Intensive Care Med (2012) 38:2007–2016
DOI 10.1007/s00134-012-2699-5

ORIGINAL

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Pathophysiology of *Escherichia coli* ventilator-associated pneumonia: implication of highly virulent extraintestinal pathogenic strains

Received: 12 February 2012
Accepted: 2 August 2012
Published online: 28 September 2012

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Electronic supplementary material
The online version of this article (doi:10.1007/s00134-012-2699-5) contains supplementary material, which is available to authorized users.

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Abstract *Purpose:* To characterize *Escherichia coli* ventilator-associated pneumonia (VAP) in intensive care unit (ICU) patients by determining antibioresistance and genotypic characteristics of *E. coli* isolates responsible for VAP or lung colonization, by comparing them with their oropharyngeal and rectal counterparts and by assessing representative isolates' virulence in a pneumonia mouse model. *Methods:* Patients under mechanical ventilation for more than 72 h were screened for simultaneous presence of *E. coli* in rectal, oropharyngeal, and respiratory samples (colonization or VAP). If present, *E. coli* isolates were characterized by antimicrobial susceptibility, phylogenetic grouping, and virulence factor (VF) gene content determination. BALB/c mice were challenged intranasally with 3.6×10^8 colony-forming units (CFU) of patients' *E. coli* isolates. *Results:* Multisite *E. coli* colonization was observed in 19 % of patients (25 patients, 12 with *E. coli* VAP). One hundred fifteen distinct *E. coli* isolates were analyzed. B2 phylogenetic group was predominant, with high VF gene content and low antimicrobial resistance. Antimicrobial resistance diversity was observed in four patients with VAP. *E. coli* isolates from VAP patients were more frequently B2 isolates, with significantly greater VF gene content than lung colonization isolates. Among screened VF genes, *iroN* and *sfa* appeared important for lung infection. A very strong correlation ($R^2 = 0.99$) was found between VF gene content and mortality in the mouse model. *Conclusions:* This is the first study establishing antibioresistance and genotypic characteristics of *E. coli* isolates responsible for VAP in adult ICU patients. These isolates are highly virulent specific extraintestinal pathogenic *E. coli* strains expressing virulence factors, representing potential targets for new therapies.

Keywords Nosocomial infection · Ventilator-associated pneumonia · *Escherichia coli* · Virulence factor genes · Antibiotic resistance · Animal model

Introduction

Lung is the first site of infection in intensive care unit (ICU) patients [1], and ventilator-associated pneumonia (VAP) is the most common life-threatening hospital-acquired infection despite considerable efforts to implement guidelines for its prevention [2, 3]. Consequences of VAP include increased duration of mechanical ventilation and length of ICU and hospital stay [4, 5], high morbidity and mortality [6], and increased antibiotic consumption and hospital costs [7]. Gram-negative bacteria (GNB) predominate in hospital-acquired pneumonia, and among them, *Escherichia coli* is the main *Enterobacteriaceae* involved in VAP [6]. *E. coli* is the most abundant aero-anaerobic commensal intestinal bacterium [8], with extraintestinal pathogenicity (extraintestinal pathogenic *E. coli*: ExPEC) [9]. Although a huge amount of data has accumulated on *E. coli* pathogenicity in intestinal, urologic, central nervous system, and bloodstream infections, there is, surprisingly, little data on lung infection [10], despite *E. coli* now being as often responsible for ICU infections as *Pseudomonas aeruginosa* [1]. Given the worrisome increase in multidrug resistance among GNB including *E. coli* [11], future therapeutic approaches will have to rely on other strategies including vaccine and immunotherapy [12, 13]. Greater knowledge of genotypic and phenotypic characteristics of *E. coli* responsible for VAP is the necessary first step in the search for potential vaccine candidates, as for *P. aeruginosa* [14, 15].

Phylogenetic analysis shows that *E. coli* population can be divided into four major groups (A, B1, B2, and D). Strains belonging to groups A and B1 are known to carry few genes encoding virulence factors (VF), with weak extraintestinal pathogenicity [16], and are commonly multidrug resistant [17]. On the other hand, group B2 and D strains typically express numerous VF mediating bacterial adhesion and protection, cell host invasion, toxicity, and iron uptake [10], usually exert high extraintestinal pathogenicity [16], and are generally more susceptible to antibiotics [17].

Phenotypic and genetic characteristics of ExPEC isolates have been extensively studied except for oropharyngeal and respiratory *E. coli* isolates in the ICU, for which there are no data. We, therefore, aimed to characterize *E. coli* respiratory isolates of ICU mechanically ventilated patients and compare them with their oropharyngeal and digestive counterparts. To better understand *E. coli* lung infection pathophysiology, an in vivo animal model of lung infection was used to assess pulmonary virulence of various representatives of these isolates.

Patients and methods

Patients

This prospective study was conducted in Louis-Mourier University Hospital medico-surgical ICU (Colombes, France) and approved by the ethics committee of the French National Society of Intensive Care (SRLF), which did not require informed consent since there was no change in practices and all procedures were already routinely performed. Patients or family/relatives were informed of the nature of the study, its purpose and objectives. During 22 months, all patients mechanically ventilated for 3 days or more were systematically sampled for simultaneous presence of *E. coli* in the digestive, oropharyngeal, and respiratory tract (and then once a week if still under mechanical ventilation). This screening was only possible during weekdays. Patients for whom day 3 occurred during the weekend were sampled on the Monday (unless they were extubated in the meantime). For each patient whose samples showed simultaneous colonization of the three sites, clinical, epidemiological, and laboratory data were recorded. Concomitant *E. coli* pneumonia was also reported.

E. coli isolates [see electronic supplementary material (ESM)]

Fecal isolates of *E. coli* were obtained by rectal swabbing performed during routine surveillance of multidrug-resistant bacteria carriage. Oropharyngeal isolates were obtained by swabbing the oropharyngeal cavity immediately before routine oropharyngeal care performed every 6 h on average in our unit.

Respiratory isolates were obtained from respiratory secretions retrieved either during routine tracheal suctioning or during fiberoptic bronchoscopy performed for VAP suspicion.

When all three sites were positive for *E. coli*, five colonies of each site were randomly picked [18], isolated, and stored at −80 °C in brain–heart infusion broth containing glycerol 20 %.

Isolates' genetic background analysis (see ESM)

Triplex polymerase chain reaction (PCR) method [19] was used to determine the *E. coli* phylogenetic group membership (A, B1, B2, D). Multiplex PCR was used to detect genes encoding for seven frequently encountered extraintestinal VF, which belong to the main classes of VF (adhesin, toxin, and iron capture system) (see ESM Table 1) [20]. The VF score was defined as the number of VF present divided by the number of VF tested for each isolate.

Isolates' intrasample genotypic diversity was investigated based on triplex PCR profile and/or VF gene content as in [21]. Different comparisons of intrasample diversity were made: between each site, between VAP and lung colonization, and between VAP and all other samples.

Pulsed-field gel electrophoresis (PFGE) was performed as in [22] on some isolates. VAP B2 phylogroup isolates were further characterized by presence of 11 additional VF [20], PCR O-typing [23], and B2-subgrouping [24].

Antimicrobial susceptibility (see ESM)

Antimicrobial susceptibility of each isolate was determined by disk-diffusion method according to the French Society of Microbiology. Susceptibility score was defined as the sum of active in vitro antimicrobial agents for each isolate. A score of 1 was attributed for a sensitive, 0.5 for an intermediary, and 0 for a resistant isolate, a higher score thus indicating a more sensitive isolate.

Infection/colonization definitions (see ESM)

Infection was defined as a microbiologically proven VAP. Colonization was defined as *E. coli* present in the respiratory sample but in the absence of clinically suspected or microbiologically confirmed VAP.

Animal study

Experiments were performed in pathogen-free 6–8-week-old male BALB/c mice (Janvier SA, France) in compliance with the recommendations of the French Ministry of Agriculture and approved by the French Veterinary Services. *E. coli* isolates were grown overnight (LBMedium®; BIO101 Inc., Carlsbad, CA, USA), and serial dilution was performed to obtain different inocula. To assess pulmonary virulence of various isolates, these microorganisms were inoculated via nasal route [25] under light anesthesia with pentobarbital (Sigma, France) and sevoflurane (Abbott, France). Preliminary experiments using a well-known ExPEC *E. coli* isolate (strain 536) [26] served to determine an inoculum that would yield 60 % mortality. This cutoff was chosen in order to be able to detect more or less virulent strains, being obtained with a 3.6×10^8 colony forming unit (CFU) inoculum per mouse. This value was therefore used for the experiments for each isolate.

Preliminary experiments with each strain tested were used to confirm histologically and microbiologically the presence of pneumonia. Animals were closely monitored for 96 h. Death never occurred after 48 h. This duration was subsequently used to assess mortality in our experiments. Each isolate was tested in 25 mice challenged intranasally with 3.6×10^8 CFU.

Statistical methods

Results are expressed as mean ± standard deviation (SD) if not indicated otherwise. The χ^2 test or Fisher's exact test was used to compare categorical variables, and analysis of variance and *t* test were used for continuous variables. Probability of survival was assessed by Kaplan–Meier analysis. Correlation between content of VF genes and mortality was tested using nonlinear regression. GraphPad Prism 4 (GraphPad Software, San Diego, USA) was used. All tests were two-tailed, with $P < 0.05$ considered significant.

Results

Patients

During the study period, 891 patients were mechanically ventilated, a total of 381 for more than 3 days, of whom 132 patients were sampled. Others were not sampled mainly because they were extubated before the sampling could be performed. All rectal samples were positive for *E. coli*. Among those patients, 12 were also positive in the oropharynx (but negative for the lung) and 25 had positive cultures for *E. coli* at the three sites (rectum, oropharynx, and lung). Positive samples in the lung were always associated with positive oropharyngeal samples.

Five *E. coli* colonies were randomly picked from each sample. Figure 1 depicts the flowchart of the patients and the isolates. Based on the genotype and antibiotype characterization, a total of 115 distinct isolates were studied.

Patients' characteristics are displayed in Table 1. Twelve of these patients had documented *E. coli* VAP. No significant difference was found between patients with and without VAP in terms of characteristics (Table 1).

Phylogenetic groups and VF genes

Considering all three sites, B2 was the main phylogenetic group (55 %), followed by group A (28 %), D (13 %), and B1 (3 %). The distribution pattern (Fig. 2) was similar among sites, although there was a trend towards a higher proportion of B2 in the respiratory tract than in the oropharyngeal and the rectal site (66, 51, and 49 %, respectively).

Table 2 details the distribution of VF genes among phylogenetic groups. All screened VF genes were different in B2 compared with non-B2 isolates: all but *traT* and *aer* genes were more prevalent in B2 isolates. Furthermore, B2 isolates had a significantly higher virulence score than non-B2 isolates (0.70 ± 0.18 versus 0.37 ± 0.25; $P < 0.0001$). There was a trend towards more isolates with a greater virulence score at the respiratory and oropharyngeal sites than the rectal site (data not shown). When considering respiratory samples only (Table 3), there was a trend toward more B2 strains and

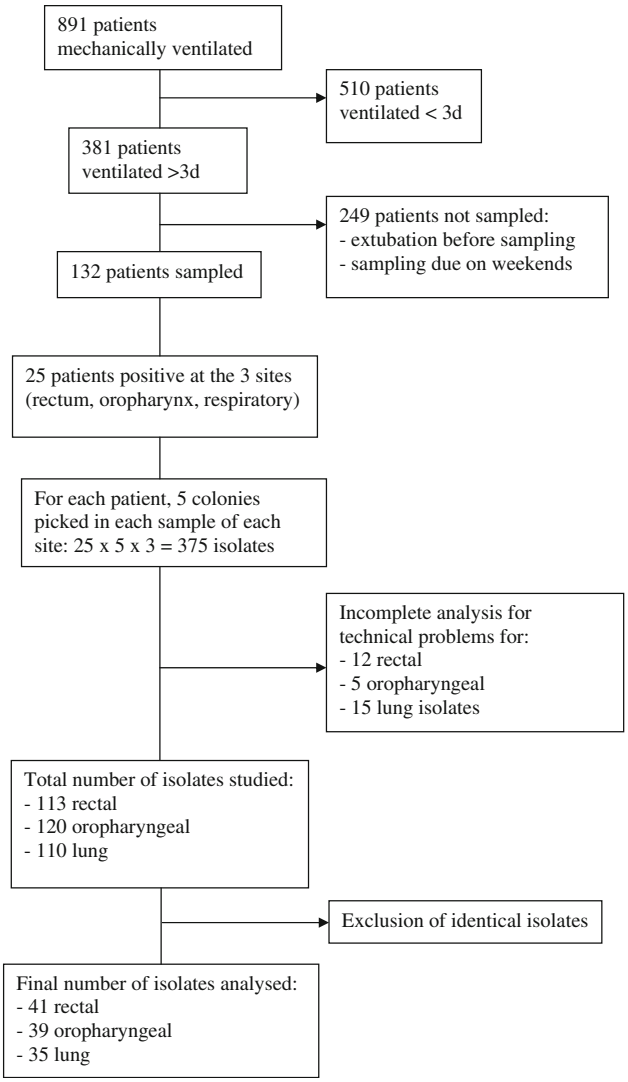

Fig. 1 Flowchart of patients and isolates. One hundred fifteen distinct *E. coli* isolates were obtained from 343 studied isolates from 25 patients. Isolates were considered as identical when they exhibited identical PCR phylotyping pattern, VF gene content, and antibiotype

Table 1 Patient demographic and clinical characteristics

	Pneumonia ($n = 12$)	Lung colonization ($n = 13$)	*P* value
Age (years)	63 (49–70.5)	62 (46.5–69.5)	0.84
Male gender, *n* (%)	9 (75)	10 (77)	1
Comorbid condition, *n*			
Neoplastic disease	2	4	
Cirrhosis	2	6	
Chronic kidney disease	2	1	
Dialysis	1	0	
COPD	4	4	
Chronic heart failure	4	1	
Chronic alcohol consumption	5	6	
Reason for ICU admission, *n*			
Community-acquired pneumonia	3	3	
COPD	3	1	
Status epilepticus	2	2	
Coma	1	3	
Cardiac arrest	0	1	
Septic shock	1	1	
Acute pulmonary edema	1	0	
Acute renal failure	1	0	
Abdominal surgery	0	1	
Hemorrhagic shock	0	1	
Severity Score (SAPS II) on admission [50]	42.5 (34.5–45.5)	45 (37–59.5)	0.35
Duration of mechanical ventilation prior to *E. coli* isolation (days)	10.5 (3.5–20)	4 (2.5–8)	0.15
Ongoing exposure to antimicrobial therapy,[a] *n* (%)	7 (58)	9 (69)	0.57
Prior exposure to antimicrobial therapy,[a] *n* (%)	8 (67)	4 (31)	0.07
ICU mortality	6 (50 %)	4 (30 %)	0.33

COPD chronic obstructive pulmonary disease, *SAPS* Simplified Acute Physiology Score

[a] An exhaustive list of ongoing and prior antimicrobial therapy can be found in ESM Table 2

fewer A strains in isolates from patients with VAP than from those without. In addition, VAP isolates had a significantly higher virulence score than those coming from patients with colonization only. This also held true when only considering non-B2 isolates (data not shown). Among VF genes, *iroN* and *sfa* were significantly more present in VAP isolates than colonization isolates (Table 3). Further characterization of the 16 VAP B2 isolates showed that only two belonged to the frequently recovered subgroup II [multilocus sequence type complex (STc)73)] [27]. Of note, five isolates from four patients belonged to subgroup III (STc127), which encompasses the highly virulent archetypal strain 536 [26] (Table 4).

Individual characteristics of the respiratory isolates of the 12 VAP patients are given in ESM Table 3. Two patients were considered to have VAP despite bacterial count below threshold. Antimicrobial agents were given in these patients because VAP was strongly suspected in view of a low PaO_2/FiO_2 ratio with new radiological infiltrate, no other site of infection, and favorable outcome with appropriate anti-*E. coli* agents. Of note, lung microbiological sampling in these patients was performed while patients had already started receiving antibiotics for VAP suspicion, a situation in which thresholds of bacterial count should be reduced to maintain validity [28].

Isolates' intrasample genotypic diversity was assessed, and no difference was found; For example, the mean and range of phylogenetic group/subgroups among the five

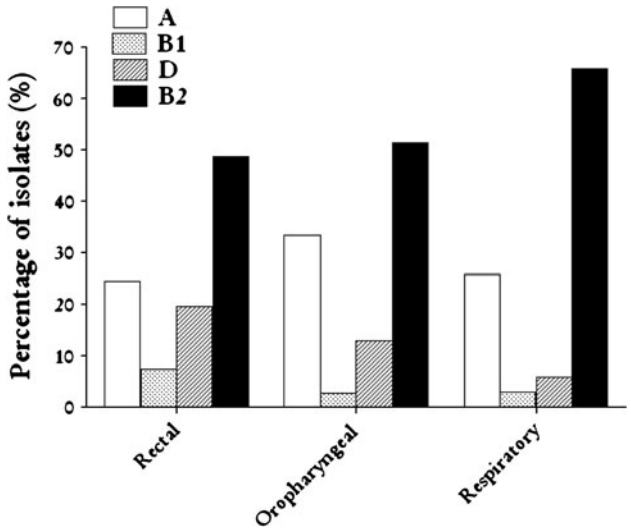

Fig. 2 Distribution of phylogenetic groups A, B1, D, and B2 at the three sites where *E. coli* was sampled: rectum, oropharynx, and respiratory tract. The distribution pattern was similar among sites, although there was a trend towards a higher proportion of B2 in the respiratory tract than in the oropharyngeal and the rectal site (66, 51, and 49 %, respectively)

isolates originating from rectum, oropharynx, and lung were 1.14 (1–3), 1, and 1, respectively.

Antimicrobial susceptibility

Antimicrobial susceptibility to seven clinically relevant antibiotics is summarized in ESM Table 4. Of all isolates, 11 % were piperacillin–tazobactam resistant or intermediate and third-generation cephalosporin susceptible.

Two patients had extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates at the three sites. B2 isolates were significantly more sensitive than non-B2 isolates with a greater susceptibility score (15.0 ± 2.7 versus 8.6 ± 3.7; $P < 0.0001$).

In 12 patients, all rectal, oropharyngeal, and respiratory isolates held the same antimicrobial susceptibility profile. In the remaining 13, intrasite diversity was observed (ESM Fig. 1). Four patients with various antibiotic susceptibility profiles in respiratory samples (ESM Table 5) had VAP. To differentiate further, PFGE were performed, and for three of them, similar genomic identities were evidenced (less than 11 % difference), suggesting a monoclonal population with microheterogeneity. For the last patient, the PFGE pattern evidenced polyclonal infection. No other case of antimicrobial heteroresistance was seen in the respiratory samples.

Animal study

Representative photos of histological features of pneumonia obtained during preliminary experiments are displayed in ESM Fig. 2. Characteristics of the six representative isolates that were used are detailed in Table 5. Twenty-five mice were used per isolate, thus a total of 150 mice were tested. No death occurred before 6 h after inoculation. We found significant differences in survival rates in mice challenged with these isolates (Fig. 3a). The B2 isolate with the greatest content in VF genes ($n = 7$) induced the highest mortality. A B2 isolate carrying five VF genes had significantly higher mortality power than A, B1, or B2 isolates with fewer VF. We found a very strong positive correlation ($R^2 = 0.99$) between the numbers of VF genes carried by *E. coli* isolates and mortality induced in the pneumonia model (Fig. 3b).

Discussion

To the best of our knowledge, this is the first study showing genotypic and phenotypic characteristics of *E. coli* isolates responsible for VAP in ICU patients and

Table 2 Distribution of virulence factor genes according to the phylogenetic group of the 115 *E. coli* isolates sampled from the three sites

Virulence factor genes	Phylogenetic group					
	A $n = 32$	B1 $n = 5$	D $n = 15$	Non-B2 $n = 52$	B2 $n = 63$	P*
papC, *n* (%)	8 (25 %)	0	2 (13 %)	10 (19 %)	49 (77 %)	<0.0001
hlyC	0	3 (60 %)	0	3 (6 %)	48 (76 %)	<0.0001
aer	20 (63 %)	1 (20 %)	11 (73 %)	32 (62 %)	24 (38 %)	0.0123
sfa	nd	nd	nd	nd	52 (83 %)	–
cnf1	nd	nd	nd	nd	47 (75 %)	–
traT	15 (47 %)	1 (20 %)	15 (100 %)	31 (60 %)	26 (41 %)	0.0502
iroN	13 (41 %)	1 (20 %)	3 (20 %)	17 (33 %)	60 (95 %)	<0.0001
Virulence score	0.4 ± 0.3	0.24 ± 0.2	0.41 ± 0.2	0.37 ± 0.3	0.70 ± 0.2	<0.0001
Antimicrobial susceptibility score	8.3 ± 3.9	5.0 ± 4.0	10.6 ± 1.4	8.6 ± 3.7	15.0 ± 2.7	<0.0001

sfa was searched for in B2 isolates, and *cnf1* in B2 isolates carrying *hlyC*

* Comparisons made between B2 and non-B2 isolates

Table 3 Phylogenetic group, content in virulence factor genes, and antibiotic susceptibility score of *E. coli* isolates from VAP and lung colonization

	VAP, $n = 21$	Lung colonization, $n = 14$	P
Phylogenetic group, *n* (%)			
A	4 (19 %)	5 (36 %)	0.236
B1	0	1 (7 %)	0.100
D	1 (5 %)	1 (7 %)	0.740
B2	16 (76 %)	7 (50 %)	0.109
Virulence factor, *n* (%)			
papC	15 (71 %)	6(43 %)	0.163
aer	8 (38 %)	5 (36 %)	0.886
hlyC	12 (57 %)	6 (43 %)	0.406
traT	10 (48 %)	6 (43 %)	0.781
iroN	18 (86 %)	7 (50 %)	0.022
sfa	15 (83 %)	5 (36 %)	0.006
cnf1	12 (92 %)	4 (29 %)	0.007
Virulence score	0.651 ± 0.196	0.435 ± 0.317	0.018
Antimicrobial susceptibility score	15.8 ± 1.55	10.5 ± 4.74	<0.0001

sfa was screened in 18 pneumonia and 8 colonization isolates, and *cnf1* was screened in 13 pneumonia and 6 colonization isolates

comparing them with their rectal and oropharyngeal counterparts. We also assessed pathogenicity and virulence of representative isolates in a pneumonia mouse model. The main results can be summarized as follows: (1) multisite colonization with *E. coli* is frequent, observed in 19 % of our patients; (2) the majority of isolates belong to phylogenetic group B2, characterized by higher susceptibility to antimicrobial agents and greater content of VF genes than the other phylogenetic groups; (3) VAP isolates belong more often to group B2 and have higher VF content than colonization isolates; (4) VAP B2 isolates exhibit infrequent ExPEC sequence types; (5) antimicrobial resistance diversity is observed in respiratory samples of patients with *E. coli* VAP, an unprecedented finding; (6) high extraintestinal virulence of these clinical isolates responsible for VAP is confirmed in a mouse model of *E. coli* pneumonia, and content of VF genes and mice mortality are strongly correlated. These results may have significant impact in VAP prevention and treatment, given the growing prevalence of hospital-acquired infections due to GNB [29], by providing for the first time a detailed analysis of *E. coli* strains colonizing and infecting ICU patients. They may

Table 4 Extended characteristics of the 16 VAP B2 *E. coli* isolates

Strain ID	Patient	Phylogenetic group[a]	B2 sub-group[b]	STc[c]	O-type[d]	K1 antigen (*neuC*)[e]	*sfa/foc*	*iroN*	*aer* (*iucC*)	*iha*	*papC*	*papG*	*hly*	*cnf1*	*hra*	*fyuA*	*irp2*	*sat*	*ireA*	*usp*	*ibeA*	*ompT*	*traT*	Antibiotype[f]
T145	12	B2	IV	141	NT	+	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	+	A
T146	12	B2	IV	141	NT	+	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	−	B
T147	12	B2	II	73	O6a	−	+	+	+	+	+	II	+	+	+	+	+	+	+	+	−	+	−	C
T148	12	B2	IV	141	NT	+	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	−	C
T149	12	B2	IV	141	NT	+	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	+	I
T189	15	B2	II	73	NT	−	+	+	−	−	+	III	+	+	+	+	+	−	+	+	−	+	−	A
T204	16	B2	III	127	O6a	−	+	+	−	−	+	III	+	+	+	+	+	−	+	+	−	+	+	D
T205	16	B2	UG		NT	−	+	+	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	E
T206	16	B2	UG		NT	−	+	+	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	F
T208	16	B2	III	127	O6a	−	+	+	−	−	+	III	+	+	+	+	+	−	+	+	−	+	+	A
T232	18	B2	III	127	O6a	−	+	+	−	−	+	III	+	+	+	+	+	−	+	+	−	+	+	A
AT261	20	B2	UG		O6a	−	−	+	+	−	−	−	−	−	−	+	+	−	−	+	+	+	+	G
PDP272	21	B2	II	73	NT	−	+	+	+	−	−	−	−	−	+	+	+	−	−	+	−	+	+	H
PDP291	22	B2	UG		NT	−	+	+	−	−	+	III	+	+	+	+	+	−	−	+	+	+	−	A
PDP302	23	B2	III	127	O6a	−	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	−	I
PDP317	24	B2	III	127	O6a	−	+	+	−	−	+	III	+	+	+	+	+	−	−	+	−	+	−	B

UG ungrouped, *NT* non O1, O2a, O4, O6a, O7, O12, O15, O16, O18, O25a, O75, O157

[a] Determined by the triplex PCR method of Clermont et al. [19]

[b] According to Ref. [24]

[c] Sequence type complex (STc) according to the Achtman multilocus sequence typing (MLST) scheme (http://mlst.ucc.ie)

[d] According to Ref. [23]

[e] The virulence genes were grouped in pathogenicity islands (PAIs) and plasmid as in Ref. [51] as follow: grey = plasmid, blue = PAI I_{CFT073}, orange = PAI II_{J96}, green = PAI III_{536}, violet = high-pathogenicity island (PAI IV_{536})

[f] Antibiotypes defined for each patient according to a given pattern of antibioresistance

Table 5 Characteristics of the six isolates assessed for mortality in the mouse pneumonia model

Sample site	Phylogenetic group	Number of virulence factor genes	Name of virulence factor genes
Rectal	B1	0	None
Respiratory	A	1	*aer*
Rectal	B2	3	*aer*, *traT*, *iroN*
Respiratory	A	4	*papC*, *aer*, *traT*, *iroN*
Respiratory	B2	5	*papC*, *aer*, *hlyC*, *traT*, *iroN*
Rectal	B2	7	*papC*, *aer*, *hlyC*, *traT*, *iroN*, *sfa*, *cnf1*

provide potential leads to investigate future therapeutic strategies such as those based on vaccine candidates [30–33].

A large-scale epidemiological study on infections in the ICU showed for the first time that *E. coli* was more frequent than *P. aeruginosa* in North America and as frequent in Western Europe [1]. More recent data even suggest that *Enterobacteriaceae* (and among them *E. coli*) are now more often responsible for VAP than *P. aeruginosa* and *Staphylococcus aureus* [34, 35]. Our local epidemiology of VAP is in agreement with these recent findings. Paradoxically, little is known about *E. coli* lung pathogenicity, apart from the inflammatory role of *E. coli* capsule and O-antigen [30] and hemolysin-induced surfactant dysfunction [36].

In order to investigate potential differences in *E. coli* isolates between intestinal commensalism, oropharyngeal colonization, respiratory tract colonization, and subsequent lung infection, we included in our study only patients whose samples were simultaneously positive for *E. coli* at these three sites. With this restriction in mind, we found that 25/132 (19 %) of our patients had multisite positive samples. Among them, 12 were diagnosed with *E. coli* VAP. Because of the specific aims of our study and therefore its design, comparison with existing data is difficult. However, our results expand those previously published dealing with GNB colonization, indicating increasing levels of colonization with increasing patient severity and subsequent risk for VAP [37–39].

The B2 group was the most frequent phylogenetic group at all sites (Fig. 2) with a trend towards a higher proportion in respiratory samples than oropharyngeal and

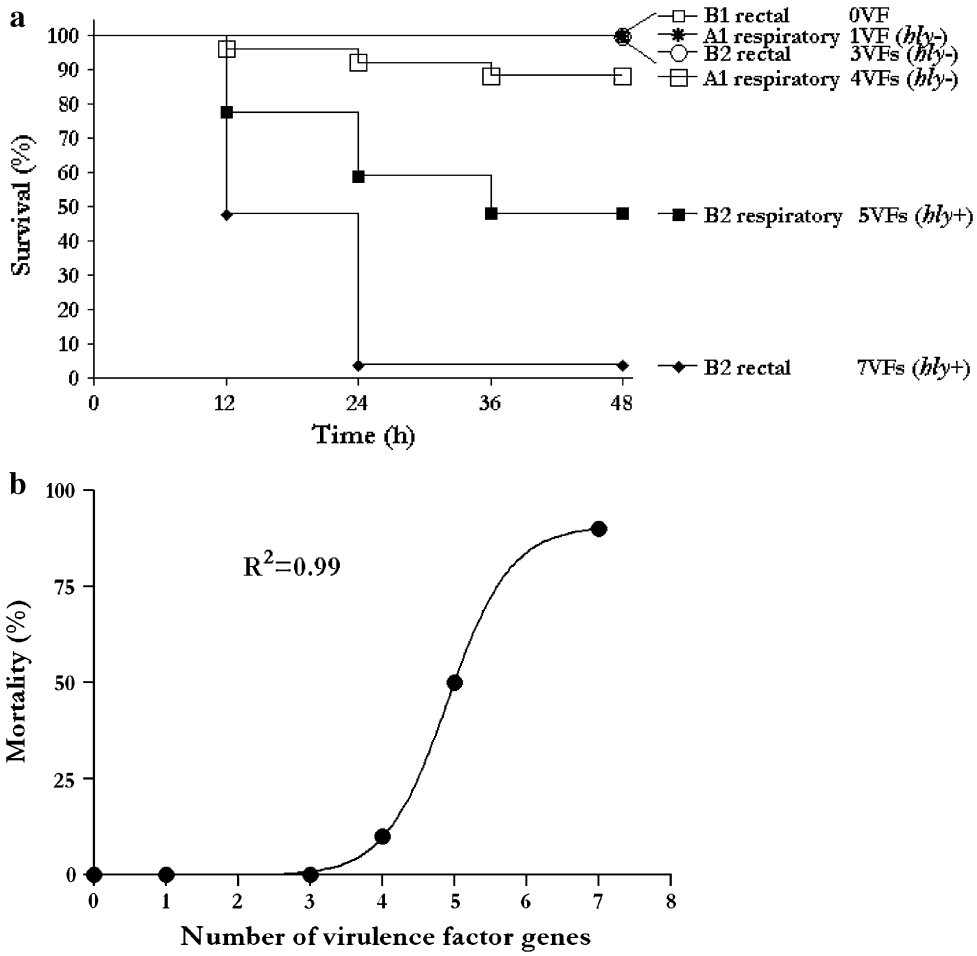

Fig. 3 **a** Survival curves of mice instilled with 3.6×10^8 CFU of different *E. coli* clinical isolates (25 mice per isolate). A significant difference was found ($P < 0.001$) among survival rates. B1, A, and B2 isolates with zero, one, and three virulence factor genes, respectively, exerted significantly less mortality that one group A or two group B2 isolates with, respectively, four, five, and seven virulence factor genes. **b** Relationship between mortality and content of virulence factor genes: a very strong correlation ($R^2 = 0.99$) was found between the number of virulence factor genes carried by *E. coli* isolates in the pneumonia mouse model and mortality. The best fit was observed with a sigmoidal curve, indicating that a minimum of virulence factor genes are required to induce mortality, and that maximum mortality was observed with seven virulence factor genes

rectal samples. Furthermore, these B2 isolates harbored more VF genes than isolates from other phylogenetic groups, in agreement with former findings [16]. This distribution is similar to what has been described in urinary tract infection, septicemia or neonatal meningitis [16, 40, 41], and reflects the greater virulence of B2 strains.

Testing of antibiotic susceptibility showed significantly higher antimicrobial susceptibility in B2, compared with non-B2, for both respiratory and nonrespiratory isolates. This is also in agreement with former findings in other settings [17]. Furthermore, this trade-off between virulence and resistance was also observed within the B2 isolates (ESM Fig. 3). Importantly, we identified 11 % of isolates that were resistant to the combination piperacillin–tazobactam but were still susceptible to third-generation cephalosporin (in relation to secretion of a high level of penicillinase). This was observed in both respiratory (2/35; 6 %) and nonrespiratory isolates (11/80; 14 %) and confirms previous reports on this phenomenon [42]. This finding bears potential therapeutic impact if piperacillin–tazobactam is used as first-line empirical therapy for Gram-negative VAP. Interestingly, we found that *E. coli* isolates retrieved from a single respiratory sample could yield different antimicrobial susceptibility patterns, highlighting antimicrobial resistance polymorphism in *E. coli*. Of note and bearing potential impact on VAP treatment, this diversity was only evidenced in respiratory samples from patients with pneumonia (4/12 patients; 33 %). PFGE was performed in order to test the clonality of the isolates and confirmed that three of these patients had monoclonal infection. In all other respiratory samples, colonies had the same antimicrobial resistance phenotype. Antimicrobial diversity resided in sensitivity to β-lactams in three patients, and in antimicrobial agents of lesser medical interest (trimethoprim or chloramphenicol) for two of these patients and for the remaining patient (ESM Table 5), and resulted probably from plasmid loss. This finding may explain clinical failure of antimicrobial treatment when the more susceptible strain has been picked for the antibiogram. We recently described the same phenomenon in other *E. coli* extraintestinal infections [43], and it has been reported for other microorganisms [44–47]. We, therefore, believe that several colonies of a single respiratory sample should be tested for antimicrobial susceptibility in case of GNB VAP.

We found among the respiratory isolates that those responsible for VAP had greater content of VF genes than those implicated in simple colonization. This held true for both B2 and non-B2 isolates (data not shown). VF genes of interest included *iroN* and *sfa*, indicating that, alongside host factors, pathogen factors also play a major role in lung infection development. *iroN* codes for a siderophore receptor of extraintestinal pathogenic *E. coli*, and has been implicated in the virulence of these strains. Studies have shown that *iroN* may be a potential vaccine candidate to prevent urinary tract infections [48]. We found that *E. coli* isolates responsible for VAP harbored *iroN* more often than those responsible for respiratory tract colonization, showing the unprecedented potential role of *iroN* in lung pathogenicity. Further studies are required to confirm and clarify the respective roles played by these VF, but our findings are consistent with the choice of iron uptake proteins as potential candidates for vaccines against *E. coli* as recently published [32].

Given the scarcity of experimental data with clinical respiratory isolates of *E. coli* (since the bulk of in vitro and in vivo extraintestinal experimental data focus on bloodstream, meningitis, and uropathogenic isolates), we decided to test representative strains in an animal model of pneumonia. We found that isolates with the greatest content of VF genes were associated with the highest mortality. Interestingly, even a rectal isolate was capable of very high mortality, in agreement with the notion that virulence is a by-product of commensalism [24] (Fig. 3a). Not surprisingly [16], but never reported before in the setting of pneumonia, we found a very strong correlation between the number of VF genes carried by isolates and mortality (Fig. 3b). The additive effects of VF have been recently reported experimentally using mutant strains [49].

Limitations and strength of the study

One limitation of the study is the number of patients screened, and therefore studied. This screening (which includes extended-spectrum β-lactamase GNB and methicillin-resistant *S. aureus*) is part of our routine ICU admission procedure. Any incoming patient (whether under mechanical ventilation or not) is screened for the pathogens listed above on ICU admission and then once a week. As mean duration in our ICU is 6 days, a limited number of patients were screened repeatedly during their stay ($n = 20$, 15 %), thus it is difficult to assess the dynamics of colonization, and the sequential acquisition of various isolates at the different sites. We can only speculate on the qualitative order of *E. coli* acquisition sequence: rectum, then oropharynx, then lung. The quantitative order (exact time to acquisition) requires another study, which we are about to perform. Because studies on *E. coli* VAP are very limited (and because *P. aeruginosa* has been already extensively studied), we deliberately focused solely on *E. coli*. Despite a limited number of patients, a major strength of the study is that it provides for the first time an in-depth phenotypic and genotypic analysis of *E. coli* VAP in comparison with their oropharyngeal and rectal counterparts.

Taken together, our results show that respiratory isolates and especially those responsible for VAP are more often B2 *E. coli*, with greater VF gene content than other isolates, including iron uptake genes such as *iroN*. Targeting these proteins by way of vaccines or monoclonal antibodies may help establish novel therapeutic strategies.

Acknowledgments We wish to thank Julie Bex, Marie-Claire Hipeaux, Maggy Grossin and Catherine Deschamps for technical assistance, and all the nurses and assistant-nurses of Louis Mourier ICU for their help in obtaining the microbiological samples. This work was supported by a grant from the Fondation pour la Recherche Médicale to J.M.

Conflicts of interest None.

References

1. Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y, Reinhart K (2009) International study of the prevalence and outcomes of infection in intensive care units. JAMA 302:2323–2329
2. Anonymous (2005) Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 171:388–416
3. Ricard JD, Conti G, Boucherie M, Hormann C, Poelaert J, Quintel M, Rubertsson S, Torres A (2012) A European survey of nosocomial infection control and hospital-acquired pneumonia prevention practices. J Infect 65(4):285–291
4. Markowicz P, Wolff M, Djedaini K, Cohen Y, Chastre J, Delclaux C, Merrer J, Herman B, Veber B, Fontaine A, Dreyfuss D, ARDS Study Group (2000) Multicenter prospective study of ventilator-associated pneumonia during acute respiratory distress syndrome. Incidence, prognosis, and risk factors. Am J Respir Crit Care Med 161:1942–1948
5. Dreyfuss D, Ricard JD (2005) Acute lung injury and bacterial infection. Clin Chest Med 26:105–112
6. Chastre J, Fagon JY (2002) Ventilator-associated pneumonia. Am J Respir Crit Care Med 165:867–903
7. Leu HS, Kaiser DL, Mori M, Woolson RF, Wenzel RP (1989) Hospital-acquired pneumonia. Attributable mortality and morbidity. Am J Epidemiol 129:1258–1267
8. Tenaillon O, Skurnik D, Picard B, Denamur E (2010) The population genetics of commensal Escherichia coli. Nat Rev Microbiol 8:207–217
9. Russo TA, Johnson JR (2000) Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC. J Infect Dis 181:1753-1754
10. Croxen MA, Finlay BB (2010) Molecular mechanisms of Escherichia coli pathogenicity. Nat Rev Microbiol 8:26–38
11. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10:597–602
12. Lynch SV, Wiener-Kronish JP (2008) Novel strategies to combat bacterial virulence. Curr Opin Crit Care 14:593–599
13. Ricard JD (2012) New therapies for pneumonia. Curr Opin Pulm Med 18:181–186
14. Sawa T, Yahr TL, Ohara M, Kurahashi K, Gropper MA, Wiener-Kronish JP, Frank DW (1999) Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury. Nat Med 5:392–398
15. Frank DW, Vallis A, Wiener-Kronish JP, Roy-Burman A, Spack EG, Mullaney BP, Megdoud M, Marks JD, Fritz R, Sawa T (2002) Generation and characterization of a protective monoclonal antibody to Pseudomonas aeruginosa PcrV. J Infect Dis 186:64–73
16. Picard B, Garcia JS, Gouriou S, Duriez P, Brahimi N, Bingen E, Elion J, Denamur E (1999) The link between phylogeny and virulence in Escherichia coli extraintestinal infection. Infect Immun 67:546–553
17. Johnson JR, Kuskowski MA, Gajewski A, Sahm DF, Karlowsky JA (2004) Virulence characteristics and phylogenetic background of multidrug-resistant and antimicrobial-susceptible clinical isolates of Escherichia coli from across the United States, 2000-2001 J Infect Dis 190:1739-1744
18. Escobar-Paramo P, Grenet K, Le Menac'h A, Rode L, Salgado E, Amorin C, Gouriou S, Picard B, Rahimy MC, Andremont A, Denamur E, Ruimy R (2004) Large-scale population structure of human commensal Escherichia coli isolates. Appl Environ Microbiol 70:5698–5700
19. Clermont O, Bonacorsi S, Bingen E (2000) Rapid and simple determination of the Escherichia coli phylogenetic groups. Appl Environ Microbiol 66:4555–4558
20. Lefort A, Panhard X, Clermont O, Woerther PL, Branger C, Mentre F, Fantin B, Wolff M, Denamur E (2011) Host factors and portal of entry outweigh bacterial determinants to predict the severity of Escherichia coli bacteremia. J Clin Microbiol 49:777–783
21. Escobar-Paramo P, Clermont O, Blanc-Potard AB, Bui H, Le Bouguenec C, Denamur E (2004) A specific genetic background is required for acquisition and expression of virulence factors in Escherichia coli. Mol Biol Evol 21:1085–1094
22. Branger C, Bruneau B, Lesimple AL, Bouvet PJ, Berry P, Sevali-Garcia J, Lambert-Zechovsky N (1997) Epidemiological typing of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae isolates responsible for five outbreaks in a university hospital. J Hosp Infect 36:23–36
23. Clermont O, Johnson JR, Menard M, Denamur E (2007) Determination of Escherichia coli O types by allele-specific polymerase chain reaction: application to the O types involved in human septicemia. Diagn Microbiol Infect Dis 57:129–136
24. Le Gall T, Clermont O, Gouriou S, Picard B, Nassif X, Denamur E, Tenaillon O (2007) Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group Escherichia coli strains. Mol Biol Evol 24:2373–2384

25. Byrd W, Mog SR, Cassels FJ (2003) Pathogenicity and immune response measured in mice following intranasal challenge with enterotoxigenic Escherichia coli strains H10407 and B7A. Infect Immun 71:13–21
26. Brzuszkiewicz E, Bruggemann H, Liesegang H, Emmerth M, Olschlager T, Nagy G, Albermann K, Wagner C, Buchrieser C, Emody L, Gottschalk G, Hacker J, Dobrindt U (2006) How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic Escherichia coli strains. Proc Natl Acad Sci USA 103:12879–12884
27. Gibreel TM, Dodgson AR, Cheesbrough J, Fox AJ, Bolton FJ, Upton M (2011) Population structure, virulence potential and antibiotic susceptibility of uropathogenic Escherichia coli from Northwest England. J Antimicrob Chemother 67:346–356
28. Souweine B, Veber B, Bedos JP, Gachot B, Dombret MC, Regnier B, Wolff M (1998) Diagnostic accuracy of protected specimen brush and bronchoalveolar lavage in nosocomial pneumonia: impact of previous antimicrobial treatments. Crit Care Med 26:236–244
29. Peleg AY, Hooper DC (2010) Hospital-acquired infections due to gram-negative bacteria. N Engl J Med 362:1804–1813
30. Russo TA, Davidson BA, Carlino-MacDonald UB, Helinski JD, Priore RL, Knight PR 3rd (2003) The effects of Escherichia coli capsule, O-antigen, host neutrophils, and complement in a rat model of Gram-negative pneumonia. FEMS Microbiol Lett 226:355–361
31. Durant L, Metais A, Soulama-Mouze C, Genevard JM, Nassif X, Escaich S (2007) Identification of candidates for a subunit vaccine against extraintestinal pathogenic Escherichia coli. Infect Immun 75:1916–1925
32. Wieser A, Romann E, Magistro G, Hoffmann C, Norenberg D, Weinert K, Schubert S (2010) A multiepitope subunit vaccine conveys protection against extraintestinal pathogenic Escherichia coli in mice. Infect Immun 78:3432–3442
33. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HLT (2009) Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS Pathog 5:e1000586
34. Hamet M, Pavon A, Dalle F, Pechinot A, Prin S, Quenot JP, Charles PE (2012) Candida spp. airway colonization could promote antibiotic-resistant bacteria selection in patients with suspected ventilator-associated pneumonia. Intensive Care Med 38(8):1272–1279
35. Ricard JD, Roux D (2012) Candida colonization in ventilated ICU patients: no longer a bystander! Intensive Care Med 38(8):1243–1245
36. Russo TA, Wang Z, Davidson BA, Genagon SA, Beanan JM, Olson R, Holm BA, Knight PR 3rd, Chess PR, Notter RH (2007) Surfactant dysfunction and lung injury due to the E. coli virulence factor hemolysin in a rat pneumonia model. Am J Physiol Lung Cell Mol Physiol 292:L632–L643
37. Johanson WG, Pierce AK, Sanford JP (1969) Changing pharyngeal bacterial flora of hospitalized patients. Emergence of gram-negative bacilli. N Engl J Med 281:1137–1140
38. Johanson WG Jr, Pierce AK, Sanford JP, Thomas GD, Johanson WG (1972) Nosocomial respiratory infections with gram-negative bacilli. The significance of colonization of the respiratory tract. Ann Intern Med 77:701–706
39. de Latorre FJ, Pont T, Ferrer A, Rossello J, Palomar M, Planas M (1995) Pattern of tracheal colonization during mechanical ventilation. Am J Respir Crit Care Med 152:1028–1033
40. Bingen E, Picard B, Brahimi N, Mathy S, Desjardins P, Elion J, Denamur E (1998) Phylogenetic analysis of Escherichia coli strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. J Infect Dis 177:642–650
41. Sannes MR, Kuskowski MA, Owens K, Gajewski A, Johnson JR (2004) Virulence factor profiles and phylogenetic background of Escherichia coli isolates from veterans with bacteremia and uninfected control subjects. J Infect Dis 190:2121–2128
42. Meybeck A, Ricard JD, Barnaud G, Eveillard M, Chevrel G, Mounier R, Dreyfuss D (2008) Incidence and impact on clinical outcome of infections with piperacillin/tazobactam resistant Escherichia coli in ICU: a retrospective study. BMC Infect Dis 8:67
43. Levert M, Zamfir O, Clermont O, Bouvet O, Lespinats S, Hipeaux M, Branger C, Picard B, Saint-Ruf C, Norel F, Balliau T, Zivy M, Le Nagard H, Cruvellier S, Chane-Woo-Ming B, Nilsson S, Gudelj I, Phan K, Ferenci T, Tenaillon O, Denamur E (2010) Molecular and evolutionary bases of within-patient genotypic and phenotypic diversity in Escherichia coli extraintestinal infections. PLoS Pathog 6:e1001125
44. Morand B, Muhlemann K (2007) Heteroresistance to penicillin in Streptococcus pneumoniae. Proc Natl Acad Sci USA 104:14098–14103
45. Rinder H, Mieskes KT, Loscher T (2001) Heteroresistance in Mycobacterium tuberculosis. Int J Tuberc Lung Dis 5:339–345
46. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L (2006) Heteroresistance to colistin in multidrug-resistant Acinetobacter baumannii. Antimicrob Agents Chemother 50:2946–2950
47. Yamazumi T, Pfaller MA, Messer SA, Houston AK, Boyken L, Hollis RJ, Furuta I, Jones RN (2003) Characterization of heteroresistance to fluconazole among clinical isolates of Cryptococcus neoformans. J Clin Microbiol 41:267–272
48. Russo TA, McFadden CD, Carlino-MacDonald UB, Beanan JM, Olson R, Wilding GE (2003) The Siderophore receptor IroN of extraintestinal pathogenic Escherichia coli is a potential vaccine candidate. Infect Immun 71:7164–7169
49. Tourret J, Diard M, Garry L, Matic I, Denamur E (2010) Effects of single and multiple pathogenicity island deletions on uropathogenic Escherichia coli strain 536 intrinsic extra-intestinal virulence. Int J Med Microbiol 300:435–439
50. Le Gall JR, Lemeshow S, Saulnier F (1993) A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 270:2957–2963
51. Bingen-Bidois M, Clermont O, Bonacorsi S, Terki M, Brahimi N, Loukil C, Barraud D, Bingen E (2002) Phylogenetic analysis and prevalence of urosepsis strains of Escherichia coli bearing pathogenicity island-like domains. Infect Immun 70:3216–3226