közötti kapcsolat vizsgálatának ötlete a következőkben részletezett megfigyeléseken alapult. Az antibiotikum rezisztencia emelkedése az egész emberiséget érintő probléma, mely a húgyúti infekciókban (HI) használatos szinte összes antibiotikumot érinti. Kísérletesen igazolt, hogy a

húgyúti infekciókban magasabb a hipermutátorok aránya, mint más lokalizációjú fertőzésekben [5, 25], mellesleg a vizsgálatok arra is rámutatottak, hogy a mutációs frekvenciában földrajzi különbségek is megfigyelhetők. Továbbá, mind a biofilm képződésnek, mind az antibiotikum rezisztencia kialakulásának széles irodalma van a HI-k terén. Létezik egy klinikai entitás, melyben a fent említett hipermutáció és biofilm képződés, illetve hipermutáció és antibiotikum rezisztencia között szignifikáns összefüggést mutattak ki. A betegség nem más, mint a cisztás fibrózis tüdő érintettsége. Mucoviscidosisban az alveolusok, brochiolusok a betegség kapcsán keletkező sűrű szekrétum miatt elzáródnak, melyben baktérium kolóniák, és ezzel összefüggésben fertőzések alakulnak ki. Hosszú távon az említett nyákban biofilm képződik, mely biztonságos mikrokörnyezetet biztosít a benne élő kórokozóknak, megnehezíti az antibiotikumok penetrációját, és a fertőzés gyógyítását.

Krónikus fertőzés, bakteriális adaptáció, un. perziszter klónok, tranziens mutátorok jelenléte, védelem az antibiotikumokkal szemben, antibiotikum, mint szelekciós tényező. Ezek azok a jelenségek, amelyek cisztás fibrózisban és húgyúti fertőzések egy részében is jelen vannak, és ezért bizonyos tekintetben hasonlóvá teszik a két betegséget. Ez adta az ötletet, hogy megvizsgáljuk a hipermutáció és biofilm képződés, illetve antibiotikum rezisztencia kapcsolatát húgyúti fertőzésekben.

Hipotézisünk szerint az *Enterobacteriaceae* családba, mint a leggyakoribb urológiai kórokozók közé tartozó egyedek hipermutátor fenotípusai nagyobb valószínűséggel lesznek antibiotikumokra rezisztensek. Továbbá, minél magasabb a patogén mutációs frekvenciája, annál gyakrabban szerez biofilm képző képességet. Céljaink között szerepelt (1) a mutációs frekvencia meghatározásának kiterjesztése *Enterobacteriaceae* családba tartozó, húgyúti fertőzések során vizeletből és hemokultúrából kitenyészett kórokozókra. (2) Az izolátumok biofilm inicializáló kapacitásának vizsgálata. (3) A mutációs frekvencia és biofilm képző képesség között lehetséges kapcsolat feltárása. (4) A kórokozók HI-ban leggyakrabban használt antibiotikumokkal szembeni rezisztenciájának meghatározása. (5) A hipermutátorok és fokozott antibiotikum rezisztencia között lehetséges kapcsolat feltárása.

10.2 Beteganyag és módszer

A vizsgálat első fázisában egy bretagne-i egyetemi klinikán (Equipe Microbiologie, Université de Rennes 1, France) a 2007-es évben beérkezett négyezer klinikai mintából *Enterobacteriaceae* családba tartozó vizelet mintákat szelektáltunk. Az izolátumok tisztaságát ellenőriztük, a polimikróbás anyagokat a vizsgálatból kizártuk, majd speciális, többszöri mintavételre alkalmas hordozóban, mélyfagyasztva tároltuk a kórokozókat. A klónokat gél elektroforézis után dendogram analízisnek vetettük alá annak érdekében, hogy csak egyedi izolátumok kerüljenek a vizsgálatba.

A kórokozók antibiotikumokkal szembeni érzékenységet diszk diffúziós teszt illetve E teszt segítségével határozták meg a klinikai megjelenés kapcsán, melyekből a HI-ban leggyakrabban használt hatóanyagokat vettük figyelembe. Az AmpC típusú β-laktamáz termelő és ESBL (extended spectrum β-laktamase) termelő izolátumokat is meghatároztuk.

A mutációs frekvencia becsléséhez az egyes törzsekben a mutáció következtében létrejött rifampicin rezisztencia mértékét használtuk fel, rifampicin dilúciós teszt segítségével. A mutációs frekvenciák osztályozását kétféleképpen végeztük el, egyrészt Baquero definíciója szerint négy kategóriát határozva meg, úgymint hipomutábilis ($f \leq 8x10^{-9}$), normomutábilis ($8x10^{-9} < f < 4x10^{-8}$), a hipermutátorokat két csoportba sorolva: gyenge mutátor ($4x10^{-8} \leq f < 4x10^{-7}$), és erős mutátor ($f \geq t\ 4x10^{-7}$) fajok [25], illetve Denamur csoportosítását használva. Utóbbi szerint mutátor az az izolátum, melynek rifampicinnel szembeni rezisztenciája kapcsán jelentkező mutációinak száma tízszerese a vizsgált populáció medián mutagenezisének. Ezeket tízszeres mutátoroknak nevezte el, amely klónok > 50x mutációs frekvenciát mutattak, azokat pedig erős mutátoroknak tekintette [5].

A kutatás harmadik lépcsőjében a kórokozók adherencia képességének, mint a biofilm képződés kezdeti lépésének meghatározását végeztük el, Stepanovic módszerét némi módosítással alkalmazva [37].

A statisztikai elemzéshez kétoldalú Fischer's exact tesztet, és a Mantel–Haenszel khi-négyzet tesztet, illetve a Student-féle *t* és khi-négyzet tesztet alkalmaztunk.

A hemokultúrából származó egyedeket a klinika alkalmazottai vizsgálták a fent leírt módszereknek megfelelően, végül 213 izolátummal kapcsolatos adatbázist bocsátottak rendelkezésünkre az összehasonlító vizsgálatokhoz.

10.3 Eredmények

Háromszázhatvankilenc húgyúti fertőzésből izolált, *Enterobacteriaceae* családba tartozó kórokozóból a PFGE 222 egyedi klónt határozott meg, melyek eloszlását az 1. táblázat tartalmazza.

A Baquero által meghatározott négy kategóriának megfelelően a törzsek 10.3%-a volt hipomutábilis, 64.8% normomutábilis, 23% gyenge mutátor és 1.8% erős mutátor. Az *Enterobacteriaceae* családra, mint egészre vonatkozó eloszlási adatok közeli hasonlóságot mutattak a fent említett beosztáshoz akkor is, ha az *E. coli*-kat kihagytuk a számításból. A Denamur-féle felosztás szerint a húgyúti mintákból származó *E. coli*-k esetében a 10x és 50x mutátorok megoszlása 4.1% és 0.9%, míg hemokultúrákban 1.9% volt 10x mutátor, és nem találtunk 50x mutátort.

Szignifikáns különbség nem mutatkozott a mutációs frekvencia és biofilm iniciáló képesség között, függetlenül az izolátum forrásától vagy a speciestől. Vizeletből származó mintákban minden biofilm kategóriában nagyobb volt az esetszám a hemokultúrákhoz képest. Összességében a húgyúti minták 63.1%-a, míg a hemokultúrák 42.3%-a mutatott biofilm inicializáló tulajdonságot. A vizeletből származó *P. mirabilis* egyedek mindegyike biofilm képző volt, és a tízből hét gyenge mutátor tulajdonsággal is rendelkezett.

A vizsgált antibiotikumokkal szembeni rezisztencia növekedése egy három évvel korábbi felméréshez képest a következő értékeket mutatta: nalidix-sav 8.7%, ciprofloxacin 9.1%, norfloxacin 13.6%, harmadik generációs cefalosporinok 3.8%, fosfomycin 6.1%, gentamicin 5.7%, amoxicillin 21%, amoxicillin-klavulánsav 9% és trimethoprim-sulfamethoxazol 15.5%.

A mutátor (erős és gyenge mutátorok csoportja) és non-mutátor (normo- és hipomutábilis kórokozók) fenotípusok között az érzékeny és rezisztens tulajdonság tekintetében egyik vizsgált antibiotikum esetében sem mutatkozott szignifikáns különbség. Ha az erős mutátorok csoportját

állítottuk szembe a gyenge mutátorok, normo- és hipomutábilis kórokozók csoportjával, akkor a kinolonoknál szignifikáns összefüggés mutatkozott az erős mutátor fenotípus és az antibiotikum rezisztencia jelenléte között (P<0,001). Azonos betegből származó, azonos PFGE mintázatot mutató kórokozók között változatos mutációs frekvenciájú klónokat mutattunk ki.

10.4 Megbeszélés

Vizsgálatunkban a mutációs frekvencia eloszlás megegyezett a Baquero által közölt adatokkal, mi több, az *Enterobacteriaceae*-k családját tekintve is azonos volt, akkor is, ha az *E. coli* izolátumokat kihagytuk a számításból. Erre vonatkozó adat korábban nem szerepelt az irodalomban.

Hipermutáció és biofilm képző sajátosság között nem sikerült szignifikáns kapcsolatot kimutatni, de összefüggés mutatkozott egy kis csoport tekintetében. Ahogy az várható volt, minden *P. mirabilis* izolátum biofilm képző volt a húgyúti mintákban, ugyanakkor tízből hét kórokozó gyenge mutátornak bizonyult. Alapfelvetésünket, mely szerint bizonyos tekintetben azonosság mutatkozik a cisztás fibrózis és bizonyos húgyúti infekciók körülményei között, utóbbi összefüggés alátámaszthatja. Nagyobb esetszámú vizsgálat tovább finomíthatná a jelenséget.

Bár a hipermutáció és az antibiotikum rezisztencia kialakulása közötti logikusnak tűnő kapcsolat igazolására számos vizsgálatot végeztek, az eredmények ellentmondásosak. Vizsgálatunkban statisztikai különbség mutatkozott a kinolon rezisztencia, és az erős mutátorok verzusz nem erős mutátorok (ide értve a gyenge mutátorok, normál populáció és hipomutábilis törzsek csoportját) között, azaz kimutathatóan több rezisztens kórokozó volt az erős mutátorok csoportjában, mely összecseng a *gyrA* génben létrejövő pont mutáció okozta fluorokinolon rezisztencia előfordulásával. Ugyanezen, de nem szignifikáns összefüggést igazoltuk gentamicinnel szemben is, mely nem várt eredmény, és bíztató újdonságnak számítana, de a kis esetszám statisztikai ereje nem elegendő az egyértelmű összefüggés kimondásához.

Azonos PFGE mintázatú, ugyanattól a betegtől származó kórokozók változó mutációs frekvenciája és antibiotikum rezisztenciája felhívja a figyelmet az elhúzódó bakteriális jelenlét és a tranziens mutátor státus jelentőségére, melyek további vizsgálata hozzásegíthet a baktériumok adaptációs mechanizmusainak megértéséhez.

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