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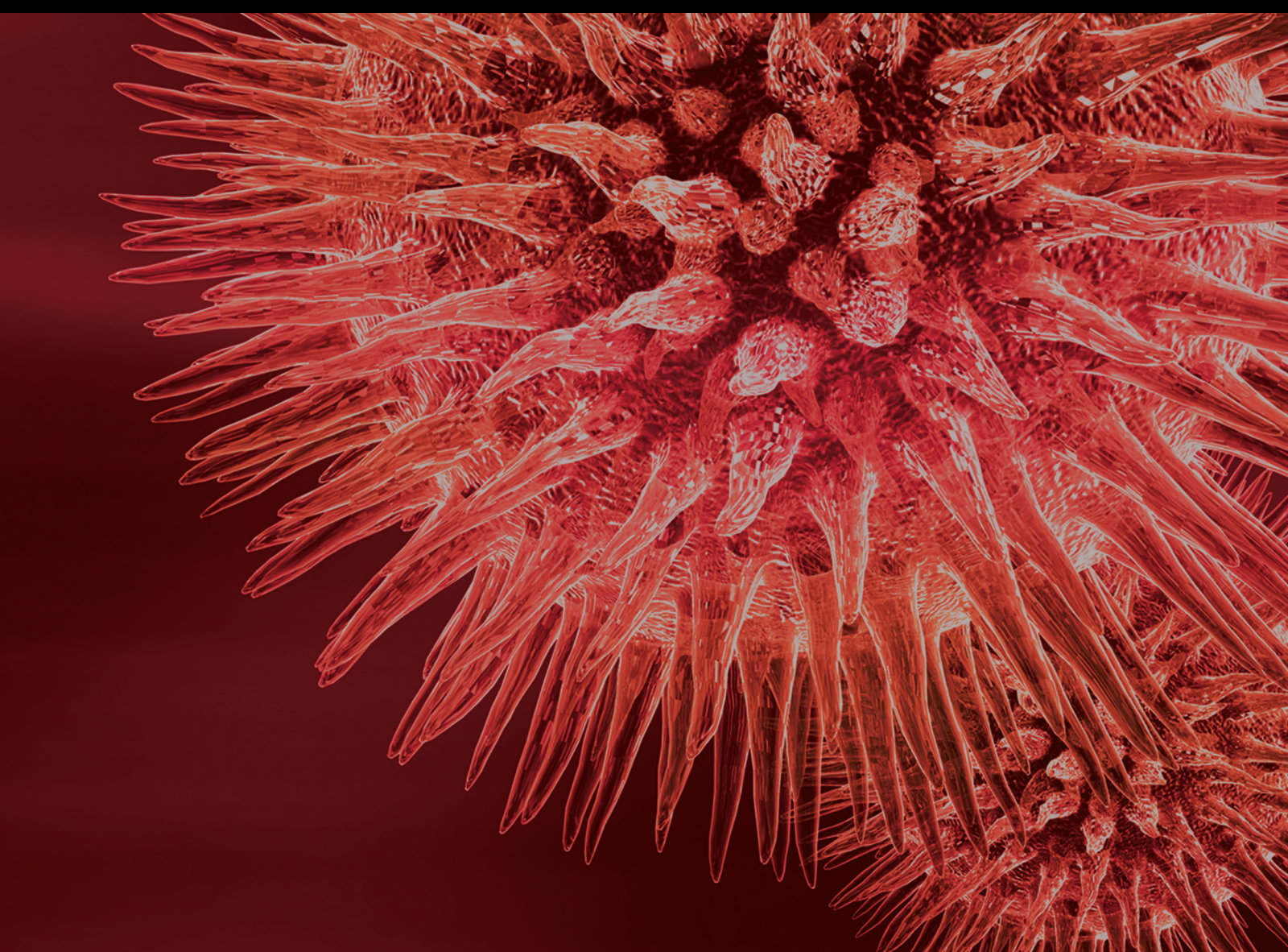

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Editorial

New Insights into Infections due to Multidrug Resistant Gram Negative Bacteria: The Interplay between Lab and Clinic

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The rapid spread of multidrug-resistant bacteria (MDR) such as carbapenem-resistant (CR)-*Klebsiella pneumoniae* and other *Enterobacteriaceae*, CR *Acinetobacter baumannii* (CRAB), and MDR *Pseudomonas aeruginosa* has become a public health concern, especially in some countries where the diffusion of carbapenem-resistant microorganisms is endemic [1].

The knowledge of the local epidemiology and the early identification of patients at risk for MDR Gram negative (GN) acquisition/infection are crucial for prompting an appropriate empirical antimicrobial therapy, whose adequacy is a key factor for reducing mortality [2].

In the absence of randomized clinical trial data, the optimal treatment of infections caused by CR GN is a real challenge for physicians [3]. In observational studies, combination therapy seemed to bring more survival benefits than monotherapy for severe infections due to CR *Enterobacteriaceae* (CRE), whereas this is less clear for CRAB infections, with a recent randomized clinical trial suggesting no advantages of colistin-meropenem combination vs. colistin monotherapy for severe CRAB infections [4]. New agents targeting MDR GN, showing restricted/preferential activity against certain type of carbapenemases, seem to exert potent activity, but should not be used indiscriminately, in line with antimicrobial stewardship principles [5]. For instance, while the first observational data for ceftazidime-avibactam indicate high survival rates in case of CRE infections [6], the possible development of resistance is of concern and resistance strains have already been reported from different geographic areas [7]. In case of resistance to both old and novel agents, alternative revolutionary approaches such as the combination of two carbapenems [8] [i.e., the double-carbapenem (DC) regimen] might still retain an important place in therapy, especially in patients with high risk of mortality, pan-drug resistant organisms, and lack of therapeutic options [9].

Since the choice of the best regimen for the treatment of MDR GN remains a matter of debate [3], the contribution of both microbiology and pharmacology laboratories is crucial for the optimization of the available treatments. In fact, several studies suggested a correlation between the carbapenem MICs of the MDR GN and the clinical effectiveness, with regimens containing high-dose carbapenems being possibly associated with better outcomes [3]. Furthermore, clinicians should be aware that traditional antimicrobial susceptibility reports do not longer suffice to provide optimal information. Thus, the evolution of susceptibility profiling that also includes synergy testing [10] or fast and molecular microbiology is nowadays needful [2]. By using advanced technology such as comprehensive genomic analysis, the investigation and characterization of the genetic background and horizontally transferable MDR resistance in GN might be determined. However, it is also important to note that whether or not to introduce advanced (but often also costly) technologies into the laboratory workflow is a choice that should always be carefully balanced locally, taking into

account also the local availability of personnel and resources, in order to both obtain and maximize the diagnostic advantage compared to standard methods [11].

Diffusion of MDR GN has also started to affect the community, thereby putting patients at risk of developing uncomplicated but difficult-to-treat infections due to MDR GN that may severely impair their quality of life and also sometimes become life-threatening because of ineffective initial treatment.

Innovative therapeutic strategies aiming at inhibition of microbial growth or virulence factors of MDR GN, such as essential oils [12] or small molecule inhibitors, are highly attractive as they may show potential as part of antimicrobial combination against MDR GN or reduce the severity of clinical manifestations and improve antibacterial immune responses. In this regard, critical components of mucosal immune system might be involved in the prevention of infection and, if altered in the expression, might correlate with infection progression.

Given the complexity of management of MDR GN infections, which need to be tailored to the severity of patients' underlying conditions and infection, to the type of MDR GN isolates with their specific antimicrobial susceptibility profiles, and to the knowledge of the local epidemiology, a multidisciplinary approach involving clinicians, the laboratory, and clinical pharmacologists as well as an antimicrobial stewardship program is recommended.

Among the articles received in response to the call for papers and after a rigorous refereeing process, 6 papers were accepted for publication in this special issue. The articles dealt with the emerging and threatening problem of MDR in GN microorganisms from both clinical and laboratory point of view.

H. Frickmann et al. thoroughly discussed the use of advanced diagnostic point-of-care options during military operations, a peculiar setting where MDR GN may be responsible for colonization and wound infections, as well as transmission to close contacts.

In their review, D. S. Lee et al. explored and enlisted essential baseline aspects for dealing with community-acquired urinary tract infections due to *Escherichia coli* in order to prevent or at least delaying development of resistance.

F. Cancelli et al. showed that the double-carbapenem regimen might represent a valid and effective therapeutic option in patients with infections due to K. pneumoniae carbapenemase (KPC) producing CR *K. pneumoniae*, including those with bacteremic infection and more severe clinical conditions. Of note, the clinical effectiveness was maintained even in the presence of extremely high meropenem MIC.

In the study conducted by S. Alousi et al., a deep genetic analysis of a strain of OXA-48 producing *E. coli* causing bloodstream infection was performed, thus highlighting the need of comprehensive genomic characterization of MDR GN for infection control and antimicrobial stewardship purposes.

In the paper by A. B. Sheremet et al., the authors showed the ability of a new small molecule, Fluorothiazinon, inhibiting type three secretion system of some Gram-negative bacteria, to reduce bacterial load and decrease lung pathology and systemic inflammation in a mouse model of lung infection due to drug-resistant *P. aeruginosa*. The reduced mortality rate of mice treated with Fluorothiazinon strongly suggested a potential use of this molecule as a therapeutic approach for the pulmonary treatment of MDR *P. aeruginosa* infections.

The review of Y.-A. Tsou et al. deals with the role of BPIFA1 (bactericidal/permeability-increasing fold 4 containing family A, member 1), a protein of innate immune system expressed in the upper airway and nasopharyngeal region including the trachea and nasal epithelium, that exhibits a bactericidal and antibiofilm activity against both Gram-positive and Gram-negative bacteria. By binding LPS, BPIFA1 is also able to inhibit the growth of *P. aeruginosa* and *K. pneumoniae*.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue. Outside the special issue, DR Giacobbe reports personal fees from Stepstone Pharma GmbH and an unconditioned grant from MSD Italia and N. Miller has these disclosures regarding commercial entities: Grant Support (Roche Molecular), Contract speaker (BioFire Diagnostics).

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Research Article

Role of Double-Carbapenem Regimen in the Treatment of Infections due to Carbapenemase Producing Carbapenem-Resistant *Enterobacteriaceae*: A Single-Center, Observational Study

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Purpose. (i) To compare infections caused by carbapenem-susceptible (CS) and carbapenemase producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE); (ii) to evaluate the clinical effectiveness of the double-carbapenem (DC) regimen in comparison with the best available treatment (BAT) in infections caused by CP-CRE; and (iii) to determine the exact minimal inhibitory concentrations (MICs) of meropenem/ertapenem (MEM/ETP) and the degree of *in vitro* ETP+MEM synergism in subjects receiving the DC. *Methodology*. Over a 3-year period (2014-2017), patients with infections due to *Enterobacteriaceae* were included in a single-center, retrospective, observational study. According to the susceptibility to carbapenems, subjects were divided into CSE and CP-CRE groups. CP-CRE group was further divided into subjects receiving the DC regimen and those treated with other regimens (BAT group). Clinical characteristics and the presence of 5th-day response and 60-day outcome were evaluated for DC and BAT groups. The determination of MEM and ETP actual MICs and the MEM+ETP synergistic activity were performed on strains obtained from subjects receiving the DC regimen. *Results*. A total of 128 patients were included in the study: 55/128 (43%) with infections due to CP-CRE and 73/128 (57%) with infections due to CSE. Among CP-CRE (n=55), 21 subjects (39%) were treated with the DC regimen whereas 34 (61%) received BAT. No differences in terms of severity of infection, presence/absence of concomitant bacteremia, type of infection, and resolution of infection were found; in contrast, DC group tended to have a higher rate of sepsis or septic shock at the onset of infection and a higher rate of 5th-day response. MICs 50/90 were 256/512 and 256/256 μg/mL for MEM and ETP, respectively. Overall, complete *in vitro* synergism was found in 6/20 strains (30%). *Conclusion*. The DC regimen is a valid and effective therapeutic option in patients with infections due to KPC producing CRE, including those with bacteremic infection and more severe clinical conditions. The clinical effectiveness is maintained even in the presence of extremely high MEM MICs.

1. Background

The rapid spread of multidrug-resistant bacteria has become a public health concern, especially in some countries where the spread of carbapenem-resistant microorganisms is endemic [1]. In particular, infections caused by CP-CRE are associated with a high treatment failure and consequent high mortality, given the limited therapeutic options and the lack of worldwide availability of new drugs such as ceftazidime/avibactam [2].

Risk factors for CP-CRE infections have been widely investigated and serve as possible drivers of prompting an appropriate antimicrobial therapy, aiming at improving the infection cure and reducing mortality [3].

Although the combination therapy is preferred over monotherapy, the optimal management of CP-CRE systemic infections remains a real challenge, which seems even more complicated given the emergence of resistance to ceftazidime/avibactam [4] and the rising diffusion of strains harbouring enzymes other than carbapenemases [1].

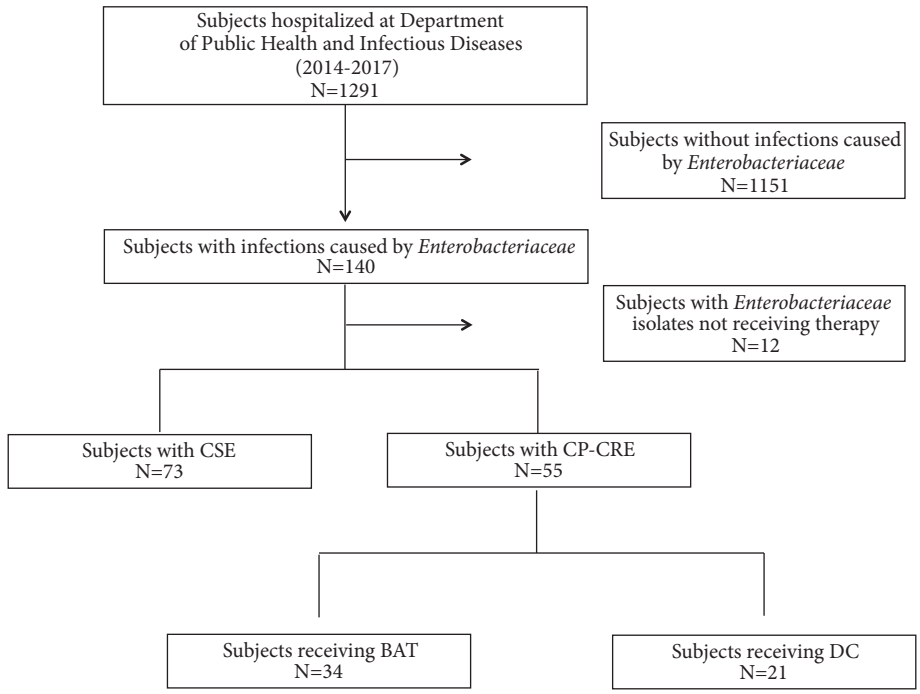

Figure 1: Flow chart of the study. CSE: carbapenem-susceptible *Enterobacteriaceae*; CP-CRE: carbapenemase producing carbapenem-resistant *Enterobacteriaceae*; DC: double-carbapenem; BAT: best available treatment.

Recently, several efforts have been made with the aim of finding the most appropriate antimicrobial regimen according to the susceptibility profile of the microorganisms and the severity of infection [5, 6]. In this setting, the double-carbapenem regimen retains a place in therapy in patients with high risk of mortality, pan-drug resistant organisms, and lack of therapeutic options [5].

Based on these premises, aims of the study were (i) to compare infections caused by CSE with those caused by CP-CRE, (ii) to evaluate the clinical effectiveness of the DC regimen in comparison with BAT in infections caused by CP-CRE, and (iii) to determine the exact MICs of MEM/ETP and the degree of *in vitro* ETP+MEM synergism in subjects receiving the DC.

2. Materials and Methods

2.1. Study Population. This was a single-center, retrospective, observational study including patients hospitalized over a 3-year period (2014-2017), at the Department of Public Health and Infectious Diseases (Sapienza University, Rome) with infections due to *Enterobacteriaceae*.

Carbapenem susceptibility was determined using VITEK-2 system and interpreted in accordance with EUCAST breakpoint [6] whereas CP-CRE were defined following CDC case definition [7].

Accordingly, subjects were divided into 2 groups: CP-CRE and CSE. CP-CRE group was further divided into subjects receiving the DC regimen and those treated with other regimens, defined as BAT group (Figure 1).

The DC consisted of ertapenem (1 g/day) followed by high doses of meropenem (6 g/day) or modified according to creatinine clearance. BAT was defined as the definitive therapy chosen by the Infectious Diseases specialists according to susceptibility profile of the microorganisms and the clinical conditions of the patients.

Demographic, clinical, and laboratoristic parameters were collected for each subject. Inclusion criteria were age >18 and patients with infections due to *Enterobacteriaceae* receiving antimicrobial therapy. Isolates collected from all sites of infection were also included. However, in case of multiple cultures from the same patient, only the first isolate causing infection was considered in the study. Exclusion criteria were age <18 and pregnancy.

Given the unconventionality of the treatment, all study participants receiving the DC regimen gave informed written consent. The study was approved by the local Ethics Committee.

2.2. Definitions. The clinical presentation of infection (sepsis, sepsis shock) was defined in accordance with the international guidelines [8].

The clinical and/or microbiological response at day 5 was defined as resolution of signs and symptoms of infections (i.e., defervescence, improvement of clinical conditions and imaging upon antimicrobial treatment) and/or negativity of cultures performed after 5 days of antimicrobial treatment, respectively, and expressed as a nominal variable. In addition, time to clinical response was defined as time (days) to resolution of fever and improvement in clinical or radiological status, expressed as a continuous variable. As for outcomes,

clinical cure was defined as survival at 60 days, resolution of signs and symptoms of infection, and absence of recurrence at 60 days following the onset of infection [9]. Infection relapse was defined as recrudescence of infection after an initial response [10].

2.3. Microbiological Studies. The antimicrobial susceptibility pattern of *Enterobacteriaceae* was obtained through the VITEK-2 system (bioMerieux, Marcy l'Etoile, France).

Strains obtained from subjects receiving the DC regimen underwent additional microbiological analyses, including the phenotypic determination of carbapenemases [11]. The determination of meropenem and ertapenem actual MICs was obtained by the macrobroth dilution method [12] whereas the synergistic activity of meropenem plus ertapenem was performed by the checkerboard method and the fractional inhibitory concentration index (FICI) calculation. Briefly, a 96-well microtitre plate containing antibiotic combinations at different concentrations and a final inoculum of ~5 x 10^5 CFU/ml of CP-CRE was incubated at 37°C for 24h under static conditions in Mueller Hinton Broth. The FICI of each combination was defined as follows: ∑FIC: FICA + FICB= MICA+B/MICA alone + MICB+A/MICB alone. A FICI ≤0.5 indicated synergism [13]. Experiments were performed in triplicate and the results were averaged.

2.4. Statistical Analysis. Results were expressed as mean ± standard deviation (SD) or median (range) and as percentages for continuous and categorical variables, respectively. Categorical variables (such as clinical and/or microbiological response at day 5) were compared by using the X^2 or Fisher's exact tests, as appropriate, whereas continuous data (such as time to clinical response) were analyzed with Student's t-test and the nonparametric Mann–Whitney test. Statistical analyses were performed using STATA 9 software (STATA Corp. LP, College Station, Texas, USA) and GraphPad Prism version 7 for Windows (Graphpad Software MacKiev), as appropriate. All statistic tests were 2-tailed and a *p value* <0.05 was considered statistically significant.

3. Results

3.1. Carbapenemase Producing Carbapenem-Resistant vs Carbapenem-Susceptible Infections. A total of 128 patients were included in the study: 55/128 (43%) with infections due to CP-CRE and 73/128 (57%) with infections due to CSE (Table 1). Although not statistically significant, length of hospitalization before the onset of infection was higher in CP-CRE than in CSE (median 29.5 vs 17 days, p=0.13).

Clinical presentation was more severe (sepsis and/or septic shock) in CP-CRE than in CSE [10/55 (18.1%) vs 1/73 (1.4%) for sepsis, p=0.0009, 1/55 (1.8%) vs 0/73 (0%) for septic shock, p=0.42, respectively].

Although the time for obtaining clinical response did not differ between the 2 groups (median 4.5 vs 4 days, p=0.16), patients in CP-CRE group tended to have a lower rate of 5^{th}-day response than subjects in CSE group. Compared with CSE, the overall length of hospitalization and mortality were higher in CP-CRE [median 31 vs 16 days, p<0.0001 and 6/55, 10.9% vs 2/74, 2.7%, p=0.07, respectively], with a global lower rate of infection cure at 60-day follow-up (43/55, 78.2% vs 67/73, 91.8%, p=0.03).

With regard to bacterial species, all the CP-CRE were *K. pneumoniae* whereas among CSE 58 (79.5%) were *E. coli* and 15 (20.5%) *K. pneumoniae*.

3.2. Carbapenemase Producing Carbapenem-Resistant Infections: DC Regimen vs BAT. Among CP-CRE (n=55), 21 subjects (39%) were treated with the DC regimen, with 3 subjects having received colistin and/or aminoglycosides prior to switch to the DC for 2, 2, and 3 days, respectively. The remaining 34 (61%) received other regimens [colistin-based combinations: 14 (colistin plus carbapenems±a third *in vitro* active drug: 9; colistin plus tigecycline±a third *in vitro* active drug: 2; colistin monotherapy: 1; colistin plus gentamicin: 1; colistin plus rifampin: 1) and other colistin-free regimens: 20 (aminoglycosides monotherapy: 13; high doses of carbapenems plus aminoglycosides: 6; high doses of meropenem plus fluoroquinolones: 1)].

Patients treated with DC tended to have a more severe clinical presentation (sepsis and/or septic shock) [6/21 (28.6%) vs 4/34 (11.7%) for sepsis, p=0.16, 1/21 (4.8%) vs 0/21 (0%) for septic shock, p=0.38, respectively]. Bacteremic infections were 7/21 (33.3%) and 7/34 (20.5%, p=0.34) for DC and BAT groups, respectively. As expected, in the DC group, colistin and aminoglycosides resistance rates were higher than those found in BAT group [10/21 (47.6%) vs 6/34 (17.6%) and 8/21 (38.1%) vs 4/34 (11.7%), p=0.04, 0.01, respectively] (Table 2).

Although DC patients tended to have a higher 5^{th}-day response rate [13/21 (61.9%) vs 14/34 (41.1%), p=0.13], with a shorter time to clinical response (median 3 vs 6 days, p=0.25), the infection cure at 60-days did not differ between the two groups [16/21 (76.1%) vs 27/34 (79.4%), p=0.73]. In particular, mortality was 2/21 (9.5%) vs 4/34 (11.7%, p=0.99). A total of 6 patients had a recurrence of infection, equally distributed between DC and BAT groups [3/21 (14.2%) vs 3/34 (8.8%), p=0.66] (Table 2).

3.3. Microbiological Analyses. Microbiological analyses were performed on strains collected from subjects receiving DC regimen (n=20; 1 strain was not available) and are represented in Table 3.

All the isolated CP-CRE harboured KPC enzymes, which is in accordance with the local epidemiology [14].

MICs 50/90 were 256/512 and 256/256 μg/mL for meropenem and ertapenem, respectively. Overall, complete *in vitro* synergism (expressed as FICI ≤0.5) was found in 6/20 strains (30%).

Among subjects with meropenem MIC was ≤128 μg/mL (n=7), which has been found as the best *in vitro* MIC value for predicting the highest activity of the DC [15] the clinical outcome at 60 days was cure or relapse in the totality of cases (5 cure, 2 relapse) whereas in patients with meropenem MIC >128 μg/mL (n=13) death occurred in 2 cases and cure in 11 (Table 4).

Table 1: General characteristics of study population, according to the carbapenem susceptibility of *Enterobacteriaceae*. CP-CRE: carbapenemase producing carbapenem-resistant *Enterobacteriaceae;* CSE: carbapenem-susceptible *Enterobacteriaceae*;COPD: chronic obstructive pulmonary disease; HA: hospital-acquired; CA: community-acquired. ◦: Only subjects with active urinary tract infection requiring antimicrobial therapy were included; §: defined as resolution of signs and symptoms of infections (i.e., defervescence, improvement of clinical conditions and imaging upon antimicrobial treatment) and/or negativity of cultures performed after 5 days of antimicrobial treatment, respectively. ∗∗: sepsis and septic shock were defined according to international guidelines [8].

	CP-CRE (n=55)	CSE (n=73)	*p-value*
General characteristics			
Age (years), mean (± SD)	61.15 (± 15.4)	64.7 (± 19.5)	0.2595
M:F, n	36:19	48:25	1
Charlson comorbidity index, mean (±SD)	5.24 (± 2.97)	5.82 (± 3.5)	0.3223
Comorbidity, n (%)			
Cancer	20 (36.4)	22 (30.1)	0.5687
Chronic Kidney disease	10 (18.2)	14 (19.2)	1.0000
Diabetes mellitus	12 (21.8)	13 (17.8)	0.6543
Heart failure	24 (43.6)	24 (32.9)	0.2689
Liver disease	5 (9.1)	8 (11)	0,7769
COPD	5 (9.1)	10 (13.7)	0.4143
Modality acquisition of infection, n			
HA:CA	50:5	38:35	< **0.0001**
Risk factors			
Hospitalization day before infection, mean (± SD), median	36.12 (± 27.6), 29.5	23.46 (±19.5), 17	0.1376
Hospitalization in the last year, n (%)	51 (92.7)	46 (63)	**0.0001**
Urinary catheter, n (%)	34 (61.8)	21 (28.7)	**0.0003**
Central venous catheter, n (%)	26 (47.3)	9 (12.3)	< **0.0001**
Tracheostomy, n (%)	10 (18.2)	0 (0)	**0.0001**
Previous antibiotic therapy (90 days), n (%)			
Cephalosporins	8 (14.5)	7 (9.6)	0.4171
Penicillin	16 (29.1)	13 (17.8)	0.1418
Carbapenems	19 (34.6)	4 (5.5)	**0.0002**
Fluoroquinolones	18 (32.7)	15 (20.5)	0.2311
Colistin	8 (14.5)	2 (2.3)	**0.0190**
Clinical presentation, n (%)			
Sepsis**	10 (18.1)	1 (1.4)	**0.0009**
Septic shock**	1 (1.8)	0	0.4297
Site of infection			
Lung	12 (21.8)	8 (11)	**0.0005**
Urinary tract°	37 (62.3)	52 (71.2)	0.6995
Soft tissue	12 (21.8)	11 (15.1)	0.3588
Bacteremic infection	14 (25.5)	12 (16.4)	0.2680
Primary bacteremia	4 (7.3)	7 (9.6)	0.7566
Type of Enterobacteriaceae, n (%)			< **0.0001**
Escherichia coli	0 (0)	58 (79.5)	
Klebsiella pneumoniae	55 (100)	15 (20.5)	
Antibiotic resistance profile, n (%)			
Carbapenem	55 (100)	0 (0)	< **0.0001**
Fluoroquinolones	53 (96.4)	47 (64.4)	< **0.0001**
Aminoglycosides	12 (21.8)	24 (32.9)	0.2333
Colistin	15 (27.3)	1 (1.4)	< **0.0001**
Tigecycline	30 (54.5)	4 (5.4)	< **0.0001**
Therapy			
Time to clinical response, days, mean (± SD), median	6.6 (± 4.65), 4.5	5.2 ± 4.04, 4	0.1674
5th day response§, n (%)	28 (50.9)	47 (64.4)	0.1487

Table 1: Continued.

	CP-CRE (n=55)	CSE (n=73)	*p-value*
Length of hospitalization, mean (± SD)	39.2 ± 29.5	20.4 ± 14.1	< **0.0001**
median	31	16	
Outcome, n (%):			
Clinical cure	43 (78.2)	67 (91.8)	**0.0393**
Infection relapse	6 (10.9)	4 (5.4)	0.4297
Death	6 (10.9)	2 (2.7)	0.0739

Table 2: Comparison between subjects treated with the double-carbapenem regimen (DC) and those treated with the best available treatment (BAT). ∗: two infections were present in some patients. ∘: only subjects with active urinary tract infections requiring antimicrobial therapy were included; §: defined as resolution of signs and symptoms of infections (i.e., defervescence, improvement of clinical conditions and imaging upon antimicrobial treatment) and/or negativity of cultures performed after 5 days of antimicrobial treatment, respectively. ∗∗: sepsis and septic shock were defined according to international guidelines [8].

	Group DC (n= 21)	**Group BAT (n= 34)**	***p* value**
Demographic characteristics			
Age (years), mean (± SD)	62.28 (± 12.1)	61.18 (± 17)	0.7971
M:F	14:7	21:13	1
Charlson comorbidity index, mean (±SD)	5.14 (± 2.76)	5.39 (± 3.78)	0.7940
Clinical presentation, n (%)			
Sepsis**	6 (28.6)	4 (11.7)	0.1619
Septic shock**	1 (4.8)	0 (0)	0.3889
Site of infection, n (%)			
Lung	4 (19)	8 (23.5)	0.7466
Urinary tract°	11 (52.4)	26 (76.4)	0.0702
Soft tissue	7 (33.3)	5 (14.7)	0.1795
Bacteremic infection	7 (33.3)	7 (20.5)	0.3453
Antibiotic resistance, n (%)			
Fluoroquinolones	21 (100)	31 (91.1)	0.5157
Aminoglycosides	8 (38.1)	4 (11.7)	**0.0424**
Colistin	10 (47.6)	6 (17.6)	**0.0136**
Tigecycline	11 (52.4)	18 (52.9)	1.0000
Therapy:			
Time to clinical response, days, mean (± SD), median	5.5 (± 4.22), 3	7.3 (± 4.87), 6	0.2570
5th day response§, n (%)	13 (61.9)	14 (41.1)	0.1351
Outcome, n (%):			
Clinical cure	16 (76.2)	27 (79.4)	0.7329
Infection relapse	3 (14.2)	3 (8.8)	0.6660
Death	2 (9.5)	4 (11.7)	0.9980

4. Discussion

Infections caused by CP-CRE are characterized by a higher morbidity and mortality than those caused by carbapenem-sensitive strains [2]. Given the worldwide spread of CP-CRE and the growing emergence of resistance to antimicrobials such as colistin and aminoglycosides, which have been used as last resort drugs, there is a growing literature investigating the best therapeutical regimen according to prognostic scores and/or antimicrobial susceptibility pattern of the microorganisms [16].

Furthermore, new agents with activity against CP-CRE show preferential activity against certain type of carbapenemases [17] and unfortunately their availability is still restricted to some countries, with obvious therapeutic limitations. The recent use of drugs such as ceftazidime/avibactam led to the consideration that it might be considered as a valid option in the setting of CRE infection [17]; however, its use might be undermined by the emergence of resistance, especially in strains harbouring KPC-3 enzymes and even during treatment [18].

Table 3: Microbiological studies on strains isolated from patients treated with the double-carbapenem regimen and correlation with clinical outcome. MEM: meropenem; ETP: ertapenem. ∘: complete synergy was defined as FICI ≤ 0.5, indifference as FICI > 0.5–4.0, and antagonism as FICI > 4.0 [13]. ∗: one strain was not available for additional microbiological studies. NA: not applicable.

Pt	*MIC MEM, VITEK-2(μg/mL)*	*MIC ETP, VITEK-2(μg/mL)*	*Actual MIC MEM (μg/mL)*	*Actual MIC ETP (μg/mL)*	*Synergism MEM+ETP*∘	*Outcome*
1	>16	>16	256	256	complete	died
2	>16	>16	512	128	indifference	cured
3	>16	>16	512	256	complete	cured
4	>16	>16	512	256	indifference	cured
5	>16	>16	128	256	indifference	relapsed
6	>16	>16	128	256	indifference	cured
7	>16	>16	128	256	indifference	cured
8	>16	>16	256	256	complete	cured
9	>16	>16	32	64	indifference	cured
10	>16	>16	128	128	indifference	cured
11*	>16	>16	NA	NA	NA	relapsed
12	>16	>16	256	128	indifference	cured
13	>16	>16	256	128	indifference	cured
14	>16	>16	256	256	indifference	cured
15	>16	>16	128	128	indifference	cured
16	>16	>16	256	256	indifference	died
17	>16	>16	512	512	indifference	cured
18	>16	>16	512	512	complete	cured
19	>16	>16	256	128	complete	cured
20	>16	>16	256	128	indifference	cured
21	>16	>16	128	256	complete	relapsed
MIC50/90			**256/512**	**256/256**		

Table 4: Association between meropenem actual MIC (obtained with macrobroth dilution) and clinical outcome after stratification according to meropenem MIC.

Actual MIC meropenem (μg/mL)	**Subjects, n (%)**	**Outcome at 60-days, n (%)**
32	1 (5)	Cure: 1/1 (100)
128	6 (30)	Cure: 4/6 (66.7) Relapse: 2/6 (33.3)
256	8 (40)	Cure: 6/8 (75) Death: 2/8 (25)
512	5 (25)	Cure: 5/5 (100)

In these challenging scenarios, the double-carbapenem regimen has been proposed as a possible therapeutic option in selected cases [14, 19–21]. While there have been positive clinical outcomes studies with double-carbapenem use and while *in vitro* studies have demonstrated bactericidal activity with the combination, the exact mechanism of action is not fully understood [10, 22–26].

In the present study, all consecutive patients with infections caused by *Enterobacteriaceae* hospitalized at the Department of Public Health and Infectious Diseases (Sapienza University, Rome) over a 3-year period were included. Apart from the observed differences between CP-CRE and CSE infections, which confirmed the widely reported data in the literature regarding epidemiology (with *K. pneumoniae* being the most frequent CR-CPE), a more severe clinical presentation and a lower rate of infection cure in CP-CRE, we were able to analyze a consistent number of patients treated with the DC regimen in comparison with the BAT group. As a matter of fact, no differences in terms of severity of infection, presence/absence of concomitant bacteremia, type of infection, and resolution of infection were found; in contrast, subjects treated with the DC tended to have a higher rate of sepsis/septic shock at the onset of infection and a higher rate of 5^{th}-day response. Taken together, these findings confirm that the DC regimen represents a valid therapeutic option when no other alternatives are possible, with a global high clinical cure, similar to that observed with the BAT. However, it should be pointed out that performing the source control (i.e., catheter/stent removal, abscesses drainage) whenever possible as part of infection treatment might have contributed to the overall observed high clinical cure. Of note, the presence of bacteremic infections in one-third of subjects receiving the DC regimen strengthens the

clinical effectiveness of this therapeutic option, which seems to retain its efficacy even in the presence of high bacterial inoculum, typically characteristic of bloodstream infections.

All the CP-CRE strains were *K. pneumoniae*: since KPC represents the most widely spread carbapenemase in our country, the results on the efficacy of DC might be translated even against KPC producing *Enterobacteriaceae* other than *K. pneumoniae* (i.e., *E. coli*) [23].

The results of the present study are in line with some recent investigations evaluating the clinical role of the DC regimen when no other options are available or after failure of first-line regimens [22] or in critically ill patients [27]. In the first study, the authors found a high clinical and microbiological success in a cohort of patients with complicated urinary tract infections (with or without secondary bacteremia), bloodstream infections, pneumonia, and external ventricular drainage infection [22] whereas in the second case-control study conducted in two Italian Intensive Care Units subjects receiving DC regimen presented with more severe clinical condition and had an improved 28-day mortality compared with those treated with standard regimen including colistin, tigecycline, or gentamicin [27].

Moreover, the efficacy of the DC regimen has been demonstrated in immunocompromised patients, including kidney transplanted patients [23, 28] and a patient after allogenic hematopoietic stem cell transplantation [29].

One of the strengths of the present research is represented by the additional microbiological studies performed on the strains collected from patients receiving the DC regimen. In fact, automated systems such as VITEK-2, by indicating high carbapenems MIC as >16 μg/mL, are unable to determine the precise MIC of carbapenems and there are growing evidences supporting the concept that knowing the real MIC of meropenem might influence the therapeutic choice and the effectiveness of carbapenem-based combination [15, 30]. In particular, the DC appeared to be more effective *in vitro* if the meropenem MIC is ≤128 μg/mL [15]. In the present study, the MICs 50/90 for meropenem and ertapenem were extremely high, with only 7/21 (33.3%) strains with meropenem MIC ≤128 μg/mL; nevertheless, the overall clinical cure was as high as for strains exhibiting higher MICs. Interestingly, these results are similar to those found in a previous study [22] where the actual meropenem MIC, which was performed in 20/27 strains, was >256 μg/mL in 5/20 (20%) strains, in the absence of clinical failure. Thus, the exact role of carbapenem MIC in predicting the DC clinical success should be better understood and deserves further investigations, together with the interaction with the patients' immune system.

5. Conclusions

In conclusion, we demonstrated that the DC regimen is a valid and effective therapeutic option in patients with infections due to KPC producing CRE, including those with bacteremic infection and more severe clinical conditions. The clinical effectiveness is maintained even in the presence of extremely high meropenem MIC.

Abbreviations

CSE: Carbapenem-susceptible *Enterobacteriaceae*
CP-CRE: Carbapenemase producing carbapenem-resistant *Enterobacteriaceae*
DC: Double-carbapenem
BAT: Best available treatment
MIC: Minimal inhibitory concentration
MEM: Meropenem
ETP: Ertapenem.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Community-Acquired Urinary Tract Infection by *Escherichia coli* in the Era of Antibiotic Resistance

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Urinary tract infections (UTIs) caused by *Escherichia coli (E. coli)* are the most common types of infections in women. The antibiotic resistance of *E. coli* is increasing rapidly, causing physicians to hesitate when selecting oral antibiotics. In this review, our objective is to ensure that clinicians understand the current seriousness of antibiotic-resistant *E. coli*, the mechanisms by which resistance is selected for, and methods that can be used to prevent antibiotic resistance.

1. Introduction

Community-acquired uncomplicated urinary tract infections (UTIs) account for a large proportion of infectious diseases in females [1], and a substantial amount of oral antibiotics is prescribed on a daily basis to treat UTIs among females in community-based outpatient clinics. Because *E. coli* accounts for up to 80% of community-acquired uncomplicated UTIs, these bacteria should be targeted when choosing empirical antibiotics [2]. In 2011, the Infectious Diseases Society of America (IDSA) recommended that trimethoprim-sulfamethoxazole (cotrimoxazole), nitrofurantoin, fosfomycin, or pivmecillinam be used if local resistance rates of uropathogens causing acute uncomplicated UTIs do not exceed 20% or if the infecting strain is known to be susceptible to these drugs [3]. Fluoroquinolones or beta-lactams such as cephalosporins are recommended as alternatives. Therefore, an awareness of regional susceptibility data regarding *E. coli* (antibiograms) is very important for selecting appropriate empirical antibiotics. However, the rate at which *E. coli* strains are becoming resistant to the vast majority of antibiotics is increasing worldwide. In addition, *Enterobacteriaceae* harbor gene(s) conferring resistance to almost all antibiotics [4] and plasmids harboring these resistance determinants can be transferred between bacteria, even between species, such that the acquisition of resistance to new antibiotics may only be a matter of time. Therefore, it is much more important to recognize practical rationales, including prescribing antibiotics when there is evidence of an infection, promoting appropriate use of antibiotics and increasing efforts for preventing UTIs. Following such strategies is essential because the abuse or misuse of antibiotics can lead to resistance via the emergence of mutant strains [5], and unresolved, relapsed UTIs tend to be resistant to previously used antibiotics [6].

Herein, we searched for an antibiotic resistance patterns for the past decade, especially with regard to oral antibiotics, reviewed the mechanisms of antibiotic resistance in *E. coli*, and suggest several strategies to overcome the challenges associated with these important issues.

2. Methods

We searched several databases, including PubMed, ISI Web of Science, Scopus, and Google Scholar using the following keywords: "*Escherichia coli*" *or* "*E. coli*", "resistant" or "resistance", "urinary tract infections" or "UTI", "epidemiology", "community", and "acquired". A key word was added when searching for regional antimicrobial susceptibilities, such as "Europe" or countries in Europe, such as "England", "UK", "France", "Germany", "Russia", "Italy", or "Spain"; "Asia" or countries in Asia, such as "China", "Korea", "Japan", "Taiwan", "Hong Kong", "India", or "Pakistan"; "America" or countries in North or South America, such as "US", "Canada", "Mexico", or "Brazil"; "Mediterranean", or countries in the Mediterranean region, such as "Greece"

Table 1: The prevalence of extended spectrum beta lactamase-producing *E. coli* in community acquired urinary tract infections before and after 2010.

	Before 2010	After 2010	References
Europe			
UK[a]	4.6%	6.6%	[18]
France	1.1%	3.3%	[19, 20]
Spain	2.4~18.2%	8.9~23.6%	[21–23]
Mediterranean region			
Italy	3.5%	6.7%	[24, 25]
Turkey	8~13.1%	24%	[12, 26]
South Asia	21.7%	33.2%	[27]
Far east Asia	4.8~7.5%[b]	7.6~10.7%	[28–30]
Latin America	1.7%[c]	7.1~12.5%	[31–33]
US and Canada	7.4	1.8~8%	[34–36]

a: Possibly contaminated by a nosocomial source.
b: The source of the specimens maybe from community acquired UTIs but was not described precisely.
c: The data were collected from multinational sources.

or "Turkey"; "Middle East" or countries in the Middle East region, such as "Egypt", "Saudi Arabia", or "Iran"; or "Australia". When searching for the mechanisms of antibiotics resistance, "mechanism" was searched for with a key word, such as "co-trimoxazole" or "trimethoprim sulfamethoxazole", "fluoroquinolone", "beta-lactams" or "beta-lactamase", "inhibitor", "fosfomycin", "nitrofurantoin", or "carbapenemase". When searching for antibiotic treatment or prevention, a word such as "strategy", "treatment", "management", "preventive", or "prevention" was added. During the review of articles, related articles on this subject were also reviewed.

3. Antimicrobial Susceptibility Pattern of *E. coli* in Community-Acquired Urinary Tract Infections for Oral Antibiotics in Recent Decades

There are limited oral options for the treatment of ESBL-producing bacteria associated with lower urinary tract infections (acute cystitis). Cotrimoxazole was a typical antibiotic used to treat UTIs, but the resistance of *E. coli* to this drug has markedly increased. According to the literature published in the past decade, in Asia, a 10~15% resistance rate to this drug was reported in Japan [7], with approximately 30% resistance rates observed in China and south Korea [8, 9]. In Europe and the Mediterranean region, the resistance rates of *E. coli* to cotrimoxazole varied but were usually over 15% [10–13]. However, there was an interesting report wherein the authors emphasized the role of cotrimoxazole in empirical antibiotics because of the recent decrease in the resistance rate to cotrimoxazole in several European countries due to its low prescription rate [14]. However, it may be not possible to reuse the drug worldwide within the next several years, and close observation of surveillance data will be required.

With respect to fluoroquinolones, in Japan and Australia, the susceptibility of *E. coli* to these drugs was approximately 90% [7, 15] and varied between 70~88% in the US [16] and 74~84% in China [8]. Middle and North European countries showed a fluoroquinolone susceptibility of 80% or greater [10, 11], while other European or some Mediterranean regions showed approximately a 60% susceptibility [12, 13]. Similar to cotrimoxazole, there was evidence that escape from exposure to this antibiotic will increase antimicrobial susceptibility in UTIs. According to Lee et al., the susceptibility of gram-negative bacteria to ciprofloxacin was much higher in patients less than 20 years old than in patients more than 20 years old. The reason for this observation may be the lower exposure to fluoroquinolones in young individuals because these drugs are not recommended for use in those under 20 years old [17].

One recent issue of importance is the increasing prevalence of extended spectrum beta lactamase- (ESBL-) producing *E. coli*. The prevalence of ESBL-producing *E. coli* has been increasing globally, as shown in Table 1 [12, 18–36]. Before 2010, the vast majority of countries showed less than a 5~10% prevalence of ESBL-producing *E. coli*, whereas the prevalence exceeded 10% in the local communities of many countries. Therefore, the increase in ESBL-producing *E. coli* is no different than that of cotrimoxazole-resistant *E. coli* or fluoroquinolone-resistant *E. coli*, and the prevalence of ESBL-producing *E. coli* is likely to increase soon.

Fosfomycin is an oral antibiotic agent that has broad activity against multidrug resistant (MDR), pathogens including ESBL-producing *E. coli*. Fosfomycin inhibits the synthesis of peptidoglycan at an earlier step than beta-lactam or glycopeptide antibiotics and has a broad spectrum of activity against various gram-positive and gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp. Fosfomycin has been shown to have advantages in the treatment of UTIs due to its high concentration in the urinary tract, which exceeds 2,000 mg/L after the initial administration and remains at high levels for a prolonged period, over 24 hours. However, fosfomycin should not be used for pyelonephritis or in patients with bacteremia due to inadequate concentrations within the bloodstream [37, 38]. Fosfomycin susceptibility in uropathogens, including *E. coli*, is currently greater than 90%, even in ESBL-producing *E. coli* [39–41].

Another oral antimicrobial agent that can be considered for the treatment of ESBL-producing *E. coli* cystitis is nitrofurantoin. Nitrofurantoin is a drug that has been used since 1950s to treat uncomplicated UTIs and works by damaging bacterial DNA in its highly active reduced form. Now, and even in earlier eras of widespread use, the baseline resistance to nitrofurantoin was low (0–5%) [42, 43]. Nitrofurantoin should only be used for lower UTIs, and its use should be avoided in patients with a creatinine clearance of less than 60 mL/minute, as reduced renal function results in decreased active drug within the urine [44].

Figure 1 [7–13, 15, 18, 20, 21, 23, 27, 33, 35, 36, 45–55] shows the efficacies of several oral antibiotics against *E. coli* in community-acquired uncomplicated UTIs. Because fosfomycin and nitrofurantoin have not been included in the antimicrobial formularies of many institutes, it is difficult to achieve previous susceptibility data. Furthermore, because of the history of disappointing in vitro results at the beginning

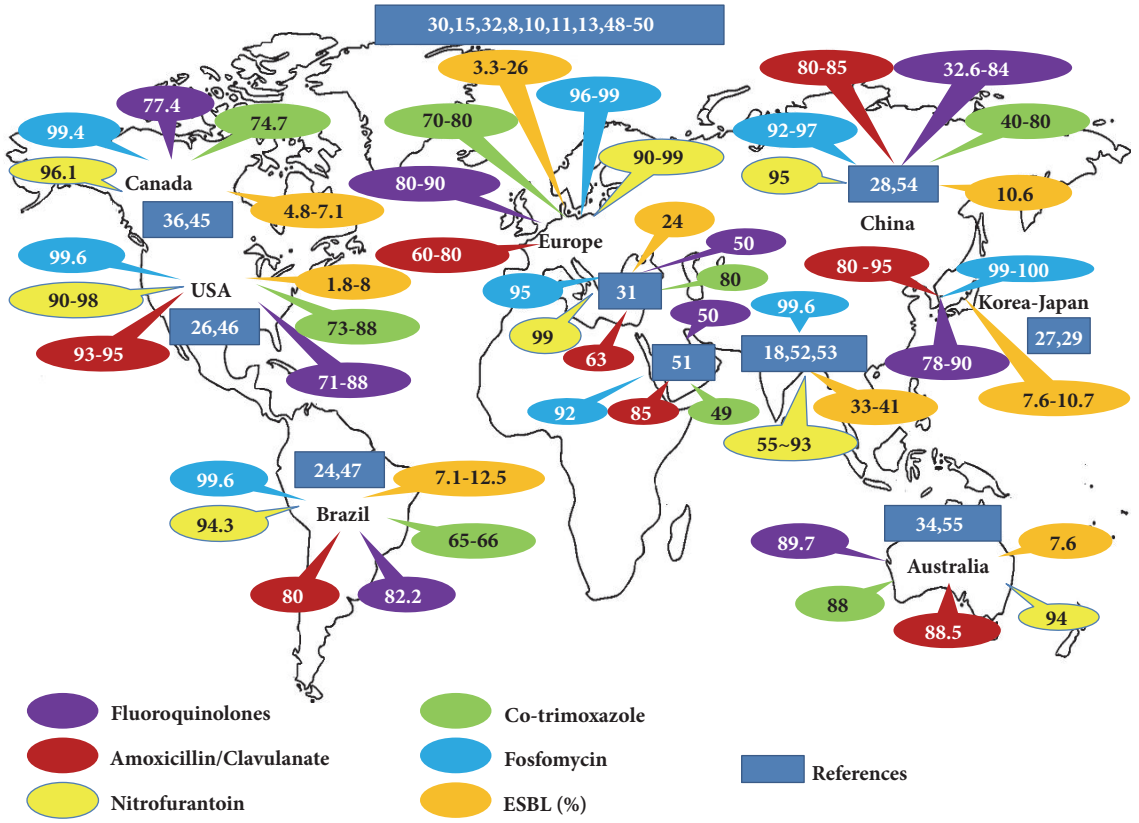

Figure 1: Worldwide susceptibilities of *E. coli* to oral antibiotics in community-acquired urinary tract infections in the last decade.

of fosfomycin-susceptibility testing, the use of the drug has been limited in the US and in many other countries [56]. Interestingly, these older medicines have become more important because of the high sensitivity of *E. coli* to these drugs in the era of antibiotic resistance. Recent studies have shown that these antibiotics have over 90~95% efficacies in almost all areas studied (Figure 1), although there may be no way to predict another decrease in the use of these drugs for UTI treatment.

4. Plasmid-Mediated Dissemination of Antibiotic Resistance Determinants

Plasmid is a generic term for DNA molecules other than chromosomes that can independently replicate in bacterial cells [57]. Although not necessary under most circumstances, plasmids can encode genes that promote bacterial survival and can be transferred to their descendants. Antimicrobial resistance determinants are some of the most important elements carried by plasmids. Although plasmids can be transmitted to other species via conjugation without becoming integrated into DNA [58], sometimes plasmids can integrate into chromosomal DNA for replication. Types of plasmids associated with ESBL-producing *E. coli* include IncFII, IncN, and IncI1, among which IncI1 resistance plasmids are known to contribute to CTX-M type ESBL dissemination in *E. coli* [59]. Resistance determinants against most antimicrobial agents can be conferred from species to species via plasmids, which is why systematic monitoring for antibiotic resistance in communities is important, as is the education of staff concerning infection control and prevention, especially in intensive care units. The next section will describe the details of epidemiological studies of plasmid-mediated resistance in *E. coli* infections.

5. Mechanisms of Action and Resistance to Anti-*E. coli* Drugs and Its Microbiological Epidemiology

Because tetrahydrofolate is required to make both purines and pyrimidines, its synthesis is important for understanding the mechanism of cotrimoxazole, which is a combination of trimethoprim and sulfamethoxazole. Trimethoprim is a structural analog of dihydrofolic acid that competitively inhibits the synthesis of tetrahydrofolic acid. Sulfamethoxazole, which has a sulfonyl group instead of a carbonyl group, is an analog of para-aminobenzoic acid that competitively inhibits the synthesis of dihydrofolic acid. Over two decades after its first use in 1974 [60], this drug has remained the first-line treatment for uncomplicated UTIs in adults [61]. Because of the widespread resistance to the drug, cotrimoxazole has been gradually replaced by fluoroquinolones since approximately the year 2000 [62]. The mechanism of bacterial resistance to cotrimoxazole is due to (1) drug efflux pumps, (2) the degradation of the antibiotics by enzymes, (3) the alteration of antibiotic binding targets, and (4) the loss of

drug entry points, all of which can occur via chromosomal mutations or the acquisition of plasmids [63].

Fluoroquinolones have a keto acetic acid group where fluoroquinolone–topoisomerase binding is facilitated through a water–metal ion bridge [64]. Eventually, fluoroquinolone–topoisomerase complex inhibits topoisomerase activity, and subsequently DNA replication is blocked. Acquisition of resistance to fluoroquinolones is from both chromosome and plasmid. Chromosomal-mediated resistance decrease in fluoroquinolone uptake and the expression of efflux pumps. Plasmid-encoded proteins which are associated with fluoroquinolones resistance are (1) Qnr proteins which decrease topoisomerase-DNA binding and protects enzyme-DNA complexes from quinolones, (2) Aac(6′)-lb-cr which acetylate the free nitrogen of the C7 ring of the quinolones, and (3) plasmid-encoded efflux pumps such as QepA1 and QepA2 [64, 65].

As shown in Figure 1, β-lactams, which are typically prescribed clinically in medical offices, are losing their efficacy in many areas because of the loss of their activity against *E. coli*. The mechanism of resistance of *E. coli* to β-lactams is essentially due to plasmid-mediated transmission of genes encoding β-lactamases. In contrast, in *Klebsiella* species, another species that causes uncomplicated urinary tract infection encodes a β-lactamase (e.g., SHV) on its chromosome [66]. It is not easy for general physicians to understand the classification of β-lactamases. In brief, according to the preferences of researchers, two classifications have been used to describe β-lactamase, including molecular classification from A to D and functional classification with regard to activity against β-lactamase inhibitors [67]. In general, class C corresponds to functional group 1, classes A and D correspond to group 2, and class B corresponds to group 3. ESBLs comprise group 2be (molecular class A) and can hydrolyze penicillin and first generation cephalosporins but can be inhibited by clavulanic acid and tazobactam. ESBLs can hydrolyze at least one of the following antibiotics at a 10% increased rate over that of benzylpenicillin: cefotaxime, ceftazidime, and/or aztreonam [67, 68]. TEM, a common genotype of β-lactamase, is named by a patient whose name was Temoneira, although the origin of TEM has not been identified precisely [69]. TEM-1 and TEM-2 belong to the group 2b (class A) β-lactamase genotype and have the ability to hydrolyze penicillin and/or first-generation cephalosporin, whereas CTX-M-15, which has recently become the most well-known genotype among β-lactamases, is common in ESBL-producing *E. coli* and is affiliated with group 2be (class A). The origin of CTX-M β-lactamases is believed to be from chromosomal ESBL genes in *Kluyvera* spp. [70]. A large-scale investigation (72 hospitals) in the US conducted in 2012 revealed that CTX-M-15 was the most common genotype in ESBL-producing *E. coli* [71]. Meanwhile, many recent studies have emphasized that the relative proportion of sequence type 131 *E. coli* (*E. coli* ST131) is predominant among all ESBL-producing *E. coli* [72, 73] and have shown that CTX-M-15 was encoded in plasmids and was transferred horizontally [74, 75]. When we compared research on non-ESBL-producing and fluoroquinolone-resistant ST131 *E. coli* isolates [76] to another study in which ST131 *E. coli* produced CTX-M-15 beta-lactamase and was resistant to fluoroquinolone [77], it appears that CTX-M-15 may be a β-lactamase that is acquired from plasmids in fluoroquinolone-resistant *E. coli* [78]. AmpC-type β-lactamases (group 1, class C) are encoded in chromosomes and are inducible by antibiotic pressure, such as amoxicillin. Beta-lactam antibiotics such as cefoxitin induce AmpC expression by binding to transpeptidases (penicillin-binding proteins), which results in a balance shift to murein degradation that subsequently activates the transcriptional regulator AmpR and increases its promoter activity [79]. These β-lactamases are generally resistant to clavulanic acid, cephalosporins, and cephamycin and can be expressed by *Citrobacter* spp., *Serratia* spp., and *Enterobacter* spp. but are rarely observed in *E. coli* [80, 81]. Dissemination of AmpC can occur via the mobilization of chromosomal AmpC genes from different enteric bacteria, such as C. freundii and E. cloacae, and their subsequent horizontal transfer to other species [82]. The first plasmid-borne AmpC gene identified was CMY-1 [83], followed by MIR-1 and CMY-2. Currently, CMY-2-type genes have been suggested to be one of the most common plasmid-borne AmpC enzymes [84]. According to a previous study by Sidjabat et al., a single IncI1 plasmid carrying *bla*CMY-2 was predominant among different clones of *E. coli*, suggesting the occurrence of horizontal transfer of this IncI1, *bla*CMY-2-carrying plasmid [85].

OXA family β-lactamases (group 2d, class D) hydrolyze oxacillin at a faster rate (> 50%) than that observed for benzylpenicillin. OXA-related β-lactamases have recently been identified in plasmids from *E. coli* [86] that exhibit low-level resistance to imipenem and resistance to ertapenem. Plasmid-mediated dissemination of OXA-48-like carbapenemases in *E. coli* has been observed in many European countries [87].

Besides OXA family β-lactamases, *K. pneumoniae* carbapenemase (KPC: group 2f, Class A) and metallo-beta-lactamases (MLBs: group 3, class B) are important types of carbapenemases. KPC enzyme in clinical isolate was first identified in 1996 [88]. In recent decade, the KPC determinants are identified world-widely and the incidence rate is rapidly increasing. KPC producers have been reported mostly from hospital-acquired *K. pneumoniae* isolates, but KPC-producing *E. coli* and other enterobacterial species have also been described [89]. In a report from Italian nationwide surveillance from outpatients, 93.2% of carbapenemases were associated with *bla*KPC type carbapenemase where the majority came from *K. pneumoniae* and 4.2% from *E. coli* [90]. Especially with respect to *E. coli*, Kalyan et al. emphasized that spread of *bla*KPC producing *E. coli* is mainly caused by horizontal transfer of *bla*KPC harboring plasmids such as IncFIA, $\text{IncFII}_{\text{K1}}$, $\text{IncFII}_{\text{K2}}$, or IncN [91].

Although MBLs were originally known as chromosomally encoded enzymes, the most frequently identified MBLs (IMPs and VIMs) have been observed to be encoded in plasmids from the family *Enterobacteriaceae* [92]. MBLs of the IMP-type appear to be primarily restricted to the Asian continent and are only rarely identified in Europe among enterobacterial isolates. In contrast, VIM-type MBLs have been identified worldwide in enterobacterial isolates responsible for large hospital outbreaks [93, 94]. NDM-1

(New Delhi metallo-ß-lactamase) is highly prevalent in the Indian subcontinent but has also been identified in many countries worldwide, demonstrating its rapid dissemination. The *bla*NDM-1 gene is primarily plasmid-associated.

Fosfomycin, which was discovered in 1969 [95], inhibits bacterial wall (peptidoglycan) biosynthesis by acting as an analog of phosphoenolpyruvate and binding UDP-GlcNAc enopyruvyl transferase, inactivating the enzyme [96]. Fosfomycin resistance has been identified in some bacteria that resulted from the mutation of UDP-GlcNAc enopyruvyl transferase [97]. However, in *E. coli*, both mutation-induced resistance and acquired resistance can occur. Several fosfomycin modifying enzymes, including FosA, encoded by plasmid-borne genes can confer fosfomycin resistance in *E. coli*.

Nitrofurantoin is one of the few drugs that can be used during pregnancy [98]. By oxygen-insensitive nitrofuran reductase, active intermediates of the drug can transferred into the bacteria where they act upon ribosimes and DNA [99], although the precise mechanisms of action of these intermediates have not yet been identified. The nfsA and nfsB genes, encoding nitroreductases, are encoded in *E. coli* [100]. Mutation in these genes in bacteria can lead to resistance to this drug. According to recent report, bacterial resistance to nitrofurantoin was shown be mediated by OqxAB efflux pumps [101], and the authors described that mutation in the resistant determinant genes nfsA and nfsB could be transmitted by plasmid.

Colistin is sensitive to most of gram negative bacteria, even to *bla*NDM-1 producing *Enterobacteriaceae* [102]. The action of colistin is known that cationic structure of the drug binds with anionic lipopolysaccharides causing displacement of cationic (calcium and magnesium) peptides from the outer cell membrane of gram negative bacteria, leading to disruption of the outer membrane and permeability change [103]. The mechanism of resistance has not been clearly understood, but several mechanisms such as outer membrane modification, over-expression of efflux pump, and overproduction of capsule polysaccharide have been suggested [104]. Recent data shows that plasmid mediated gene such as MCR-1 plays a great role on horizontal dissemination of colistin resistance in community [105]. In addition, Mao et al. emphasized that exposure to antibiotics elicited the emergence of MDR *E. coli* harboring MCR-1 [106].

6. Risk Factors for Acquisition of Antimicrobial Resistance in *E. coli*

Antibiotic exposure is the most important factor for the selection of antimicrobial resistance. Lee et al. described that increased exposure to fluoroquinolones/cephalosporins made bacteria more resistant to fluoroquinolone/cephalosporins [30]. Although it is not fully understood in detail how antibiotic resistance arises in microorganisms after their exposure to antibiotics, Baquero suggested that exposure to very low antibiotic concentrations can select for low-level resistant mutants, which serve as stepping stones to the strains with high-level resistance [5]. Similarly, Cantón et al. suggested that the use of an antibiotic at a concentration capable of preventing the generation of mutants, above the minimal inhibitory concentration, would restrict the emergence of such first-step mutants within a susceptible population [107]. Undesirable exposure to antibiotics typically occurs due to the abuse or misuse of antibiotics. In many countries, antibiotics can be obtained over the counter and are as easy to obtain as aspirin and cough medicine [108], which is a major contributing factor to antibiotics abuse. Recently, a study from India, where resistant rate of antibiotics has been relatively high, investigated antibiotics misuse where participants with limited access to an allopathic doctor, either for logistical or economic reasons, were observed to be more likely to purchase medications directly from a pharmacy without a prescription [109]. In the United States 20 years ago, experts estimated that at least half of the human therapeutic use of antibiotics in the United States was unnecessary or inappropriate [4].

Colonization has also been suggested to be risk factor for the selection of antimicrobial resistance. Most clinical factors associated with colonization and infection by ESBL-producing organisms involve healthcare exposure, such as hospitalization, residence in a long-term care facility, hemodialysis use, and the presence of an intravascular catheter [110, 111]. In a study of Dutch individuals who had no ESBL colonization prior to international travel, 34 percent overall and 75 percent of individuals who travelled to southern Asia became colonized by ESBL-producing strains following their travels [112]. Another report showed an ESBL prevalence of 49.0-64.0% for residents and 5.2-14.5% for staff [113]. Thus, travelers to endemic areas, hospitalized patients, care-givers in health care unit, guardians of in-patients, and hospital workers, including residents, are at an increased risk of colonization by antimicrobial resistant bacteria, showing the importance of environmental hygiene and taking precautions against contact with MDR bacteria. Once a cluster of resistant bacteria colonizes any part of the human body, it is possible that the bacteria will grow and horizontally transfer plasmid-encoded resistance genes to other susceptible bacteria or to different species [58].

Another important route of slow encroachment by resistant bacteria is the dispensing of antibiotics into ecosystems. Harrison et al. demonstrated that human ingestion of animal and plant food products carries a strong potential for the spread of antibiotic resistance genes via the consumption of antibiotic residues and antibiotic-resistant bacteria [4]. The authors concluded that the continued use of antibiotics in livestock and other agricultural endeavors may soon make these drugs ineffective for human therapeutic use.

Finally, indwelling catheters, which lead to complicated UTIs, are a known a risk factor for the acquisition of MDR bacteria [114].

7. Complicated UTIs and MDR Gram-Negative Bacteria

Classically, UTIs with functional or anatomical abnormalities of the urinary tract are named complicated UTIs [115]. With respect to complicated UTIs, treatment of asymptomatic bacteriuria has not been shown to be beneficial; it

could increase the risk of the development of antimicrobial-resistant uropathogens [116]. Antimicrobial resistance is more common in complicated UTIs [42], which may be because patients with complications are more vulnerable to UTIs and are more likely to be exposed to antibiotics, catheterization, and hospital sources.

Meanwhile, a major point in complicated UTIs is the concept of drainage of infected materials to reduce treatment periods and prevent infections from ascending to upper tract. Lee et al. emphasized that drainage of prostatic abscesses would reduce the period of antibiotic administration [117]. A report of long-term bladder management in spinal cord injury showed that condom catheter use (passive drainage) increases the vulnerability of patients to severe infection rather than intermittent catheterization (active drainage) [118].

Catheter-associated UTIs have multiple confounding factors associated with emerging antimicrobial resistance, that is, hospital factors due to frequent hospitalization, foreign body bridges between the urinary bladder and the outside of the body, and frequent antibiotic exposure. A previous study conducted with outpatient UTIs showed that catheter-associated UTIs were more closely associated to exposure to antibiotics and exhibited a higher occurrence of infection caused by an atypical organism, such as *Citrobacter* species, *Proteus mirabilis*, *Morganella morganii*, *Enterobacter species*, and *Pseudomonas aeruginosa* rather than *E. coli* [17]. Those atypical organisms can harbor MDR determinant and can transfer the resistance determinants (e.g., AmpC gene) to *E. coli* (see Section 5). Therefore, regarding plasmid-mediated resistance determinants, catheter associated UTIs obviously contribute to the emergence of resistant strains. Therefore, in managing neurogenic bladder with high bladder volume or high bladder pressure, avoidance of unnecessary catheterization and/or a change in the catheterization method used, from indwelling catheters to clean intermittent catheterization, must be considered [42].

A recent study recommended the use of amoxicillin/clavulanate (or amoxicillin plus aminoglycoside), cefixime, ceftibuten, levofloxacin, ciprofloxacin, and fosfomycin as empirical antibiotics against catheter-associated UTIs, whereas recommended regimens for empiric treatment of uncomplicated UTIs were fosfomycin, nitrofurantoin and pivmecillinam [119]. However, cultivation should be performed prior to the use of empirical antibiotics, especially in complicated UTIs because atypical and/or MDR microorganisms are more likely to be isolated.

8. Antibiotic Treatment of UTIs by MDR Gram-Negative Bacteria

In regard to laboratory cut-off values of microbial load (10^3 cfu/ml, 10^4 cfu/ml, or 10^5 cfu/ml), it is very difficult to determine treatment initiation in certain cut-off value. However, bacterial count is usually $10^2 \sim 10^4$ cfu/ml in many patients with UTIs, and half of women with symptomatic cystitis have bacteriuria lower than 10^5 cfu/ml [120]. Franz et al. also suggested antibiotic treatment in symptomatic patients with microbial load between 10^2 and 10^5 cfu/ml [121]. Furthermore, when severe infection such as urinary sepsis was suspected, physicians should start antibiotic therapy before the cultivation report (no time to wait laboratory microbial count) because of its high mortality. Therefore, symptoms (or signs) may be more important factor than the cut-off values of laboratory microbial load to initiate the treatment of UTIs.

Clinical studies have indicated that delayed treatment with inappropriate antibiotics for MDR bacteremia would exert a negative influence on patient mortality [122, 123]. Therefore, when a MDR bacterial infection is suspected and previous cultivation reports are not available, or when a patient has a systemic illness, physicians may be obligated to choose parenteral agents such as piperacillin/tazobactam or carbapenems empirically. In these circumstances, patients require hospitalization.

Currently, the emergence of many types of carbapenemases has caused a sense of crisis for physicians. However, several new drugs have been developed that have allowed physicians to save the use of carbapenems. Ceftazidime/avibactam is a new cephalosporin β-lactamase inhibitor combination targeting to *Enterobacteriaceae* and *Pseudomonas aeruginosa*, which can be used as an alternative to carbapenems for infections caused by ESBL- or AmpC-producing gram-negative bacteria [124, 125]. Ceftolozane/tazobactam can also be used as an alternative to carbapenems to treat ESBL-producing gram negative infections [126].

A recent study suggested a treatment algorithm for MDR gram-negative bacterial infections [127]. If an MDR bacterial infection is suspected, and there was no past carbapenem resistance, it is recommended to use parenteral amoxicillin/clavulanate or piperacillin/tazobactam followed by oral fosfomycin, nitrofurantoin or pivmecillinam plus amoxicillin/clavulanate, unless patients have systemic illness, whereas if there was no susceptibility data or patients have systemic illness, it is recommended to use carbapenems, temocillin, or ceftolozane/tazobactam. If carbapenem resistance had been noted, specific antibiotics should be considered according to the local policy of avoiding the development of antibiotic resistance. Therefore, investigations concerning the types of carbapenemases and their surveillance are very important. For example, if a cultivation assay result identifies a strain as being resistant to carbapenemase and metallo-β-lactamases is known as a prevalent type in the local area, physicians can use colistin or tigecycline [128].

9. Contradiction between the Use of and Resistance of Antibiotics, Focusing Acute Cystitis

The fact that exposure to antibiotics increases resistance is a problem for clinicians who need to continue to treat infected patients. It is true that the use of antibiotics should be reduced to decrease the development of strains that are resistant to antibiotics. However, excessive limitation of antibiotics for treating symptomatic UTI or for prophylaxis may lead to another cost increase due to recurrence. Therefore, we need to develop a strategy to adequately control urinary tract infections while minimizing the increase in antibiotic resistance.

First, a management strategy should be developed for systemic and localized infections. In the case of pyelonephritis that causes systemic infections, including UTI sepsis, broad spectrum antibiotics should be used intensively. When a course of antibiotics is started empirically, the choice of agent should be reevaluated once culture results are available. Continuous surveillance of antibiotic resistance patterns by region is essential for the appropriate selection of antibiotics for empirical treatment.

Reducing the total amount of antibiotic use is important for resistance control. The use of cephalosporins and quinolones and the long-term use of antibiotics have been identified as risk factors for infections caused by extended-spectrum ESBL *E. coli* and *Klebsiella* species [129, 130]. The use of broad spectrum antibiotics to treat systemic infection may be inevitable. However, total antibiotic usage is higher in non-febrile uncomplicated UTIs (in other words, acute cystitis). Finally, an effective strategy for controlling antibiotic resistance is to use antibiotics appropriately for cystitis treatment and to prevent recurrent cystitis using available means.

Furthermore, this strategy is more important and necessary because of the occurrence of collateral damage, which describes increased colonization or infection by MDR organisms with the use of broad-spectrum antimicrobials, including fluoroquinolones and cephalosporins [131, 132]. Thus, fluoroquinolones and cephalosporins are no longer recommended as the first-line treatment for acute uncomplicated cystitis in the EAU and IDSA guidelines. Instead, drugs that cause minimal resistance and have a propensity for collateral damage are recommended as the first-line treatment, such as nitrofurantoin, fosfomycin, and pivmecillinam [3, 133].

Obviously, the best way to treat UTIs should be to use optimal antibiotics instead of empirical treatment, if possible, based on culture findings [134]. This principle is the same whether it is simple cystitis or febrile UTI. Of course, it is well known that worldwide socio-economic status varies from region to region, and a full laboratory examination cannot be performed for all patients [135, 136]. Therefore, it is not possible to implement consistent guidelines with respect to laboratory diagnostics. However, we should be aware of the high incidence of infectious diseases, even if they cause low mobility or mortality, such as cystitis. It is also important to strengthen the natural defense mechanism of the human body for helping recovery from the infected condition and to prevent recurrence of the same disease, rather than blind antibiotic prescription.

10. Prevention of Infectious Disease Is the Best Option for Antibiotic Resistance Control, Especially in Recurrent UTI

Recurrent UTI is defined as recurrence of uncomplicated and/or complicated UTIs, with a frequency of at least three UTIs/year or two UTIs in the last six months. Generally, UTIs differ from other infectious diseases in which pathognomonic sources are transmitted from the outside, such as sexually transmitted infections or respiratory tract infections. The bacteria that cause UTIs have characteristics that are typically symbiotic with the human body, and when an imbalance arises for some reason, it can result in infections of the urinary tract [137]. Even individuals who do not have a urinary system abnormality are at risk to become infected, and some individuals suffer from repeated UTIs without apparent cause. Moreover, a person with a structural or functional abnormality of the urinary tract is at a higher risk for urinary tract infections. For this reason, efforts to manage the exposure of humans to infectious agents can reduce the incidence of urinary tract infections, and these efforts may help to slow the development of antibiotic resistance [58].

11. Proper Bladder Emptying

All physicians treating UTIs should have awareness of urogenital anomalies and the need for proper bladder emptying. Congenital urinary tract abnormalities should be investigated for pediatric UTIs. For adults, close history taking can identify functional factors, such as bladder outlet obstruction or underactive bladder caused by spinal cord lesions. Physicians should also pay attention to noticeable problems, such as spinal cord injury and congenital anomalies in the urogenital system, as well as inconspicuous problems, such as BPH or DM polyneuropathy. Urogenital imaging studies are performed when evaluating recurrent UTIs, but additional measurements such as postvoid residual urine volume should be considered [138, 139]. If there is a problem in bladder function, proper bladder emptying methods, such as clean intermittent catheterization (CIC), the use of urethral catheters, or the administration of some medications, such as alpha-blockers for sphincter relaxation, should be actively explored. Proper emptying of the bladder is the most important factor in recurrent UTI control.

12. Nonantimicrobial Prophylaxis

The active use of nonantimicrobial prophylaxis is often indicated and does not result in an increase in antimicrobial resistance of the commensal flora, as nonantimicrobial prophylaxis, immunoactive agents, probiotics (*Lactobacillus* spp.), cranberry-based products, D-mannose, hormonal replacement (in postmenopausal women), and others have been studied [140–144]. Among these modalities, the urinary immunopotentiator is now well documented and strongly recommended in the guidelines [145]. The oral immunostimulant OM-89 (Uro-Vaxom®), an extract of 18 different serotypes of heat-killed uropathogenic *E. coli*, stimulates innate immunity by increasing non-specific and specific humoral and cellular immune responses by stimulating the production of interferon-γ and tumor necrosis factor-γ, as well as the activities of lymphocytes and macrophages [146–148]. Uro-Vaxom® is a safe and effective medicine that can reduce recurrent UTI episodes [140, 149–151] and can effectively reduce the repeated use of antibiotics [152]. Physicians need to actively use immunoactive agents that have been proven effective rather than letting patients find a solution or take medication on their own. However, a weakness of these agents is that they cannot completely prevent the recurrence of an infection and require relatively long-term

use. Therefore, to increase compliance, it is important to provide sufficient information to the patient and to establish a good doctor-patient relationship.

13. Awareness of Asymptomatic Bacteriuria (ABU)

ABU should be distinguished from symptomatic UTI. ABU occurs in an estimated 1-5% of healthy pre-menopausal females, increasing to 4-19% in otherwise healthy elderly females and men, also occurring in 0.7-27% of patients with diabetes, 2-10% of pregnant women, 15-50% of institutionalized elderly patients, and 23-89% of patients with spinal cord injuries [153, 154]. ABU does not cause systemic influences, such as renal damage [155]. Thus, treatment of ABU is not recommended in patients without risk factors [153]. Furthermore, ABU should not be overtreated without the awareness of the physician [156]. Even in catheterized patients (also see Section 7), antibiotics should be considered only when patients with indwelling catheters present symptoms or they have any complications during placement or exchanges of catheters [157]. In contrast, considering ABU in pregnancy, many researches recommended treating the UTIs because not to treat UTIs in pregnant women can increase the possibilities of preterm labor or low birth-weight [158].

The major risk factor for ABU is diabetes mellitus. DM, even when well regulated, is reported to correlate to a higher frequency of ABU [159]. Considering the high morbidity and mortality of symptomatic UTI in DM, patients should be sufficiently treated by nonantibiotic methods. In particular, bladder dysfunction, such as diabetic cytopathy, should be carefully considered, and proper bladder emptying must be encouraged with diverse methods [160]. Even in these cases, immunopotentiators can be a good option for preventing symptomatic UTIs.

14. Pain Control for Cystitis Patients

For patients with nonfebrile uncomplicated cystitis, active pain control, and minimal use of antibiotics should be prioritized. Uncomplicated cystitis can be a self-limited disease in many cases. One study even showed that only symptomatic care using NSAID could be as effective as antibiotics in acute cystitis [161]. Pain in acute cystitis is a natural consequence of the inflammatory response, and pain-mediated urinary frequency or urgency is the chief complaint of patients. Therefore, for this self-limited disease, pain killers, including NSAIDs, may be a good option for symptomatic care as well as reducing the consumption of antibiotics. Delayed treatment is also a good strategy for antimicrobial-sparing [162]. Another caution is the abuse or misuse of overactive bladder (OAB) medicine with anticholinergic effects for acute urgency or urge incontinence in UTIs. Overuse of anticholinergic medicine can interfere with proper bladder emptying, and adverse effects with respect to UTI control may occur.

15. Antimicrobial Stewardship

Antibiotics are overused across the world through their prescription, self-medication, or over-the-counter (OTC) availability. With the quantity of antibiotic use linked to antibiotic resistance, society should seek to preserve the use of this irreplaceable resource through education and regulation [163].

Antimicrobial stewardship programs aim to optimize the outcomes of prevention and treatment of infection while curbing the overuse and misuse of antimicrobial agents [145, 164, 165]. Antimicrobial stewardship has a positive clinical impact on UTIs caused by ESBL-producing *E. coli* [166]. To this end, antimicrobial therapy should be tailored to each patient, taking into consideration the severity of disease, individual and local patterns of antimicrobial resistance and the potential for collateral damage associated with antimicrobial use. Selecting the correct drug, dose, as well the shortest clinically effective duration of therapy when possible, is key to optimal antimicrobial stewardship [134]. Some prescription strategies should be considered carefully, including the following [167]:

(i) Precise indication for antibiotic treatment

(ii) Choice of the appropriate compound

(iii) Appropriate dosage

(iv) Adequate route of administration

(v) Administration timing and treatment length

All physicians who treat UTIs should take on the responsibility of antimicrobial stewardship.

16. Conclusion

Even if new antibiotics are introduced and appear on the market, the development of resistance to these antibiotics by *E. coli* will begin immediately. The mechanisms by which *E. coli* becomes resistant to antibiotics vary greatly with the antibiotic, but genes conferring resistance can be transmitted via plasmids among species. Therefore, the implementation of antibiotic stewardship programs is crucial to minimize the chance of selecting for resistant resistance. Such programs should be founded on the following principles: (1) antibiotics should be used when there is evidence of a bacterial infection to minimize the unnecessary exposure of patients to antibiotics; (2) ABU should not be treated (if there is no risk factor) to minimize unnecessary exposure to antibiotics; (3) cultivation before using antibiotics and using appropriate antibiotics (if possible, considering using nitrofurantoin, fosfomycin, or pivmecillinam as first-line antibiotics) should be performed according to regional susceptibility data to decrease the chance of "collateral damage"; (4) the use of appropriate antibiotic doses, not underdoses, to potentially reduce mutant formation; and (5) the use of antibiotics for appropriate durations to reduce recurrence (appropriate de-escalation with repeated culture). In addition, to prevent overuse of antibiotics, self-medication, or over-the-counter (OTC) availability should be limited by education or regulation. Furthermore, paying attention to hygiene, especially individuals who travel to endemic areas or who are frequently in circumstances with a high risk of exposure to antibiotic resistant bacteria (e.g., healthcare units), could reduce the

chance of colonization by resistant micro-organisms. Finally, the use of nonantimicrobial prophylaxis could effectively reduce the total amount of antibiotic consumption.

In general, cotrimoxazole has under 80% sensitivity and fluoroquinolones have approximately 80% sensitivity, but the latter drug shows under 60% sensitivity in some parts of Asia, the Middle-East, and the Mediterranean region. Beta-lactam with inhibitor, amoxicillin/clavulanate, shows approximately 80% sensitivity, except for some European countries and the Mediterranean region. South Asia is an endemic region of ESBL producing *E. coli*. Yet, nitrofurantoin and fosfomycin show over 90% of sensitivity in the most of countries of the world.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Small Molecule Inhibitor of Type Three Secretion System Belonging to a Class 2,4-disubstituted-4H-[1,3,4]-thiadiazine-5-ones Improves Survival and Decreases Bacterial Loads in an Airway *Pseudomonas aeruginosa* Infection in Mice

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Pseudomonas aeruginosa is a cause of high mortality in burn, immunocompromised, and surgery patients. High incidence of antibiotic resistance in this pathogen makes the existent therapy inefficient. Type three secretion system (T3SS) is a leading virulence system of *P. aeruginosa* that actively suppresses host resistance and enhances the severity of infection. Innovative therapeutic strategies aiming at inhibition of type three secretion system of *P. aeruginosa* are highly attractive, as they may reduce the severity of clinical manifestations and improve antibacterial immune responses. They may also represent an attractive therapy for antibiotic-resistant bacteria. Recently our laboratory developed a new small molecule inhibitor belonging to a class 2,4-disubstituted-4H-[1, 3, 4]-thiadiazine-5-ones, Fluorothiazinon (FT), that effectively suppressed T3SS in chlamydia and salmonella *in vitro* and *in vivo*. In this study, we evaluate the activity of FT towards antibiotic-resistant clinical isolates of *P. aeruginosa* expressing T3SS effectors ExoU and ExoS in an airway infection model. We found that FT reduced mortality and bacterial loads and decrease lung pathology and systemic inflammation. In addition, we show that FT inhibits the secretion of ExoT and ExoY, reduced bacteria cytotoxicity, and increased bacteria internalization *in vitro*. Overall, FT shows a strong potential as an antibacterial therapy of antibiotic-resistant *P. aeruginosa* infection.

1. Introduction

Pseudomonas aeruginosa is an often cause of hospital pneumonia, urinary tract infections, primary bacteremia, and skin and soft tissue infections in burn, immunocompromised, and surgery patients [1–3]. It causes up to 34%-48% of all hospital infections with high mortality [4, 5]. Bacterial virulence factors affect host defenses and contribute to the immune misbalance favoring nonspecific inflammation and disturbing the initiation of protective responses towards pathogen [5, 6]. High incidence of multidrug-resistance in this pathogen adds to the severity of the situation.

Type three secretion system (T3SS) is a leading virulence system of pathogenic *Pseudomonas spp*. The proteins secreted by T3SS are toxins that induce cell apoptosis or necrosis, suppress the immune response, and inhibit macrophage and neutrophil recruitment and phagocytosis. In this context, innovative therapeutic strategies aiming at inhibition of T3SS activity are of particular interest, as they reduce the severity of clinical manifestations and improve antibacterial immune responses while preserving commensal flora [7]. These strategies also reduce the risk of selecting resistance, since they only disarm bacteria, allowing the host to employ immune mechanisms to fight the infection [8]. Different types of

T3SS inhibitors are currently reported for Gram-negative bacteria such as therapeutic antibodies against *P. aeruginosa* PcrV protein [9], hybrid antibodies against PcrV and Psl [10], salicylidene acylhydrazides and hydroxyquinolines [11], and others [12, 13]. Recently our laboratory developed a new small molecule inhibitor designated as Fluorothiazinon (FT) that belongs to the class of 2,4-disubstituted-4H-[1, 3, 4]-thiadiazine-5-ones. FT effectively suppressed T3SS in chlamydia and salmonella *in vitro* and *in vivo* [14–17]. FT significantly decreased mortality and bacteria loads in susceptible and resistant mice infected with *S. enterica serovar Typhimurium* [14]. FT inhibited the intracellular growth of different *Chlamydia* species in a dose-dependent manner and decreased the translocation of the type III secretion effector IncA [18]. FT possessed antibacterial activity *in vivo* and was able to control *C. trachomatis* serovar D vaginal shedding, ascending infection, and inflammation in the upper genital organs in DBA/2 mice [16]. Preclinical toxicological research confirmed its safety, lack of acute and chronic toxicity, mutagenicity, immunotoxicity, allergic potential, and lack of reproductive toxicity [14].

In this study, we evaluated the activity of FT towards antibiotic-resistant clinical isolates of *P. aeruginosa* expressing T3SS effectors ExoU and ExoS in an airway infection model. We found that FT reduced mortality and bacterial loads and decreased lung pathology and systemic inflammation. In addition, we showed that FT inhibited the secretion of ExoT and ExoY, reduced bacteria cytotoxicity, and increased bacteria internalization *in vitro*. Overall, FT shows a strong potential as an antibacterial therapy of antibiotic-resistant *P. aeruginosa* infection.

2. Material and Methods

2.1. Fluorothiazinon. Fluorothiazinon (FT) is N-(2,4-difluorophenyl)-4(3-ethoxy-4-hydroxybenzyl)-5-oxo-5,6-dihydro-4H-[1, 3, 4]-thiadiazine-2 carboxamide previously reported as CL-55 and synthesized as described earlier [17]. For *in vitro* studies FT stock solution was prepared by dilution of 20 mg of FT, 44 mg of NaOH, and 77 mg of ammonium acetate in endotoxin-free deionized water, final pH – 7.0±0.2, to the final concentration of FT – 2 mg/ml.

2.2. Bacteria. *P. aeruginosa* clinical isolates used in this study were obtained from two Moscow hospitals and are listed in the Table S1 in Supplementary Materials.

2.3. Bacterial Culture. *P. aeruginosa* bacterial strains were streaked into LB broth from frozen stocks and grown overnight at 37°C. To assess FT antimicrobial activity *in vitro* night cultures were diluted in LB medium (1:100). FT was added to cultures to final concentrations of 5, 10, 20, and 40 μg/ml. Gentamicin (Belmedpreparat, Minsk, Belarus) and Ciprofloxacin (CF) (Promed Exports, New Delhi, India) were added to final concentrations of 4 μg/ml and 0.25 μg/ml. Diluent without FT or antibiotics was used in the controls. Cultures were incubated overnight at 37°C with shaking. To assess the bacteria growth 10-fold serial dilutions of the cultures were seeded on Cetrimide agar (Cetrimide Agar Base w/o Glycerine, Himedia, Mumbai, India). Cultures were incubated at 37°C for 24 hours. The numbers of colonies were assessed as described elsewhere.

2.4. Mice and Ethics Statement. A/JsnYCit (A/Sn) mice were bred and maintained under conventional conditions at the Animal Facilities of the Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, Russia, in accordance with National Guidelines. Male mice were used between 6 and 8 weeks of age. The Gamaleya National Research Center Animal Care Committee approved all experiments.

2.5. In Vivo Lung Infection Model. Two clinical isolates 1840 and KB6 with multiple antibiotic resistance were chosen for *in vivo* experiments (Supplementary Materials Table S1). Mice were infected intranasally with 40 μl of *P. aeruginosa* culture in the doses indicated in the RESULTS section.

For *in vivo* experiments, FT was prepared by grinding of FT in 1% solution of starch. FT suspension was administered *per os* by gavage needle in a volume of 200 μl. Mice received 50 mg/kg of FT twice a day for four days.

To assess the numbers of *P. aeruginosa* in lungs and spleens the specimens were homogenized in 1 ml of saline solution and centrifuged for 10 min at 800 rpm. 10-fold serial dilutions of organ homogenates were plated on Cetrimide agar and incubated for 24 hours at 37°C. Blood specimens were collected into tubes containing sodium heparin as an anticoagulant and 10-fold dilutions in saline were plated on Cetrimide agar.

2.6. Histochemistry. Lungs were sectioned and stained with hematoxylin and eosin as described before [16].

2.7. Cytokine Analysis. The concentrations of IL-6 and TNF-α in the blood and lung homogenates were determined using a commercial enzyme-linked immunosorbent assay kits (ELISA MAX Deluxe Set, Biolegend, San Diego, CA). Optical densities were measured using BioTek plate reader at the wavelength of 450 nm.

2.8. Immunoblot. Night cultures of *P. aeruginosa* were diluted 1:100 in a fresh LB medium with 5 mM of EGTA and cultivated for 3 hours at 37°C. Bacteria were centrifuged and extracellular proteins were concentrated from supernatant by 10%-saturated trichloroacetic acid, washed with 100% acetone, resuspended in the sample buffer and subjected to a polyacrylamide gel electrophoresis as described previously [18]. After electrophoresis the proteins were transferred by a semidry blot from gel to nitrocellulose membranes using the TE70 PWR system (GE, Moscow, Russia) [19] Membranes were incubated with primary antibodies to ExoT and ExoY (in-house obtained mouse serum diluted 1:20000) overnight at 4°C. Blots were incubated with a secondary antibody linked to HRP (1:5000) for one hour at RT, and the signals were developed. The reaction was read with chemiluminometer (Vilber Lourmat, Eberhardzell Germany).

2.9. LDH Release Assay. Confluent CHO cells grown in RPMI-1640 medium supplemented with 10% fetal bovine

serum (FBS) in 96-well plates were washed and covered with RPMI-1640 containing 1% FBS. *P. aeruginosa* grown overnight in LB medium was subcultured into fresh LB and grown to the mid-log phase. CHO cells were infected with the mid-log-phase *P. aeruginosa* at an initial multiplicity of infection (MOI) of 10. Plates were incubated for 3 hours in the presence of FT or diluent in the controls at the concentrations indicated in the RESULTS section. Plates were centrifuged at 1500 rpm for 10 min to sediment bacteria, and lactate dehydrogenase (LDH) release was measured in culture supernatants using CytoTox 96 nonradioactive cytotoxicity assay (Promega, Fitchburg, WI) in accordance with the manufacturer instructions. Percent of LDH release was calculated relative to the uninfected control, which was set as 0% of LDH release, and the cells lysed with Triton X-100, which was set as 100% of LDH release.

2.10. Pseudomonas Internalization Assay. *P. aeruginosa* was grown overnight in LB medium and further subcultured in fresh LB medium for 3 hours. After that, bacteria were washed and resuspended in DMEM with 1% of FBS. FT was added to *P. aeruginosa* at the concentrations indicated in the RESULTS section and incubated with shaking for 30 min. FT or diluent treated *P. aeruginosa* isolates were added to HeLa cells grown in 6-well plates at MOI of 10. After 2 hours of incubation extracellular bacteria were removed by washing with PBS, fresh DMEM medium containing 50 μg/ml gentamicin was added, and cells were incubated for additional 2 h. After three washes with PBS, the cells were lysed in PBS containing 0.25% Triton X-100 and plated on the Cetrimide agar plates to count the number of bacteria internalized within HeLa cells.

2.11. Statistics. The results obtained from the mortality rates studies are represented as Kaplan-Meier survival curves, and the differences in survival were calculated by the log-rank test.

Significant differences of the other data were determined using the Mann–Whitney nonparametric two-tailed test using GraphPad Prism Version 6.

3. Results

3.1. Ft Promotes Survival of Animals in a Murine Model of P. aeruginosa Airway Infection Given Directly after the Onset of Infection. To investigate antibacterial effect of FT in the treatment of *P. aeruginosa* airway infection caused by antibiotic-resistant clinical isolates, we used *P. aeruginosa* clinical isolates of two different T3SS genotypes, *exoU*$^+$ and *exoS*$^+$ with multiple antibiotic resistance. A/Sn mice (n=10 per group) were infected intranasally with *exoU*$^+$ *P. aeruginosa* clinical isolate 1840 ($6.5\text{x}10^6$ and $3.2\text{x}10^6$ CFU/animal) or *exoS*$^+$ *P. aeruginosa* clinical isolate KB6 ($2.2\text{x}10^7$ and $1.1\text{x}10^7$ CFU/animal). Intranasal infection with *P. aeruginosa* clinical isolate 1840 in a dose of $6.5\text{x}10^6$ CFU/animal induced 80% of mortality (LD_{80}) and 50% of mortality (LD_{50}) in a dose of $3.2\text{x}10^6$ CFU/animal. Intranasal infection with *P. aeruginosa* clinical isolate KB6 in a dose of $2.2\text{x}10^7$ CFU/animal induced 90% of mortality (LD_{90}) and $1.1\text{x}10^7$ CFU/animal induced 70% of mortality (LD_{70}). The dose of FT and the regimen of treatment was evaluated in preliminary experiments (data is not shown). Infected animals were treated *per os* with 50 mg/kg of FT immediately after infection for 4 days twice a day, as this protocol was found the most effective (data is not shown).

As shown in Figure 1, FT provided survival of 70% of mice after infection with LD_{80} of clinical isolate 1840 (Figure 1(a)), and of 100% of mice after infection with LD_{50} (Figure 1(b)). FT protected 100% of animals after infection with LD_{70} of KB6 clinical isolate (Figure 1(d)) and 80% of animals after infection LD_{90} of the KB6 clinical isolate (Figure 1(c)). These results showed that FT administered *per os* reduced mortality of infected animals in the first 5 days postinfection.

3.2. FT Decreases Bacterial Loads in the Murine Model of P. aeruginosa Airway Infection Given Directly after the Onset of Infection. Survived animals were sacrificed at day 5 postinfection. Average $6.4\pm4.5\text{x}10^2$ CFU/lung of clinical isolate 1840 was detected in the lungs of control animals infected with a dose of $6.5\text{x}10^6$ CFU/animal and average $6.08\pm7.6\text{x}\ 10^2$ CFU/lung was detected in lungs of animals infected with a dose of $3.25\text{x}10^6$ CFU/animal. Bacteria were found in spleens and blood of the control animals (Figure 2) that revealed systemic spread of infection. Administration of FT resulted in a decrease of bacterial burden by an order in lungs,and by two orders in the spleen compared to the controls ($P \geq 0.05$). Complete clearance of bacteria from the blood was observed in 40% of mice infected with $6.5\text{x}10^6$ CFU/animal and 100% of mice infected with $3.25\text{x}10^6$ CFU/animal of clinical isolate 1840.

For clinical isolate KB6 control mice infected with $2.2\text{x}10^7$ CFU/animal had $4.5\pm2.9\ \text{x}\ 10^3$ CFU/lung and mice infected with $1.1\text{x}10^7$ CFU/animal had $1.4\pm1.08\text{x}10^3$ CFU/lung. Bacteria were also found in blood and spleens (Figure 2). Treatment with FT reduced bacterial loads in lungs. The numbers of bacteria decreased by two orders. 3 and 8 mice from high and low dose infection groups correspondingly were completely cleared from infection. All survived mice in the treated groups had no bacteria in the blood.

Thus, the results obtained in this study demonstrated the effectiveness of FT treatment in our airway infection model, induced with multiple antibiotic-resistant clinical isolates expressing various T3SS proteins. FT decreased mortality and bacterial loads in lungs and completely cleared infection from the blood.

3.3. FT Provided Survival of Animals in a Murine Model of P. aeruginosa Airway Infection Given as a Combined Prophylaxis-Treatment Regimen. *P. aeruginosa* clinical isolates 1840 and KB6 were used in this set of experiments. A/Sn mice (n=10 per group) were inoculated intranasally with *exoU*$^+$ *P. aeruginosa* cytotoxic clinical isolate 1840 with two doses: $7.0\text{x}10^6$ (LD_{80}) and $3.5\text{x}10^6$ (LD_{50}) CFU/mice; and *exoS*$^+$ *P. aeruginosa* clinical isolate KB6 in the doses of $1{,}75\text{x}10^7$ (LD_{80}) and $8\text{x}10^6$ (LD_{50}) CFU/animal.

Mice were treated with 100 mg/kg of FT *per os* once a day for 2 days before infection and with 50 mg/kg of FT twice a day for 4 days starting immediately after infection.

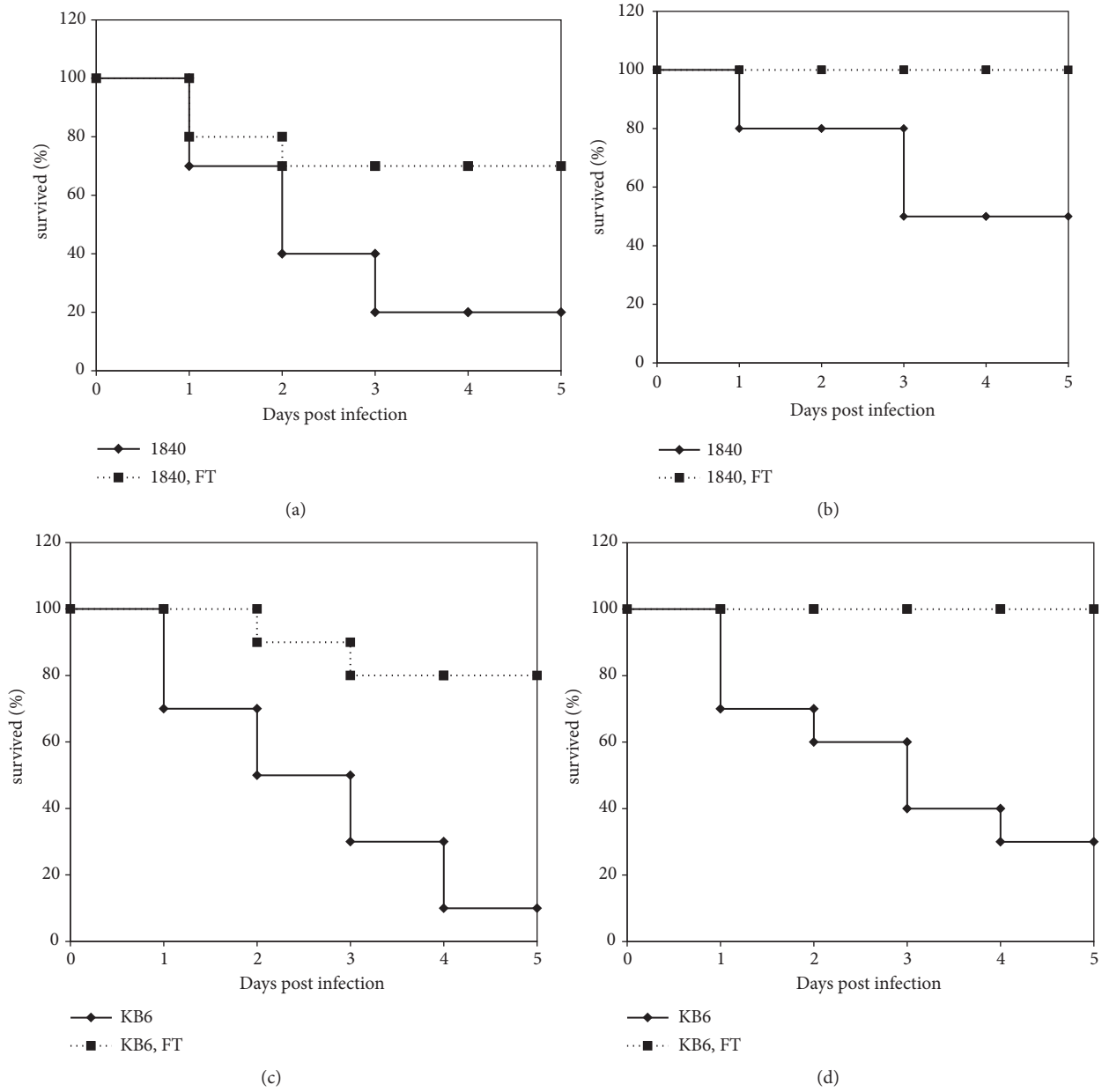

Figure 1: FT improves survival of mice infected with *P. aeruginosa* antibiotic-resistant clinical isolates of *exoU*$^+$ (1840) and *exoS*$^+$(KB6) genotypes. (a) mice infected with clinical isolate 1840, 6.45x10^6 CFU/animal; (b) mice infected with clinical isolate 1840, 3.2x10^6 CFU/animal; (c) mice infected with clinical isolate KB6, 2.2x 10^7 CFU/animal; (d) mice infected with clinical isolate KB6, 1.1x10^7 CFU/animal. Infected animals were treated with 50 mg/kg of FT *per os* immediately after infection for 4 days twice a day. Black line, control; dotted line, FT-treated mice.

Survival rates and bacterial loads in lungs, spleens, and blood of survived animals were analyzed at day 5 postinfection. The results are presented in Figure 3. As shown in Figure 3, combined prophylaxis-therapy treatment with FT in mice infected with LD_{80} and LD_{50} of *P. aeruginosa exoU*$^+$ clinical isolate 1840 led to 100% percent survival of animals. In the case of *P. aeruginosa exoS*$^+$ KB6 infection (Figures 3(c) and 3(d)) the rate of survival was 90 and 100% for LD_{80} and LD_{50}.

3.4. FT Decreased Bacterial Loads of Survivors in a Murine Model of P. aeruginosa Airway Infection Given as a Combined Prophylaxis-Treatment Regimen. To confirm eradication of bacteria, viable counts were performed on lung and spleen homogenates and blood from mice treated with FT. Survived animals were sacrificed at day 5 after the initiation of infection. The results are presented in Figure 4.

As shown in Figure 4, 2.5±0x10^2 CFU/lung of clinical isolate 1840 was detected in lungs of control animals infected with a dose of 7x10^6 CFU/animal and 3.4±4x10^2 CFU/lung was detected in lungs of animals infected with a dose of 3.5x10^6 CFU/animal. Infection was also found in spleens and blood (Figures 4(a) and 4(b)) that revealed the systemic

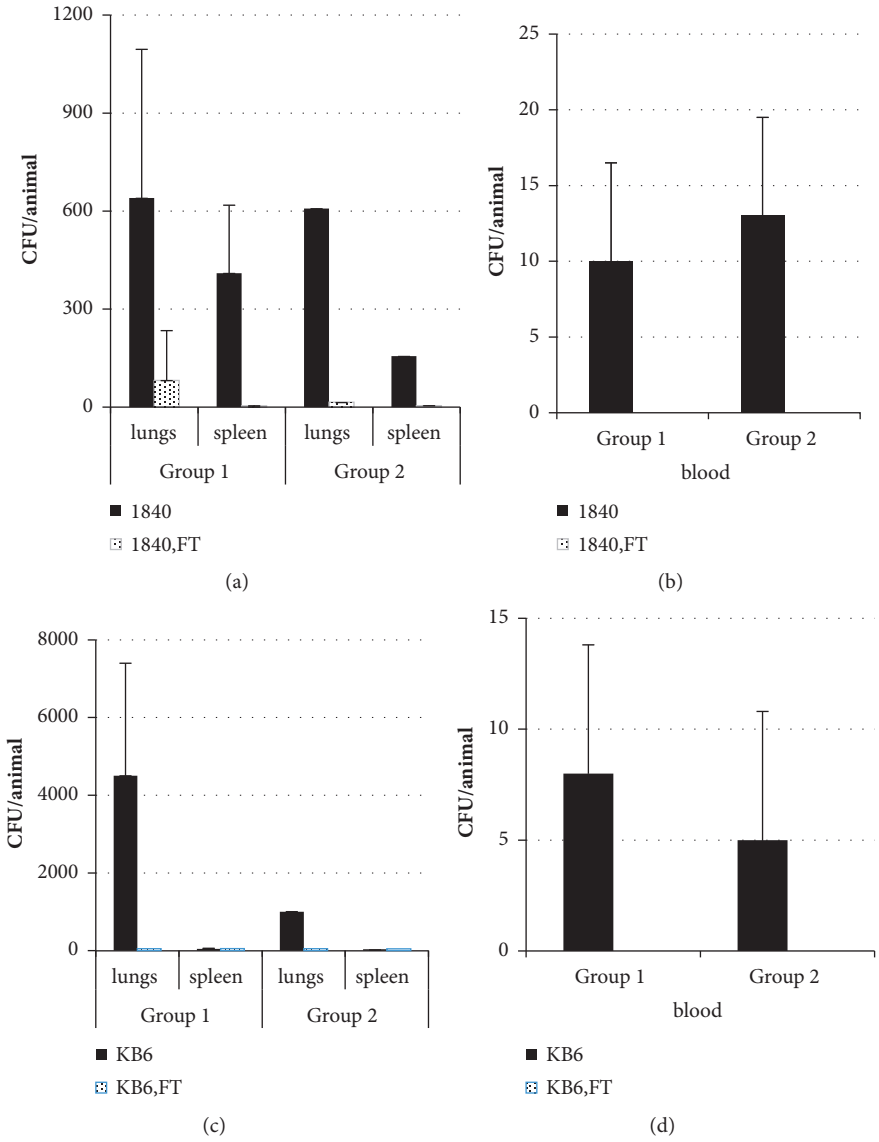

FIGURE 2: Treatment with FT reduces *P. aeruginosa* loads after infection with antibiotic-resistant *P. aeruginosa* clinical isolates of *exoU*$^+$ (1840) and *exoS*$^+$ (KB6) genotypes. A/Sn mice were infected with 6.5x10^6 CFU/mouse of *P. aeruginosa* clinical isolate 1840 ((a), group 1); with 3.25x10^6 CFU/mouse of *P. aeruginosa* clinical isolate 1840 ((b), group 2); with 2.2x10^7 CFU/mouse of *P. aeruginosa* clinical isolate KB6 ((c), group 1); with 1.1x10^7 CFU/mouse of *P. aeruginosa*$^+$ clinical isolate KB6 ((d), group 2). Infected animals were treated *per os* with 50 mg/kg of FT immediately after infection for 4 days twice a day. Bacterial loads in lungs, spleens, and blood of survived animals were analyzed at day 5 postinfection. Black bars, mice not treated with FT; dotted bars, mice treated with FT, $P < 0.05$.

spread of infection. Introduction of FT decreased bacterial burden in lungs, spleen, and blood compared to the controls ($P \geq 0.05$). Lungs of 70% of mice infected with 7x10^6 CFU/animal of clinical isolate 1840 were cleared completely and all mice had no bacteria in spleen and blood. Statistically significant increase in the number of mice that cleared infection from lungs (8 compared to 1 in the control group) was found in the group of mice infected with 3.5x10^6 CFU/animal of clinical isolate 1840. For the clinical isolate KB6 mice infected with 1.75x10^7 CFU/animal had 1.8±0x10^3 CFU/lung of bacteria and mice infected with 8x10^6 CFU/animal had 4,7±3,6x10^2 CFU/lung of bacteria. Infection was also found in blood and spleen (Figures 4(c) and 4(d)). Treatment with FT reduced bacterial loads in lungs (2.1±3.4x10^1 and 0.6±1.0x10^1 correspondingly). 7 and 9 mice from high and low dose infection groups correspondingly were completely cleared from infection. Survived mice had no bacteria in the blood.

Therefore, the results obtained give the evidence on the effectiveness of FT in the combined prophylaxis-treatment regimen of airway infection, induced with multiple antibiotic-resistant clinical isolates expressing various T3SS proteins. FT decreased mortality and bacterial loads in lungs and completely cleared infection from the blood.

3.5. Reduced Lung Damage after Treatment with FT. Lung morphology was studied in mice infected with 3.25x10^6

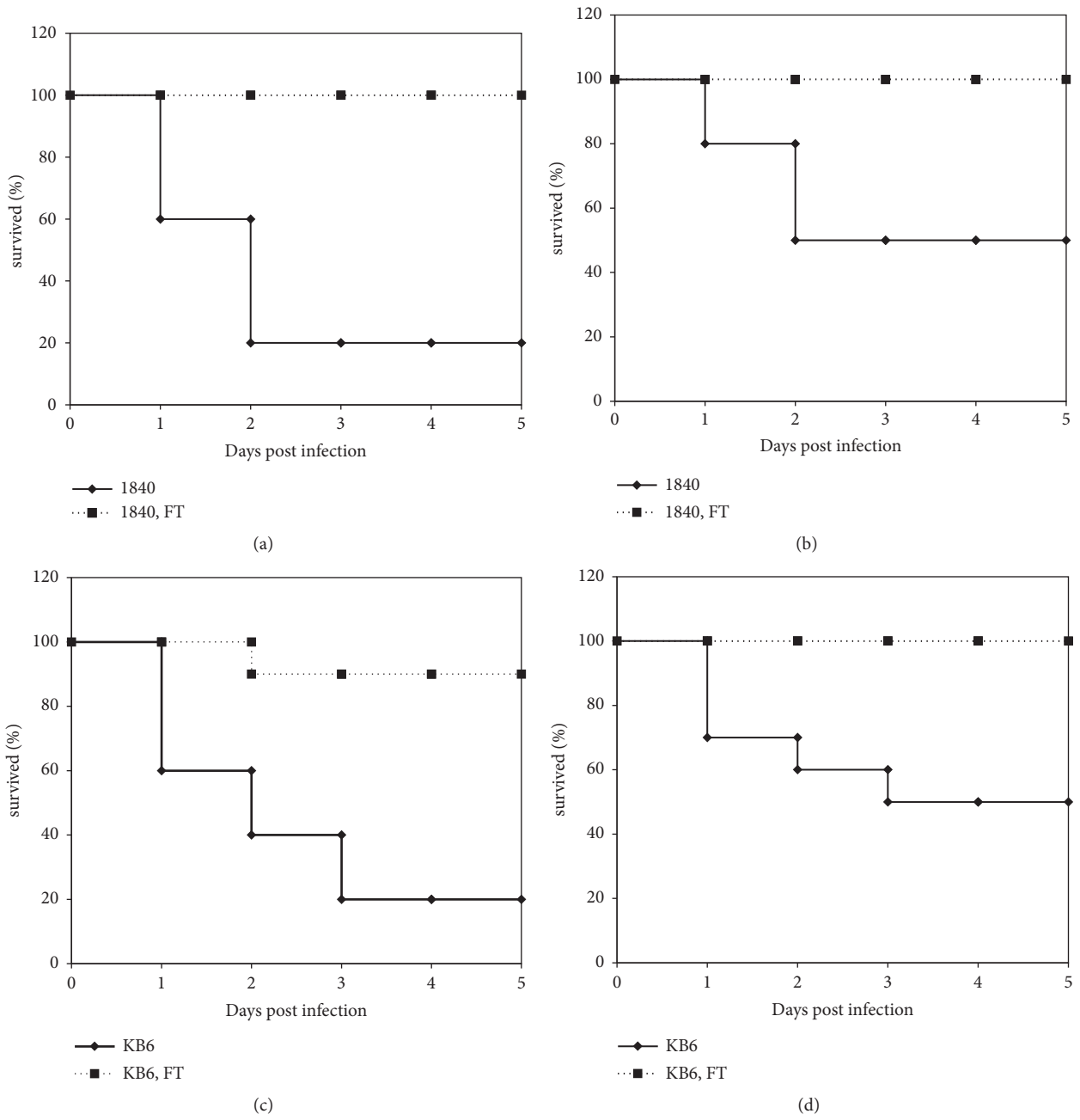

Figure 3: Combined prophylaxis-therapy treatment with FT improves survival of mice infected with *P. aeruginosa* antibiotic-resistant clinical isolates of *exoU*$^+$ (1840) and *exoS*$^+$(KB6) genotypes. (a) mice infected with clinical isolate 1840, 7.0x10^6 CFU/animal; (b) mice infected with clinical isolate 1840, 3.5x10^6 CFU /animal; (c) mice infected with clinical isolate KB6, 1.75x10^7 CFU/animal; (d) mice infected with clinical isolate KB6, 8x10^6 CFU/animal. Mice were treated with 100 mg/kg of FT *per os* once a day for 2 days before infection and with 50 mg/kg of FT twice a day for 4 days starting immediately after infection.

CFU/animal of *exoU*$^+$ clinical isolate 1840 and treated with FT. To this end, mice were treated *per os* with 50 mg/kg of FT twice a day for 3 days. The results are presented in Figure 5. Infection resulted in a pronounced damage to lungs as deduced from H&E staining. Damaged alveoli structure, peribronchial leukocyte infiltration, and dense parenchyma indicated severe lung inflammation (Figures 5(c) and 5(d)). Treatment with FT resulted in decreased cellularity in alveoli and in interstitial spaces and alveolar septal thickening. However, separate spots of infiltration were still observed in the treated groups (Figures 5(e) and 5(f)). Overall, these results suggest that FT effectively prevents lung damage in mice infected with multiple antibiotic-resistant clinical isolates of *P. aeruginosa*.

3.6. FT Modulates Proinflammatory Cytokines in the Course of P. aeruginosa Airway Infection. In this study, we have analyzed the effect of FT treatment on cytokine production

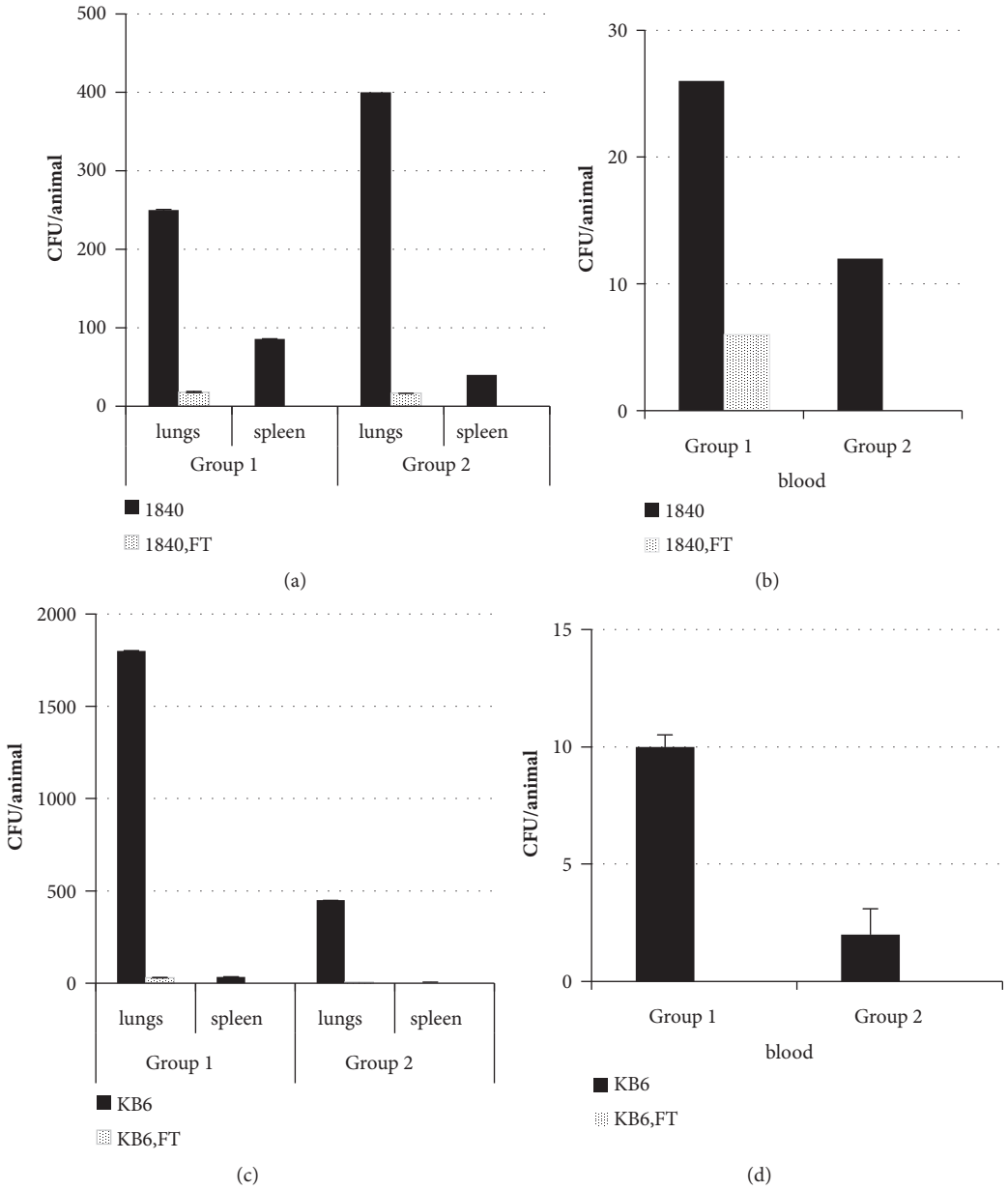

FIGURE 4: The prophylaxis-therapy regimen with FT reduces *P. aeruginosa* loads after infection with antibiotic-resistant *P. aeruginosa* clinical isolates of *exoU*$^+$ (1840) and *exoS*$^+$ (KB6) genotypes. A/Sn mice were infected with 7x10^6 CFU/mouse of *P. aeruginosa* clinical isolate 1840 ((a), group 1); with 3.5x10^6 CFU/mouse of *P. aeruginosa* clinical isolate 1840 ((b), group 2); with 1.75x10^7 CFU/mouse of *P. aeruginosa* clinical isolate KB6 ((c), group 1); with 8x10^6 CFU/mouse of *P. aeruginosa* clinical isolate KB6 ((d), group 2). Mice were treated with 100 mg/kg of FT *per os* once a day for 2 days before infection and with 50 mg/kg of FT twice a day for 4 days starting immediately after infection. Bacterial loads in lungs, spleens, and blood of survived animals were analyzed at day 5 postinfection. Black bars, mice not treated with FT; dotted bars, mice treated with FT, $P < 0.05$.

in lungs and blood during *P. aeruginosa* airway infection induced by 10^7 CFU/animal of *exoS*$^+$ clinical isolate KB6. Mice were treated *per os* with 50 mg/kg of FT twice daily before infection and once postinfection. Mice were sacrificed 24 and 48 hours after bacterial infection (n=10 in each group, two separate experiments). The levels of key cytokines involved in the regulation of inflammation were determined in lung tissue homogenates and blood serum using ELISA (Figure 6).

Treatment with FT significantly increased the levels of the proinflammatory cytokines IL-6 and TNF-alpha and IFN-gamma in lung homogenates at day 1 postinfection (Figures 6(a), 6(b), and 6(c)). The levels of these cytokines were also higher in FT-treated group compared to nontreated mice at day 2 postinfection, however, tended to decrease compared to the levels at day 1.

In contrast, in blood, we observed a significant decrease of IL-6 at day 2 postinfection in FT- treated mice infected

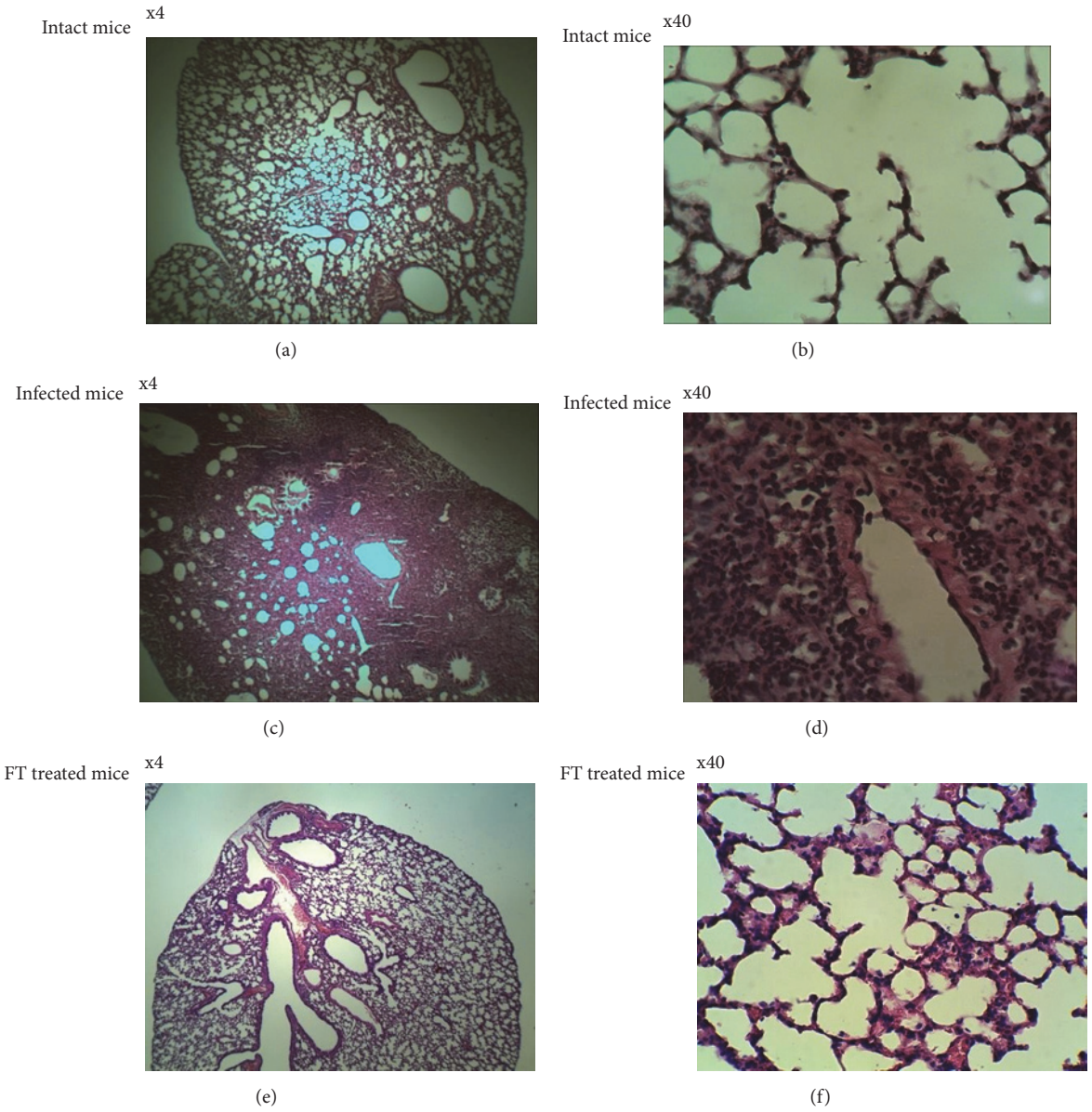

FIGURE 5: FT reduces lung pathology in mice intranasally infected with *P. aeruginosa* clinical isolate *exoU*$^+$ 1840 in a dose of 3.25x10^6 CFU/mouse. Mice were treated *per os* with 50 mg/kg of FT twice a day for 3 days starting immediately after infection. Lungs of survived animals were sectioned, stained with H&E and analyzed at day 5 postinfection. Photographs were taken at multiplication x4 (a, c, e) and x40 (b, d, f) for intact (a, b); infected (c, d) and FT-treated mice (e, f).

with KB6 compared to nontreated animals (Figure 6(d)). No alterations of TNF-alpha or IFN-gamma were observed in the blood of all experimental groups compared to controls (data is not shown).

Generally, the effects from FT were seen in all compartments investigated, suggesting both systemic effects and effects within the lung. FT increased the levels of proinflammatory cytokines in lungs at early stages of infection that probably reflects its ability to confront virulence mediated downregulation of host defenses; however, it significantly decreased the level of systemic production of IL-6 in blood that in line with a decrease in systemic bacterial loads manifests its potential to control systemic infection.

3.7. FT Inhibits Secretion of P. aeruginosa T3SS Effectors and Bacteria Cytotoxicity and Restores Bacterial Internalization. Next, we have assessed the effects of FT on the secretion of T3SS effectors. To this end, we evaluated *in vitro* secretion of ExoT and ExoY proteins in *P. aeruginosa* clinical isolates by immunoblot. Clinical isolates 1840 and KB6 were incubated *in vitro* with different concentrations of FT for 4 hours. Expression of T3SS was induced by decreasing Ca^{+2} concentration. We have found that FT inhibits secretion of ExoT and ExoY in a dose-dependent manner as shown in Figure 7. Inhibition of T3SS in clinical isolates 1840 and KB6 was observed starting with the concentration of 10 μg/ml. No difference in bacterial growth in the presence or absence of FT

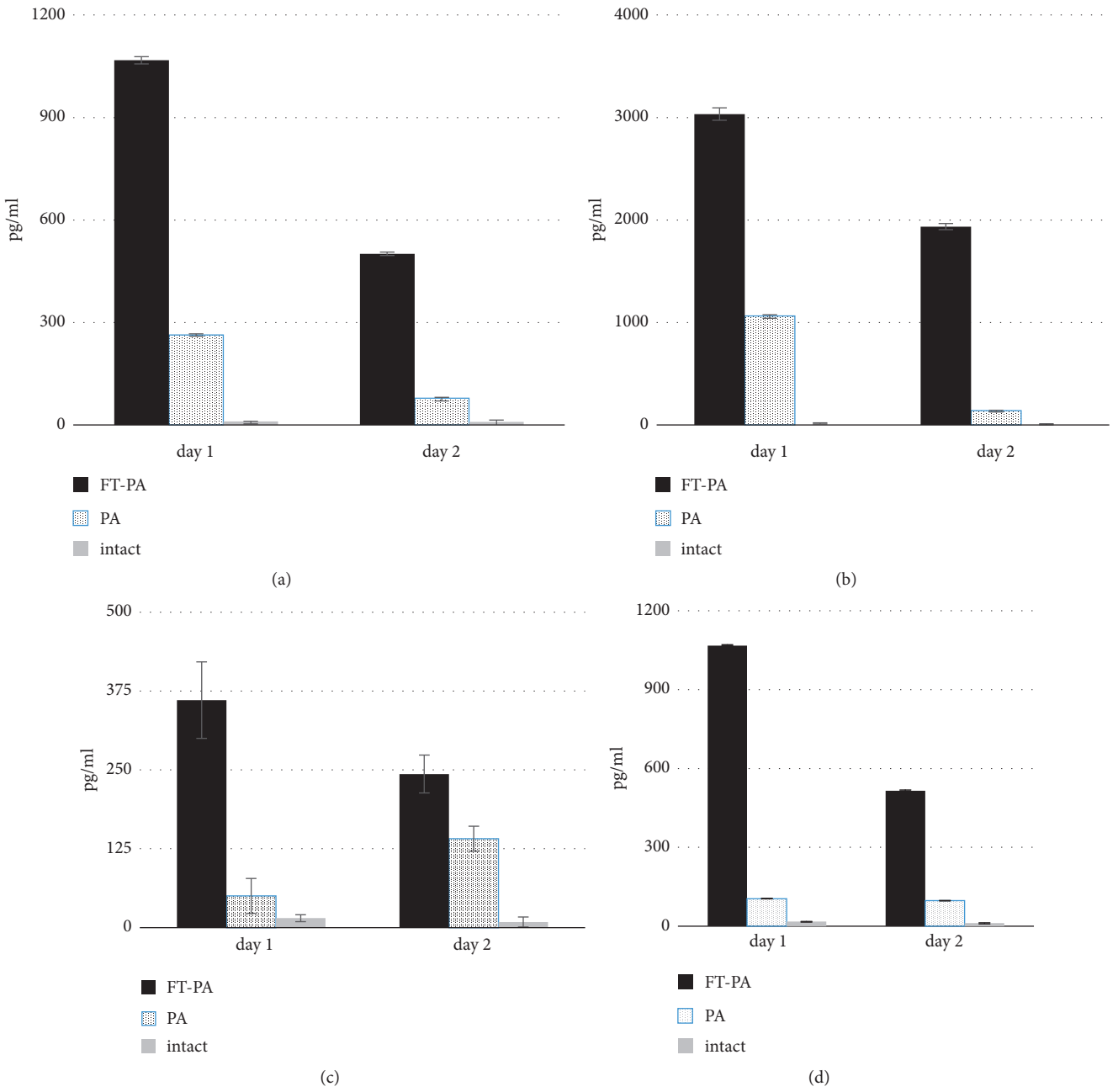

FIGURE 6: FT increases the production of inflammatory cytokines in lungs but decreases IL-6 production in blood. A/Sn mice were infected intranasally with 10^7 CFU/animal of *P. aeruginosa exoS*$^+$ clinical isolate KB6. Mice were treated with 50 mg/kg of FT *per os* twice daily before infection and once postinfection. IL-6 (a), TNF-alpha (b), and IFN-gamma (c) were tested in lung homogenates at day 1 and 2 PI. IL-6 (d) in blood was tested at day 2 PI. Black bar, treatment with FT; dotted bar, untreated infected mice; grey bar, intact controls, $P < 0.05$.

for *P. aeruginosa* reference strains as well as for clinical isolates cultured for 24 hours was observed in these experiments (Supplementary Materials, Table S2). Thus, we confirmed that FT downregulates the secretion of *P. aeruginosa* T3SS effector proteins.

Next, we assessed the effects of FT on *P. aeruginosa* induced cytotoxicity. CHO cells were infected with *P. aeruginosa* clinical isolates preliminary incubated for 30 minutes with different concentrations of FT. We found that *P. aeruginosa* clinical isolates induced profound cell cytotoxicity given in a dose of 10 MOI. Addition of FT in the doses of 10, 20, and 40 μg/ml significantly reduced cell cytotoxicity ($P \leq 0.05$). The results are presented in Figure 8. We found that FT inhibited cytotoxicity of *P. aeruginosa* clinical isolates in a dose-dependent manner. We found that FT completely inhibited cytotoxicity of ExoU expressing clinical isolate 1840 at the concentration of 20 μg/ml. The cytotoxicity of two other *exoU*$^+$ isolates was inhibited up to 50%. FT inhibition of ExoS expressing *P. aeruginosa* clinical isolates cytotoxicity was more pronounced compared to ExoU expressing strains.

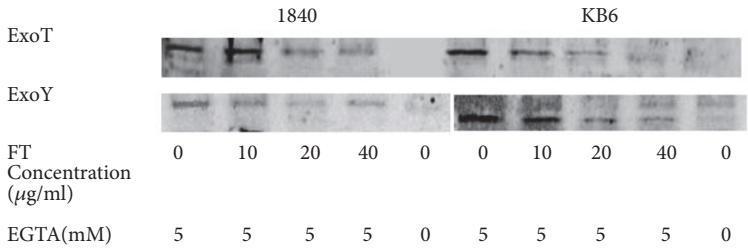

Figure 7: FT inhibits the secretion of *P. aeruginosa* T3SS effectors in the clinical isolates under the study. Expression of T3SS was induced by adding 5 mM of EGTA to culture media. FT was added in concentrations from 10 to 40 μg/ml. ExoT and ExoY were evaluated by immunoblot with polyclonal antibodies against ExoT and ExoY.

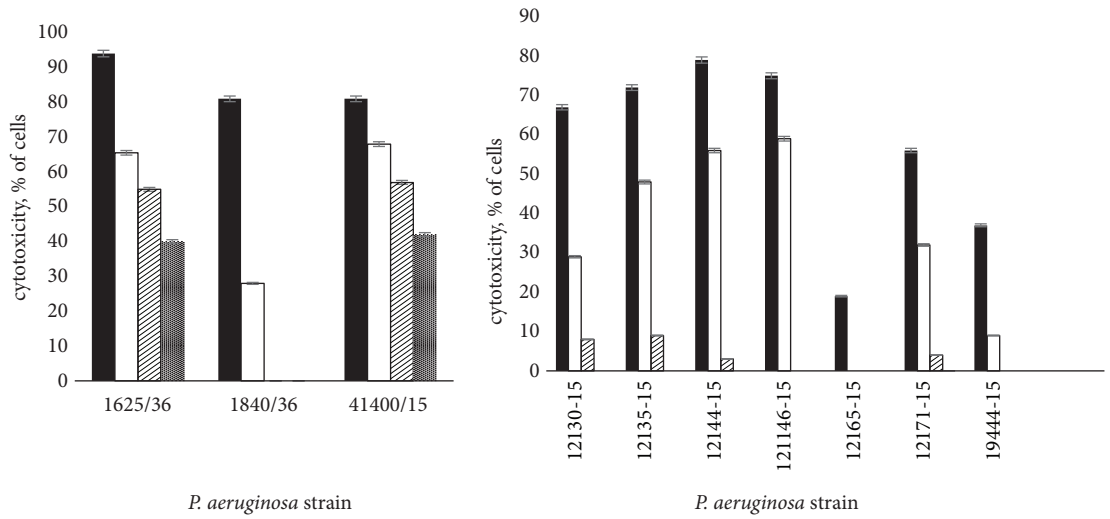

Figure 8: FT reduced *P. aeruginosa* cytotoxicity towards CHO cells. (a) ExoU expressing *P. aeruginosa* clinical isolates; (b) ExoS expressing *P. aeruginosa* clinical isolates. Dark bars, controls; white bars, 10 μg/ml of FT; crossed bars, 20 μg/ml; checkered bars, 40 μg/ml of FT, $P < 0.05$.

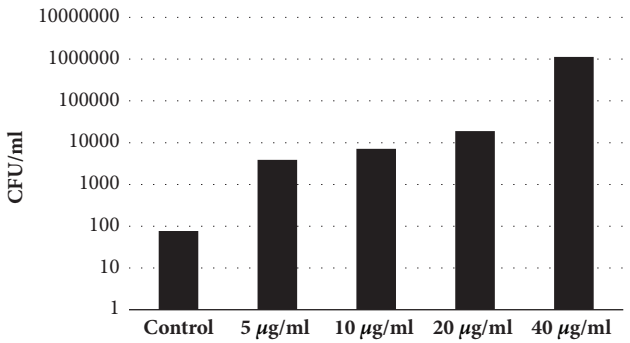

Figure 9: FT increased bacteria internalization in a dose-dependent manner. To assess the capability of FT to affect bacteria internalization, *P. aeruginosa exoS*$^+$ clinical isolate 1653, sensitive to gentamicin, was preincubated with FT for 30 min and was added to HeLa cells at MOI of 10. After incubation for 2 hours extracellular bacteria were eliminated by gentamicin. The numbers of intracellular bacteria were determined 2 hours later, $P < 0.05$.

P. aeruginosa ExoS and ExoT were shown to prevent bacteria internalization by epithelial and phagocytic cells that in turn reduces bacteria elimination by phagocytes and facilitates the spread of infection. To assess the capability of FT to affect bacteria internalization, *P. aeruginosa* clinical isolates were preincubated with FT for 30 min and were added to HeLa cells at MOI of 10. After incubation for 2 hours extracellular bacteria were eliminated by gentamicin. The numbers of intracellular bacteria were determined 2 hours later. *P. aeruginosa exoS*$^+$ clinical isolate 1653, sensitive to gentamicin, was used in these experiments. We have found that FT increased bacteria internalization in a dose-dependent manner (Figure 9). Even 5 μg/ml gave a 50-fold increase in the quantity of internalized bacteria, while 40 μg/ml of FT gave 10^4 increase in bacteria internalization.

4. Discussion

This study suggests that a small molecule compound, designated as Fluorothiazinon (FT), given as a combined prophylaxis-therapy treatment or as a therapy started after the onset of infection may improve the outcome in severe antibiotic-resistant *P. aeruginosa* airway infection.

Therapeutic agents that target virulence determinants of pathogenic bacteria have become an increasingly promising alternative to antibiotics [20, 21]. T3SS proteins are attractive targets for "anti-virulence" compounds because they are

often essential to the virulence of widely distributed Gram-negative bacterial pathogens of plants, animals, and humans. Targeting only virulence and lacking unwanted side effects such as evolvement of antibiotic-resistant variants makes this therapeutic strategy highly promising. Recently, whole-cell-based high-throughput screens performed to identify T3SS inhibitors gave several classes of small molecule compounds. Salicylidene acylhydrazides, salicylanilides, sulfonylaminobenzanilides, benzimidazoles, thiazolidinone, and some natural products were shown to be effective against a number of pathogenic bacteria that utilize T3SS, including *Yersinia, Chlamydia, Salmonella*, enteropathogenic *Escherichia coli, Shigella*, and *Pseudomonas* [11, 22–25].

A novel compound with a predicted T3SS inhibitory activity named FT, N-(2,4-difluorophenyl)- 4-(3-ethoxy-4-hydroxybenzyl)-5-oxo-5,6-dihydro-4H-[1, 3, 4]-thiadiazine2-carboxamide was previously characterized by low toxicity, high levels of solubility, stability, and specific efficiency towards *C. trachomatis* and *Salmonella in vitro* and *in vivo* [14, 16]. Besides, FT was shown to decrease the translocation of the type III secretion effector IncA of *C. trachomatis* [17].

In this study, we show that FT given as a combined prophylaxis-therapy treatment regimen or as a therapy started after the onset of infection significantly reduced mortality of mice infected with antibiotic-resistant *P. aeruginosa* clinical isolates. Besides, FT treatment significantly reduced bacterial loads in lungs and blood of experimental animals. The potential of FT to control generalized pseudomonas infection induced by antibiotic-resistant clinical isolates can be of great importance for improved clinical outcomes.

Lung infection with *P. aeruginosa* antibiotic-resistant clinical isolates of two different T3SS genotypes in our model was associated with severe lung inflammation reflected as damaged alveoli structure, peribronchial leukocyte infiltration, and parenchyma thickening. This represents an important feature of *Pseudomonas* lung pathogenesis and in line with clinical data. FT treatment resulted in the rehabilitation of alveoli structure and lesser interstitial cellularity (Figure 5); however, residual leukocyte infiltration of lungs was still observed in FT-treated groups.

Therefore improved mortality rates and decreased bacterial loads were associated with a decrease of lung pathology as analyzed at day 5 postinfection. That might be due to the lesser numbers of bacteria in lungs as shown in other studies [26]. Furthermore, diminished virulence of bacteria due to downregulation of exotoxins (Figure 7) can also contribute to the decrease in lung pathology [27].

The dysregulated host responses to bacterial toxins are of critical importance during severe infections [28]. T3SS was shown to interfere with the protective host responses. Thus, *P. aeruginosa* T3SS effector protein ExoU can inhibit activation of the NLRC4 inflammasome and caspase-1 and, as a result, downregulates rapid neutrophil recruitment and rapid infection clearance [29]. ExoS was shown to prevent neutrophil recruitment and efficient clearance of bacteria [30]. As neutrophil accumulation in lungs is under the control of tumor necrosis factor-alpha (TNF-alpha) [31] in the present study we evaluated its production in lungs of mice infected with antibiotic-resistant clinical isolates 1840 and KB6. IL-6 and IFN-gamma were also evaluated in this study. Treatment with FT significantly increased the levels of IL-6, IFN-gamma, and TNF-alpha in lungs at day 1 postinfection compared to nontreated animals. The quantity of IL-6, IFN-gamma, and TNF-alpha in FT-treated group decreased at 48 hours postinfection but still remained higher than in infected group not treated with FT. IL-6 in lungs of pseudomonas-infected animals was recently shown to contribute to the local protection against some of *P. aeruginosa* toxins [32]. At the same time, it was found to be harmful in generalized infection and sepsis [33]. In our study in contrast to elevated levels of IL-6 levels in lungs, blood IL-6 levels were decreased in FT-treated group compared to nontreated infected mice. These data suggest FT potential to control generalization of inflammatory processes along with generalized infection. This is also in line with the general concept of the roles of T3SS in the dissemination of infection [34]. Our results on reducing of lung pathology in FT-treated mice suggest that FT decreases the severity of infection induced by antibiotic-resistant *P. aeruginosa* resistant clinical isolates. In this study, we also assessed FT effects on *P. aeruginosa* T3SS toxins as it was originally described as inhibitors of T3SS in *Salmonella* and *Chlamydia* spp. [14–17]. We found that FT reduces the total amount of ExoT and ExoY toxins detected by Western blot in FT-treated cultures of *P. aeruginosa* clinical isolates 1840 and KB6 (Figure 7). Besides, FT increased bacteria internalization in HeLa cells (Figure 9) and reduced cytotoxicity of various *P. aeruginosa* clinical isolates towards CHO cells (Figure 8). Our results on FT activity on the secretion of T3SS effectors and their functions are in line with previously reported data for different T3SS inhibitors. Thus, the inhibition of the T3SS-mediated secretion and translocation of ExoS or ExoT by mutation was shown to increase internalization of bacteria (6, 15, 18, 50). Overall, the results obtained in this study suggest that FT is a promising novel T3SS inhibitor of pulmonary antibiotic-resistant *P. aeruginosa* infection.

5. Conclusions

In conclusion, in this study we found that Fluorothiazinon successfully reduced mortality and bacterial loads and decreased lung pathology and systemic inflammation in a mouse bronchopulmonary model. It inhibited the secretion of T3SS effectors ExoT and ExoY, reduced bacteria cytotoxicity, and increased bacteria internalization *in vitro*. Overall, FT shows a strong potential as an antibacterial therapy of antibiotic-resistant *P. aeruginosa* infection.

Data Availability

For more data from the article, please send a request to the mail snejpice@gmail.com, Anna Sheremet.

Disclosure

The project report is deposited on https://pharmmed2020.mipt.ru.

Conflicts of Interest

All authors state that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary 1. Table S1. *P. aeruginosa* clinical isolates.

Supplementary 2. Table S2. Impact of FT on *P. aeruginosa* clinical isolates growth.

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Review Article

The Role of BPIFA1 in Upper Airway Microbial Infections and Correlated Diseases

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The mucosa is part of the first line of immune defense against pathogen exposure in humans and prevents viral and bacterial infection of the soft palate, lungs, uvula, and nasal cavity that comprise the ear-nose-throat (ENT) region. Bactericidal/permeability-increasing fold containing family A, member 1 (BPIFA1) is a secretory protein found in human upper aerodigestive tract mucosa. This innate material is secreted in mucosal fluid or found in submucosal tissue in the human soft palate, lung, uvula, and nasal cavity. BPIFA1 is a critical component of the innate immune response that prevents upper airway diseases. This review will provide a brief introduction of the roles of BPIFA1 in the upper airway (with a focus on the nasal cavity, sinus, and middle ear), specifically its history, identification, distribution in various human tissues, function, and diagnostic value in various upper airway infectious diseases.

1. Introduction

The gene of the short palate, lung, and nasal epithelium clone 1 (*splunc1*), now referred to as BPIFA1, was first described in the palate and nasal epithelium of murine embryonic and adult lung tissue, which is referred to as *plunk* [1]. The human gene that encodes BPIFA1 contains nine exons that are located on chromosome 20q11.2, and its expression is limited to the upper airway and nasopharyngeal region, including the trachea and nasal epithelium [2, 3]. Di and his colleagues [4] determined that this gene, referred to as *spurt* (secretory protein in upper respiratory tract), was significantly induced by all-trans-retinoic acid in primary cultured human tracheobronchial epithelia.

BPIFA1 is a member of a family of seven proteins that are encoded by adjacent genes in an approximately 300 kb region of chromosome 20q11 (Figure 1). The human BPIFA1 cDNA is 1,020 bp in length and contains a leucine-rich protein of 256 amino acids weighing approximately 25 kDa [2]. BPIFA1 is also a member of the parotid secretory protein (PSP)/lipopolysaccharide-binding protein (LBP) superfamily of proteins [5]. Members of the PLUNC family fall into two groups based on their size: "short" proteins, a group comprising BPIFA1 (256 amino acids), SPLUNC2 (249 amino acids) and SPLUNC3 (253 amino acids) and "long" proteins comprising LPLUNC1 (484 amino acids), LPLUNC2 (458 amino acids), LPLUNC3 (463 amino acids), and LPLUNC4 (>469 amino acids) [6]. LPLUNC6/BPIFB6 is important in

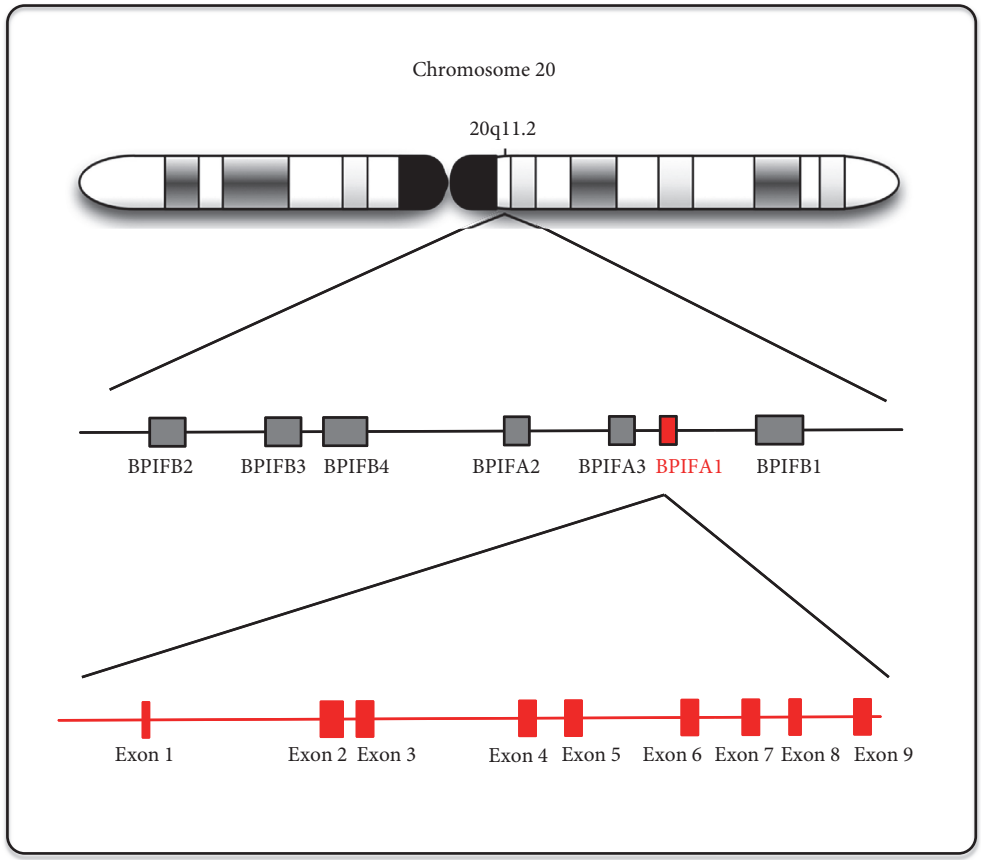

FIGURE 1: **The genomic location of *BPIFA1* and related *BPI* family members.** *BPIFA1* is located on chromosome 20q11.2 and contains nine exons. There are 7 *BPIF* gene families located in loci of *BPIFA1, BPIFA2, BPIFA3, BPIFB1, BPIFB2, BPIFB3,* and *BPIFB4*.

some virus replication as coxsackievirus B (CVB), poliovirus (PV), and enterovirus 71 (EV71) [7].

SPLUNC proteins contain domains structurally similar to the N-terminal domain of bactericidal/permeability-increasing protein (BPI), whereas LPLUNC proteins contain domains structurally similar to both domains of BPI [6, 8].

Human BPIFA1 is expressed in the salivary gland, and nose of the upper respiratory tract [21]. High expression of BPIFA1 is also found in normal adult nasopharyngeal epithelium [2, 6, 22], trachea, bronchi of the adult lung [1, 23]. Various reports have demonstrated that bactericidal/permeability-increasing fold containing family A, member 1 (BPIFA1) is present in the innate immune material that protects against various diseases and has been widely reported in humans to play a role in lower airway defense against different kinds of bacterial infection. For example, higher levels of BPIFA1 have been correlated with the presence of *Pseudomonas pneumonia* [16, 24, 25], *Klebsiella pneumonia* [26], *Mycoplasma pneumonia* [27], and *Staphylococcus aureus* [28]. BPIFA1 also plays an important role in the innate immunity of the pulmonary airway against influenza A [20, 29] and respiratory syncytial virus infection [14]. Other lower respiratory tract diseases, notably cystic fibrosis, have also demonstrated a correlation between BPIFA1 expression and disease progression [21, 30]. Several reports illustrated a correlation between BPIFA1 expression and middle ear infection [31], chronic rhinosinusitis with nasal polyps (CRSwNP) [32, 33], and sinusitis [34]. Quantitation of BPIFA1 expression could be applied as a diagnostic tool for certain upper airway diseases and may have a value for determining treatment outcomes. Although existing reviews have examined the functions of BPIFA1 and correlations with lower airway diseases, few studies have focused on its functions and correlates in the upper airway.

2. BPIFA1 Expression and Regulation

After searching the databases, the BPIFA1 in various upper airway infectious diseases WAS retrieved. Overall, BPIFA1 has been examined through specific expression in upper airway tracts, including the tongue, tonsil, nasal polyps, adenoid, and middle ear (Figure 2).

The materials that affect BPIFA1 expression were summarized to assess future therapeutic applications of this secretory protein in treating upper airway-related diseases (Table 1). Although there are currently no regimens for direct supplementation with BPIFA1, repression of BPIFA1 in patients may still be prevented by controlling or treating the patient's underlying allergic rhinitis or asthma-related disease. This approach may decrease levels of IL-13, a cytokine

Table 1: Materials that affect BPIFA1 expression.

Effector of BPIFA1 expression	Study design materials	Correlated diseases	Disease findings	Mechanism/pathway	Biofilm correlation /pathogen
IL-13	Human nasal lavage fluid	Allergic rhinitis	Lower in allergy	ERK	Not mentioned /not mentioned
	Tissue specimens by sinus surgery	LPS treated RPMI-2650	Lower in polyps tissue	JNK, AP1	Not mentioned/LPS toxin
Lactoferrin	RPMI-2650 cells	LPS treated RPMI-2650	Recovery of BPIFA1	Inhibition of MEK/ERK pathway	Not mentioned /LPS toxin
Intranasal steroid	RPMI-2650 cells	LPS treated RPMI-2650	Recovery of BPIFA1	Reactivation of AP-1	Not mentioned /LPS toxin
MMP9 (matrix metallo-proteinase 9)	Targets cells/macrophages/oral epithelia/airway epithelia	PLUNCS inhibited by MMP9	PLUNCS inhibited	PLUNC protein proteolytically cleaved by MMP9	Not mentioned /microorganism colonization

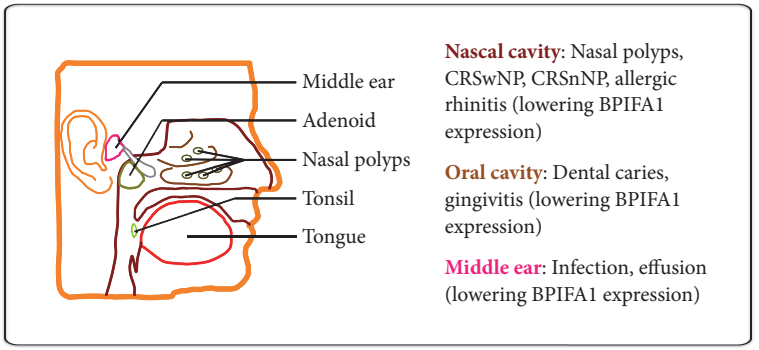

Figure 2: **Highly specific expression of BPIFA1 in the human upper airway respiratory system.** The major expressed tissues of BPIFA1 are located in the tongue, tonsil, nasal polyps, adenoid, and middle ear (as shown in the left panel). Those lowering BPIFA1 expression are affected by infections and correlated diseases of upper airway tracts (as shown in the right panel).

secreted by many cell types, primarily Th2 helper T cells, to help mediate anti-inflammatory responses to allergens and prevent downregulation of BPIFA1 through the JNK/c-Jun, API, and ERK pathways.

Another critical protein, lactoferrin, has been shown to recover expression of BPIFA1 after LPS-induced infection and is a safe, established product utilized in the treatment of various human diseases [18]. Lactoferrin is a key innate material that defends humans from respiratory syncytial virus, middle ear infections, and sinusitis, while LPS is an endotoxin present in the outer membrane of Gram-negative bacteria that is strongly immunogenic in animals. Supplementation with lactoferrin has been shown to indirectly recover BPIFA1 attenuated by LPS through downregulation of the MEK/ERK pathway, suggesting a possible therapeutic benefit for patients with lower expression of BPIFA1 caused by chronic bacterial infection. Multiple studies have noted that intranasal steroids could restore BPIFA1 to normal levels by reactivating AP-1, the transcription factor responsible for regulating expression of BPIFA1 [35, 36].

Matrix metalloproteinases (MMPs) are responsible for degradation of extracellular matrix (ECM) proteins in normal cell turnover and are upregulated in inflammatory diseases of oral tissues [37], as well as in dental caries and oral cancer [38]. It is hypothesized that elevated MMPs may inhibit the function of BPIFA1. Therefore, inflammation itself could be the reason for downregulation of BPIFA1. Reducing inflammation of the nasal epithelium is another strategy to protect BPIFA1 from inhibition mediated by infection or injury.

How these studies were executed and their materials and methods are shown in Table 2 [9, 11–13, 15, 17–19, 39]. Furthermore, Table 3 summarizes the working mechanisms utilized by BPIFA1 to protect humans from related pathogens, viral or bacterial, that infect the upper airway. The primary mode of defense induced by BPIFA1 entails neutralization of

Table 2: Summary of available BPIFA1 studies, methods of analysis utilized, and related upper airway diseases under study.

Author (year)/[Reference]	Study design	Material	Methods	Correlated upper airway diseases
Casado et al. (2005)/[9]	Human (in vivo)	Nasal lavage fluids	Proteomics	Upper airway microbial infections and unclean inhaled air
Tsou et al. (2015)/[10]	Human (in vitro)	Septum squamous carcinoma	Western blot, RT-PCR, IHC for expression levels of BPIFA1 and IL-13	Human chronic rhinosinusitis with nasal polyps
Bingle and Gorr (2004)/[11]	Human (in vitro)	Human oral, nasal and respiratory epithelia	Cell line, genomes sequencing, cDNA, cDNAarray etc.	Oral, respiratory, GNB infection
Yeh et al. (2010)/[12]	Human (in vitro)	Nasal polyp epithelial cells in air-liquid interface culture	RT-PCR, Western blot	CRSWNP (chronic rhinosinusitis with nasal polyps)
Fornander et al. (2013)/[13]	Human (in vivo)	Nasal lavage fluids	Western blot, 2D gel electrophoresis	Upper airway symptoms
Fornander et al. (2011)/[14]	Human (in vivo)	Naspharyngeal aspiration	2D gel electrophoresis and mass spectrometry	Respiratory syncytial virus infection
Ghafouri et al. (2006)/[15]	Human (in vivo)	Nasal lavage fluids	2D gel electrophoresis, and matrix assisted laser desorption/ionization time-offlight mass spectrometry	Seasonal allergic rhinitis
Tsou et al. (2013)/[16]	Human (in vivo)	Sinus polyps	Bacterial culture, RT-PCR, IHC	CRSWNP (chronic rhinosinusitis with nasal polyps)
Tsou et al. (2014)/[17]	Human (in vivo)	Sinus polyps	Bacterial culture, RT-PCR, IHC	Sinonasal infections, CRSWNP
Tsou et al. (2017)/[18]	Human (in vitro)	Nasal RPMI-2650 cells	Western blot, indirect immunofluorescence, confocal laser scanning microscopy, and quantitative fluorescence analysis	CRSWNP (chronic rhinosinusitis with nasal polyps)
Rye et al. (2012)/[19]	Human (in vivo)	Genomic DNA was extracted from blood	Single nucleotide polymorphisms	Otitis media
Teran et al. (2012)/[20]	Human (in vivo)	Nasal aspirates	2D gel electrophoresis and mass spectrometry	Seasonal influenza A virus

LPS by direct binding or action as a surfactant to increase macrophage or neutrophil recruitment to the infected area of the upper airway. These mechanisms are discussed in the following sections.

3. Upper Airway Infection and Diseases

Upper airway infections cover a wide disease spectrum in human history from flu virus pandemics to outbreaks of various bacterial infections such as tonsillitis, adenoiditis, sinusitis, pharyngitis, and laryngitis. If not properly controlled or treated, these conditions may be life-threatening. Among infections in the head and neck region, tonsillitis, adenoiditis, and chronic sinusitis are often the most difficult to cure, and they incur major medical costs around the world in both adult and pediatric groups [40]. Various antibiotic-resistant conditions make the diseases hard to treat and susceptible to relapse in current practice due to both the increased population of various penicillin-resistant strains [10] and the intractable condition caused by biofilm accumulation [41]. Therefore, curative surgical procedures such as tonsillectomy and adenoidectomy (T/A) [42], middle ear surgery, and sinus surgery are inevitable and are widely used for chronic or recurrent upper respiratory tract infection (URTI) in child and adult chronic upper airway infection-related diseases. Avoidable surgical treatment options are for patients who have failed to respond to medication, and they are strongly correlated with tissue biofilm formation that subsequently elicits lower BPIFA1 production [16, 17]. Some upper airway infectious diseases such as chronic rhinosinusitis and middle ear infection lead to biofilm formation and lower BPIFA1 expression and require avoidable surgeries. These pathologies are summarized below.

3.1. Middle Ear Infection. Current evidence of the direct relationship between middle ear infection and BPIFA1 is lacking. Middle ear effusion, or otitis media, has been shown to exhibit BPIFA1 gene expression [19, 43]. The indirect relationship of otitis media and BPIFA1 may be related to poor eustachian tube function and BPIFA1 expression. Patients with decreased BPIFA1 expression were also noted to be smokers and to present with allergic rhinitis, conditions

Table 3: Summary of mechanisms, correlated pathogens, and findings from studies of BPIFA1 and related upper airway diseases.

Author (year)/[Reference]	Mechanism	Pathogen	Findings
Casado et al. (2005)/[9]	Innate immune response in the nose against microbial infections and unclean inhaled air	Not mentioned	LC-ESI-MS/MS was involved in acquired and innate immune response in the nose against microbial infections and unclean inhaled air, e.g., rhinosinusitis
Tsou et al. (2015)/[10]	IL-13 perturbation of GNB-related bacterial infection and BPIFA1 expression in CRSwNP through JNK/c-Jun pathway	Lipopolysaccharide (LPS) related GNB such as *Haemophilus influenza*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*	IL-13 attenuated LPS (GNB) bacteria-induced BPIFA1 expression causing compromise of certain GNB bacterial infections
Bingle and Gorr (2004)/[11]	LPS neutralization	Not mentioned	PLUNC proteins mediate host defense functions in the oral, nasal and respiratory epithelia
Yeh et al. (2010)/[12]	Inhibition of BPIFA1 production	Not mentioned	IL-13 is harmful to the host innate immune response through the inhibition of BPIFA1 production
Fornander et al. (2013)/[13]	BPIFA1 is a target of human neutrophil elastase (HNE) activity	Metal working fluids (biocides, surfactants, anti-oxidants and corrosion inhibitors)	IL-1b was significantly higher in subjects with airway symptoms
Fornander et al. (2011)/[14]	Innate immune response	Respiratory syncytial virus	A decrease in BPIFA1 in the upper airways may increase the risk for severe pneumonia
Ghafouri et al. (2006)/[15]	NLF levels of the cysteine proteinase inhibitors, cystatin S and VEGP were decreased and failed to inhibit proteinase action in SAR	Pollen	BPIFA1 is significantly decreased in the NLF during nasal inflammation by allergic rhinitis
Tsou et al. (2013)/[16]	Anti-bacteria or surfactant	*Pseudomonas aeruginosa*	Sinusitis with bacterial culture positive for *P. aeruginosa* isassociated with lower expression of BPIFA1 and unfavorable sinus surgery outcomes
Tsou et al. (2014)/[17]	Anti-bacteria or surfactant	GPC: *Staphylococcus areus* GNB: *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Klebsiella pneumonia*	BPIFA1 is a novel predictive outcome biomarker for patients with CRSwNP and bacterial colonization Lower BPIFA1 related to multiple bacterial infections
Tsou et al. (2017)/[18]	Lactoferrin increases BPIFA1 expression to regulate inflammation in RPMI-2650 cells through JNK/cJun pathway	Gram-negative bacteria	Lactoferrin could be a possible treatment strategy for LPS-induced chronic rhinosinusitis
Rye et al. (2012)/[19]	Genome-wide associated study of childhood otitis media susceptibility found that decreased BPIFA1 was correlated with higher otitis media in children	Not mentioned	GWAS was performed to identify the genetic determinants of OM in childhood
Teran et al. (2012)/[20]	Lipocalin-1 can enhance the bactericidal activity of lysozyme and exhibit inherent antimicrobial function	Seasonal influenza A virus	To provide the pathogenesis of respiratory infections caused by seasonal influenza A virus in nasal fluid

which were shown to increase the risk of developing otitis media with related middle ear diseases. This decrease in BPIFA1 expression in sinus or upper airway mucosa also leads to repeated infection of the upper airway, including middle ear infection and sinusitis [44].

3.2. Sinusitis. Chronic rhinosinusitis comprises a notable portion of upper airway infections and is considered a chronic upper airway infectious disease with diffuse sinus mucosa inflammation. In a case control study, the level of expression of BPIFA1 was depressed in patients with chronic rhinosinusitis, and the protein was considered to have provided a protective function in the sinus mucosa [34]. It was found that sinusitis positive for *Pseudomonas aeruginosa* bacterial culture is associated with decreased BPIFA1 in the sinus mucosa, which may serve as a diagnostic tool to assess expression of BPIFA1 in patients [16]. Furthermore, BPIFA1 expression was found to be significantly reduced in the mucosal epithelia and submucosal glands in patients with multibacterial colonization, particularly those mediated by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [17].

3.3. Chronic Rhinosinusitis with Nasal Polyps (CRSwNP). Patients with chronic rhinosinusitis with nasal polyps (CRSwNP) were found to have lower levels of BPIFA1 expression, which was associated with bacterial colonization [16]. This evidence indicated that decreased levels of BPIFA1 might facilitate bacterial infection in a host, leading to severe disease manifestations. Repeated sinus surgery is correlated with lower BPIFA1 expression and subsequently elevated pseudomonas infection rates. This pattern of repeated surgeries in the sinuses as well as poor surgical outcomes is often observed in CRSwNP [16]. Patients with CRSwNP had higher secretion of interleukin-13 (IL-13), which appears to play a critical role in downregulating BPIFA1 expression. In nasal epithelial cells, IL-13 attenuates BPIFA1 expression by downregulating the lipopolysaccharide- (LPS-) induced activation of phosphorylated JNK and c-Jun [39].

CRSwNP is a disorder characterized by a higher trend of developing Th2-driven inflammation and tissue eosinophilia that may be induced by microbial infection [45]. IL-13, a cytokine predominately secreted by Th2, has been found to contribute to airway allergies and to suppress BPIFA1 expression in nasal epithelial cells [12].

3.4. Allergic Rhinitis. The BPIFA1 level was significantly lower in patients with persistent allergic rhinitis (AR) [15, 46]. It was reported that patients with allergic rhinitis had significantly higher chronic rhinosinusitis rates than patients without allergic rhinitis [47]. Patients with lower BPIFA1 expression were found to be more susceptible to sinus infection by certain bacterial infection, which may explain this phenomenon.

4. Mechanisms and Therapeutic Use of BPIFA1

4.1. Possible Mechanisms of Defense Function. Several studies have shown that BPIFA1 possesses antimicrobial activity and exhibits the same surfactant properties as airway secretions, a trait that may inhibit the formation of bacterial biofilm. It has also been reported that BPIFA1 plays an important role in the regulation of airway surface liquid (ASL) volume [48]. Mechanistically, BPIFA1 contributes to the innate immune response by directly binding to LPS and yielding a bactericidal or bacteriostatic effect against various bacterial infections, an anti-biofilm function, and surfactant properties. Furthermore, BPIFA1 has been shown to be secreted from chemoattracted neutrophils or macrophages to support innate mucosal immunity in the lower airway [25, 49]. It was also reported that BPIFA1 protein binding to bacterial lipopolysaccharide inhibited the growth of *Pseudomonas aeruginosa* [50], *Klebsiella pneumonia* [26], *Mycoplasma pneumonia* [27], and the Gram-positive bacteria *Staphylococcus areus* [28] by its antimicrobial and surfactant adjusting function.

4.2. Mechanism as Surfactant. The surfactant function of BPIFA1 has been well-characterized *in vitro*. BPIFA1 contains hydrophobic residues and is a pH-sensitive regulator that can specifically bind to the epithelial Na^+ channel (ENaC) [48]. This channel has been shown to be critical to the regulation of ASL and absorption of fluids in many epithelia and is frequently the rate-limiting factor. In addition, BPIFA1 has been hypothesized to act as volume sensors to inhibit ENaC and thus fluid reabsorption by preventing its cleavage and activation by serine proteases. This mechanism inhibits ENaC-dependent Na^+ absorption, resulting in preservation of airway surface liquid volume. A proteomics screen for molecules that bind trypsin-sensitive ENaC channels identified BPIFA1 as a candidate volume sensor, which was supported by subsequent RNA interference (RNAi) knockdown experiments that demonstrated an inability to regulate ENaC-mediated fluid reabsorption in bronchial epithelial cell cultures [51]. Furthermore, the addition of recombinant BPIFA1 to culture restored ASL volume and ENaC regulation to normal values. Cells coexpressing cystic fibrosis transmembrane conductance regulator (CFTR) and ENaC were incubated with rBPIFA1, which inhibited ENaC activity but did not affect CFTR [52]. This illustrates the specificity of ENaC inhibition by BPIFA1 and its subsequent restoration of ASL, demonstrating a key molecular mechanism underlying the increase in mucosal fluid secretion by elevated BPIFA1 in response to pathogen exposure.

4.3. Relation of LPS Interaction. Possible biochemical pathways giving rise to anti-pseudomonas and anti-influenza infection may be mapped by surveying elevated chemokines quantitated in BAL and lower airway cell lines or animal lower airway infection models such as higher levels of CXCL1, CXCL2, and CCL20 [50]. CXCL1 recruits neutrophils to the infection site, while CXCL2 recruits monocytes, macrophages, and granulocytes to combat microbial infection by innate immune response. BPIFA1 was found to have an immunomodulatory function through the toll-like receptor 2 (TLR2) pathway [53], which is responsible for recognition of pathogens and recruitment of innate immune cells such as those delineated above. Further, this same review

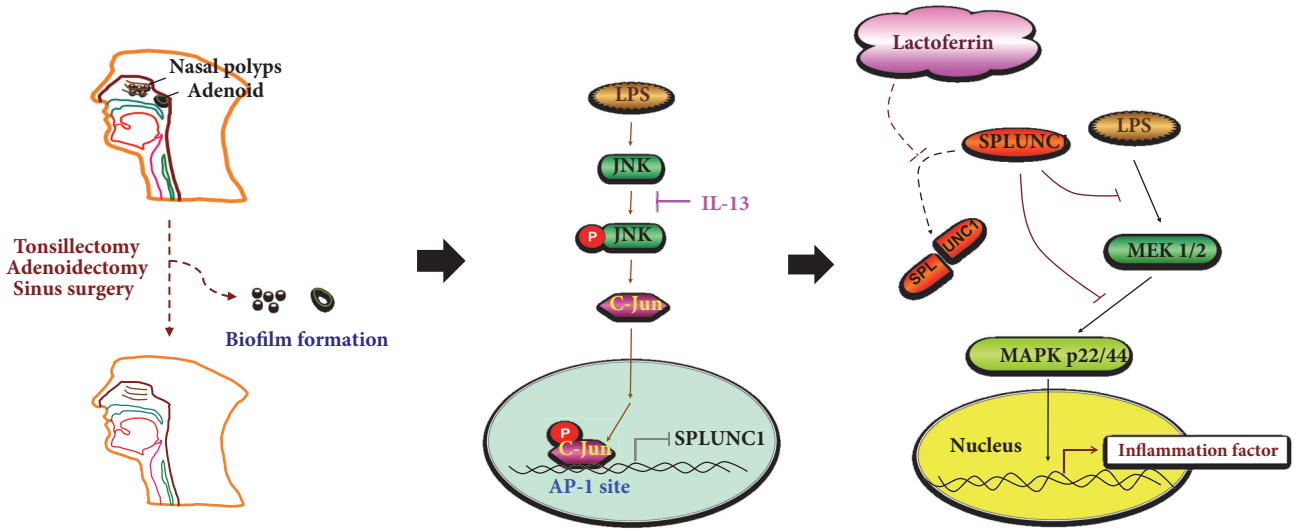

FIGURE 3: **Proposed mechanism of IL-13 inhibition of LPS-induced BPIFA1 expression in nasal polyps and adenoid tissue.** The IL-13 inhibits *BPIFA1* (*SPLUNC1*) gene expression through a JNK/c-Jun regulation pathway. Lactoferrin also interacts with BPIFA1 in the nasal polyps and adenoid tissues to avoid LPS-induced inflammation via downregulated MEK1/2-MAPK signaling.

illustrated that downregulation of TLR2 in non-transformed human bronchial epithelial cells through shRNA interference reduced BPIFA1 expression, demonstrating a positive correlation between the activation of this pathway and secretion of BPIFA1 in innate immune material. It was shown that, in defending the lower airway against *Mycoplasma pneumonia* infection, BPIFA1 signaled through the TLR2-NF-κB pathway and also through TLR2-induced (MARK)/activating protein 1 (AP-1) in human lung epithelium cells [54]. In a recent survey using a nasal epithelium (RPMI-2650) model, it was demonstrated that BPIFA1 expression was elevated after LPS-induced inflammation though the MEK/ERK pathway, and BPIFA1 expression was attenuated by IL-13 through the JNK and c-Jun signaling pathway [39] as shown in Figure 3. Lactoferrin was shown to recover the suppressed BPIFA1 through downregulation of the MEK1/2-MAPK signaling pathway by preventing BPIFA1 degradation by LPS. However, whether BPIFA1 exactly could bind with LPS is still questioned till now, since there was also a study which revealed that the LPS is not cognate ligand of BPIFA1 [55]. Besides, we will not just get the concept that BPIFA1 has a similar structure of BPI, which is a major LPS receptor. Actually, there is an allergen called dust mite Der P7 having a similar structure [56]. Therefore, we shall not only just focus on BPI but also check other ligand binding proteins in future studies.

4.4. Immune Effects. Neutrophil function was enhanced in the anti-*Mycoplasma pneumonia* immune response induced by BPIFA1 in chronic lung disease [27]. In the lower airway, knockout BPIFA1 mice were shown to have decreased mucociliary clearance and decreased innate immune function [50]. However, this same region in human patients contains human neutrophilic elastase (HNE), which could potentially degrade BPIFA1 in chronic obstructive pulmonary disease (COPD) patients with acute exacerbation of symptoms by nontypeable *Haemophilus influenzae* infection [57]. The mechanism of anti-influenza A function has yet to be elucidated, but it has been shown that increased amounts of BPIFA1 were quantitated after lung and bronchoalveolar lavage (BAL) administration [58]. The protein has been shown to function as an immunomodulatory material in the lower airway, as demonstrated in chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis. The expression of human BPIFA1 was altered in nasal lavage fluids (NLFs) after exposure to irritants and was quantitated in an increased proportion in NLFs of smokers, suggesting that it is involved in the airway inflammatory response [59, 60].

4.5. Microbial Defense. BPIFA1 was also shown to confer a resistance to *Klebsiella pneumonia* infection through its ability to adjust mucosa surface tension in contrast to the BPIFA1 knockout mouse model [26] and to decrease *Mycoplasma pneumoniae* levels that subsequently inhibited epithelial IL-8 production induced by *Mycoplasma pneumoniae*-derived lipoproteins. High levels of BPIFA1 have been shown to be expressed in normal human and mouse large airway epithelial cells. Although *Mycoplasma pneumoniae* infection increases BPIFA1, IL-13 still decreases BPIFA1 expression and *Mycoplasma pneumoniae* clearance, suggesting that BPIFA1 serves as a novel host defense protein against *Mycoplasma pneumonia* [23].

4.6. Different Mechanisms and Functions of BPIFA1 between Upper Airway and Lower Airway Infections. The major pathogens present in the lower airway respiratory tract include *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Mycoplasma pneumonia,* while the population of infectious pathogens in the upper airway includes *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia.*

Most of the Gram-negative bacilla (GNB) are correlated with LPS toxin-induced infections. BPIFA1 has been shown to neutralize the aforementioned bacterial species in LPS related interaction reported in the lower and upper airways; however, whether BPIFA1 binds to LPS is still questioned [18, 61].

The surfactant function of BPIFA1 is demonstrated mainly in cystic fibrosis in the lower airway and is not widely reported in the mucosa of the upper airway. Furthermore, LPS-induced BPIFA1 expression in the upper airway is mainly involved in JNK/c-Jun [39] and MEK1/2-MARK pathway signaling [18], while the lower airway is mediated by MARK/AP-1 activation through the TLR2 pathway [54]. The lower airway attracted neutrophils and macrophages that could also secrete BPIFA1 and aid in innate immune protection of pulmonary tissue, a phenomenon that has not been reported in the upper airway.

Patients presenting with nasal allergies and Th2-predominant upper airway allergic rhinitis had higher IL-13 expression correlated with low BPIFA1 expression and high sinusitis infection rate than patients lacking a background with allergic rhinitis. This observation also explained a trend in eosinophilic CRS patients who demonstrated higher IL-13 levels and reduced innate immunity to multiple bacterial, and specifically pseudomonas, infections compared to noneosinophilic CRS patients. This pattern was reported in the lower airway in *Mycoplasma pneumonia* clearance related to asthma pathobiology [23].

4.7. Materials or Cytokines That Affect BPIFA1 Expression. IL-13 inhibits BPIFA1 expression in upper airway infection. Lactoferrin may recover suppressed BPIFA1 expression by antagonizing LPS and could serve as a future treatment material in nasal spray treatment or sinus irrigation during postoperative care (Table 1).

5. Conclusions

The focus of this article was the secretory protein, BPIFA1, which plays a key role in the regulation of airway surface liquid volume and serves in host defense against bacterial infection. BPIFA1 expression was shown to be significantly reduced in the mucosal epithelia and submucosal glands in patients with multibacterial colonization, particularly those mediated by *S. aureus* and *P. aeruginosa*, a trend that may serve as a useful diagnostic marker should methods of BPIFA1 upregulation or supplementation be adapted for therapeutic use. CRSwNP patients requiring repeated sinus surgeries for recurrent or persistent sinusitis also presented much lower BPIFA1 expression than those who did not require repeated sinus surgery. Downregulation of BPIFA1 was shown to produce an immune defect that rendered the host more susceptible to bacterial infection, thus indicating that reduced SPLUNC1 expression might facilitate recurrent *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections in patients with CRSwNP. It was demonstrated that IL-13 plays a critical role in the regulation of BPIFA1 expression in patients by downregulating the LPS-induced activation of phosphorylated JNK and c-Jun, followed by attenuation of BPIFA1 expression in these patients.

Lactoferrin was shown to decrease LPS-induced inflammation-related protein MEK1/2 and p42/44 MAPK activation under BPIFA1 recovery. This finding might suggest that a new therapy could be developed that utilizes lactoferrin treatment to lessen inflammation and increase immune response in persistent infection of host airways.

It was noted that patients who underwent surgical procedures of T/A did not decrease chronic or recurrent upper respiratory tract infection and that repeated bacterial infections in the adenoid were shown to be caused by antibiotic resistance. Furthermore, it was demonstrated that biofilm formation played a major role in these ENT infections. In addition, the colonies of *S. aureus* and *P. aeruginosa* were associated with respiratory diseases, chronic adenoiditis, and rhinosinusitis. Further insight into the mechanism of biofilm resistance raises a useful future direction in infectious diseases and postoperative treatment.

Abbreviations

AR: Allergic rhinitis
ASL: Airway surface liquid
BPIFA1: Bactericidal/permeability-increasing fold containing family A, member 1;
CFTR: Cystic fibrosis transmembrane conductance regulator
COPD: Chronic obstructive pulmonary disease
CRSwNP: Chronic rhinosinusitis with nasal polyps
ECM: Extracellular matrix
ENaC: Epithelial Na+ channel
ENT: Ear-nose-throat
GNB: Gram-negative bacilla
HNE: Human neutrophilic elastase
IHC: Immunohistochemistry
LBP: Lipopolysaccharide-binding protein
MMPs: Matrix metalloproteinases
NLFs: Nasal lavage fluids
PSP: Parotid secretory protein
SPLUNC1: Short palate, lung, and nasal epithelium clone 1
TLR2: Toll-like receptor 2
URTI: Upper respiratory tract infection.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Yung-An Tsou, Min-Che Tung, and Chuan-Mu Chen proposed the conception and design of the article structure. Katherine A. Alexander, Wen-Dien Chang, Hsiao-Ling Chen, and Ming-Hsui Tsai analyzed the data and prepared the figures. Yung-An Tsou drafted the manuscript. Hsiao-Ling Chen and Chuan-Mu Chen edited and revised the manuscript. Yung-An Tsou and Min-Che Tung contributed equally to this work.

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Research Article

Genomic Characterization of MDR *Escherichia coli* Harboring bla_{OXA-48} on the IncL/M-type Plasmid Isolated from Blood Stream Infection

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Escherichia coli is responsible for a wide variety of community and hospital acquired extraintestinal infections, and the emergence of ESBL resistant isolates is a major clinical concern. In this study, we characterized the genomic attributes of an OXA-48 and CTX-M-3 producing *E. coli* EC-IMP153. Whole-genome initial assembly produced 146 contigs with a combined 5,504,170 bp in size and a G+C content of 50.5%. wgSNPs-based phylogenetic comparison with 36 publically available genomes was also performed. Comprehensive genomic analysis showed that EC-IMP153 belonged to sequence type ST-405 and harbored several resistance determinants including the β-lactam resistance genes bla_{OXA-48}, $bla_{CTX-M-3}$, bla_{TEM-1B}, bla_{OXA-1}, and bla_{CMY-70}, aminoglycoside *fyuA* and *aac(3)IId*, tetracycline *tet(A)* and *tet(R)*, and fluoroquinolone *gyrA, parC,* and *mfd* resistance determinants. Plasmids with the following incompatibility groups were detected *in silico* and confirmed using PBRT: IncI1-α, IncL, IncW, Col (BS512), and IncF. To our knowledge this is the first in-depth genomic analysis of an OXA-48 producing *E. coli* ST-405 isolated from a patient in Lebanon and linked to a blood stream infection. Continuous monitoring is necessary to better understand the continued diffusion of such pathogens, especially in view of the population movements triggered by unrest in the Middle East.

1. Introduction

Escherichia coli is responsible for a wide variety of community and hospital acquired extraintestinal infections. Extraintestinal pathogenic *E. coli* (ExPEC) is widely known to cause bloodstream, urinary and respiratory tract, cerebrospinal fluid, and peritoneum infections [1, 2]. Successful treatment of infections caused by pathogenic *E. coli* could be achieved through the use of β-lactams. In recent years, there has been an evident increase in β-lactamases production, including extended spectrum β-lactamase (ESBL), plasmid-mediated AmpC β-lactamase (e.g., CMY), and carbapenemases produced by ExPEC [3]. Three most significant classes of carbapenemases are the class A (e.g., KPC), class B also known as metallo β-lactamases (e.g., NDM), and class D OXA-types (e.g., OXA-48) [3].

The OXA-48 β-lactamase was first identified in *Enterobacteriaceae* in Turkey in 2001, with OXA-48 positive *Enterobacteriaceae* belonging to ESBL producers and nonproducers [4]. Subsequently *E. coli* producing OXA-48-like variants were reported in different countries around the world [5] including Turkey, Belgium, France, and Lebanon [6]. Generally, OXA-48 producing isolates are multidrug-resistant (MDR) and are able to hydrolyze antimicrobial agents at different levels, exhibiting high hydrolyzing activity towards penicillins, low activity against carbapenems, and sparing

broad-spectrum cephalosporins, and are not susceptible to β-lactamase inhibitors [7].

Genes encoding for β-lactamases are generally located on mobile genetic elements such as transposons, plasmids, and integrons [8]. The spread of the $bla_{\text{OXA-48}}$ gene is regularly linked to the dissemination of the 62-kb IncL/M-type plasmid, with most of the OXA-48-positive *Enterobacteriaceae* harboring this specific type of plasmid [9]. Previously Matar et al. (2008) [6] reported the emergence of carbapenemase OXA-48 in Lebanon in the periods 2008-2010 with *Klebsiella pneumoniae* being the major OXA-48 producing *Enterobacteriaceae*. In 2012, *E. coli* strains isolated from Lebanon represented 73% of clinical producers of OXA-48 [10].

In this study, using genome sequencing we aimed at investigating and characterizing the genetic background and horizontally transferable MDR resistance determinants in *E. coli* EC-IMP153. Particular interest was given to the IncL/M-type plasmid carrying $bla_{\text{OXA-48}}$ and to IncFII plasmid having $bla_{\text{TEM-1B}}$, $bla_{\text{CTXM-3}}$, $bla_{\text{OXA-1}}$, *tet(A)*, and *tet(R)*. The genetic environment of the plasmids carried by EC-IMP153 revealed the coexistence of multiple resistance genes on the same plasmid. Additionally, MLST, wgSNPs, and comparative genome analysis were considered to investigate the phylogeny of the isolate.

2. Materials and Methods

2.1. Sample Collection. The isolate was recovered from the blood samples of a patient admitted to the American University of Beirut Medical Center (AUBMC) in 2010.

2.2. Antimicrobial Susceptibility Testing. Antimicrobial susceptibility profile of EC-IMP153 isolate was screened through the disk diffusion method on Muller-Hinton agar for the following antibiotics: ampicillin, gentamicin, piperacillin, piperacillin-tazobactam, amoxicillin-clavulanic acid, cefepime, ceftriaxone, cefuroxime, cefotaxime, ceftazidime, aztreonam, imipenem, meropenem, ertapenem, colistin, trimethoprim-sulfamethoxazole, tetracycline, and ticaracillin. *E. coli* ATCC 25922 was used for routine quality control. Results were interpreted according to the Clinical Laboratory Standards Institutes (CLSI) criteria [11]. Screening tests for ESBL production were performed through double-disk synergy test (cefotaxime and ceftazidime disks with and without clavulanic acid) following the CLSI criteria [11]. *K. pneumoniae* ATCC 700603 was used as positive control for ESBL production.

2.3. DNA Isolation. Extraction of bacterial DNA was performed, after growing on tryptone soy broth overnight at 37°C, using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol.

2.4. Genome Sequencing. Library preparation was done using genomic DNA (gDNA). Bioruptor® NGS was used to sonicate 1 μg of sample DNA. The resulting sheared DNA was used as input for library preparation using the Illumina TruSeq DNA library preparation kit (Illumina). The gDNA was further subjected to end-repair, A-tailing, ligation of adaptors as recommended by the manufacturer. Fragments between 500 and 1000 bp were selected using the Pippin Prep™ DNA size selection system (Sage Science). qPCR was used to quantify the resulting library in triplicate at 1:1000 and using the Kapa library quantification kit (Kapa Biosystems, Woburn, MA, USA), following the manufacturer's instructions. The resultant library size was assessed using an Agilent Bioanalyzer with the High Sensitivity DNA Kit. The library was multiplexed, clustered, and sequenced on an Illumina MiSeq with paired-end 500 cycles protocol to read a length of 250 bp.

2.5. Genome Assembly. Genome assembly was performed *de novo* using A5 with default parameters [14]. This pipeline automates the processes through several steps: read quality filtering and error correction, contig assembly, permissive draft scaffolding, misassembly detection, and conservative scaffolding.

2.6. Genome Annotation and Analysis. The assembled draft genome was annotated using RAST. The RAST server identifies protein-encoding genes, rRNA and tRNA, and predicts the different subsystems within the genome [15]. The Antibiotic Resistance Database (ARDB) [16] and ResFinder server v2.1 were used to identify resistance genes using a threshold of 90% identity (ID) [17]. The multilocus sequence type (MLST) was determined using two different MLST schemes available on CGE server, i.e., MLST1; the Achtman scheme and MLST2; the Pasteur scheme. The presence of plasmids and corresponding sequence types (STs) were determined using both *in silico* PlasmidFinder 1.3 server [18] and pMLST available on CGE. IS-finder was used to identify insertion sequences (ISs) and identify IS-families [19]. PLACNETw separated chromosomal genome from accessory plasmid genomes, based on paired-end reads assembly [20].

2.7. Plasmid DNA Extraction. For plasmid extraction the isolate was inoculated on Luria Bertani broth and incubated overnight at 37°C. The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions.

2.8. Identification of Resistance Genes. The β-lactamase gene $bla_{\text{OXA-48}}$ was traced by PCR amplification, on both crude genomic DNA and bacterial plasmid DNA, using the following set of primers: OXA-48A (5′-TTGGTGGCATCG-ATTATCGG-3′) and OXA-48B (5′-GAGCACTTCTTT-TGTGATGGC-3′) as described by Aktas et al. (2008) [12]. PCR amplicons were electrophoresed on 1.5% agarose gels. Automated sequencing was performed on ABI 3500 DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). $bla_{\text{OXA-48}}$ gene sequence was compared with online sequences using the BLASTn. Similarly, bla_{AmpC} was amplified through PCR on both crude genomic DNA and plasmid DNA using the following set of primers: AmpC-F: (5′-ATGATGAAAAAATCGTTATGC-3′) and AmpC-R: (5′-TTGCAGCTTTTCAAGAATGCGC-3′) [13] (Table 1).

The locations and genetic environment of $bla_{\text{OXA-48}}$ and $bla_{\text{CTXM-3}}$ were determined through sequence alignment

Table 1: Primers sequences of $bla_{\text{OXA-48}}$ and bla_{AmpC} and PCR conditions used for the amplification along with target size in (bp).

Primer name	Target	Sequence (5′-3′)	Size (bp)	T [°C]	Reference
OXA-48A	$bla_{\text{OXA-48}}$	TTGGTGGCATCGATTATCGG	743	60	[12]
OXA-48B		GAGCACTTCTTTTGTGATGGC			
AmpC-F	bla_{AmpC}	ATGATGAAAAAATCGTTATGC	1,143	56	[13]
AmpC-R		TTGCAGCTTTTCAAGAATGCGC			

on BioNumerics v7.6.1 beta software (Applied Maths, Sint-Martens-Latem, Belgium). The sequence of IncFII plasmid was extracted and the genetic environment of resistance genes $bla_{\text{TEM-1B}}$, $bla_{\text{CTXM-3}}$, and $bla_{\text{OXA-1}}$ was annotated using RAST and IS-finder.

Fluoroquinolone resistance-determining regions (QRDR) of *gyrA* and *parC* were compared to the amino acid sequence of that in *E. coli* K-12 (GenBank accession no. NC_000913). Protein alignment was performed on EMBOSS Needle tool available on EMBL-EBI website (http://www.ebi.ac.uk/Tools/).

2.9. Plasmid Typing. EC-IMP153 isolate genomic DNA was subjected to PCR-based replicon typing analysis (PBRT) to determine plasmid incompatibility groups as described by Carattoli et al. (2011) [21]. Eight multiplex PCRs were performed for the amplification of 28 replicons: L/M, N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB-M, W, Y, P, A/C, T, K, U, R, B/O, HI1, HI2, I1, I2, X1, X2, and HIB-M representative of major plasmid incompatibility groups and replicase genes that are typically found on resistance plasmids circulating among *Enterobacteriaceae* [21, 22].

2.10. Genome Comparative Analysis. *E.coli* EC-IMP153 was compared with the following genomes of ST-405 *E. coli* isolates: 50579417 (Accession #: NZ_LNHL00000000) OXA-48 producing *E. coli* isolated from a patient in Norway with a travel history to Thailand and used as a reference strain [23], *E.coli* LAU-EC4 (Accession #: AYOP0100000000), and *E.coli* LAU-EC5 (Accession #: AYOG0100000000) isolated from Lebanon [24] and circular visualization was constructed using CGViewer [25].

2.11. wgSNPs Phylogenetic Analysis. The genome was compared against the GenBank Nucleotide database using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the closest relative genomes available on the database (Supplementary Table 1: reference genomes accession numbers). BioNumerics v7.6.1 beta software (Applied Maths, Sint-Martens-Latem, Belgium) was used to align *E. coli* genomes against reference genome *E. coli* 50579417. SNP-calling was performed by mapping the paired-end reads of isolate EC-IMP153 and 34 assembled *E. coli* genomes obtained from NCBI to the reference genome of 50579417 *E. coli* strain. *K. pneumoniae* genome was used as an outgroup. SNPs were deduced through strict SNP filtering for each genome sequence using BioNumerics Chromosome Comparisons module. A neighbor-joining (NJ) tree was generated in BioNumerics by using mutation filtering module, filtered from wgSNP data input.

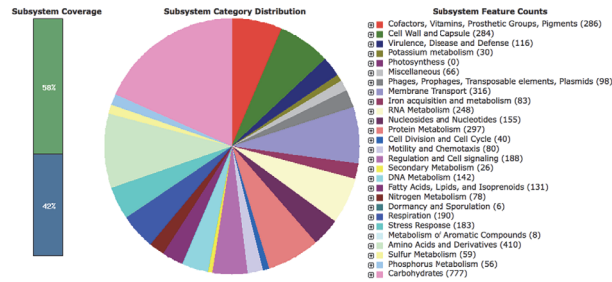

Figure 1: Subsystem categorical distribution in *E. coli* EC-IMP153.

2.12. Nucleotide Sequence Accession Number. The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number LJOJ000000000. The versions described here is LJOJ01000000.

3. Results

3.1. Antimicrobial Resistance Patterns. *E. coli* EC-IMP153 was found to be resistant to tetracycline, ticaracillin, gentamycin, ampicillin, piperacillin, piperacillin/tazobactam, amoxicillin/clavulanic acid, cefepime, ceftriaxone, cefuroxime, azetronam, ceftazidime, and cefotaxime. It additionally showed intermediate resistance to trimethoprime/sulfamethoxazole but was sensitive to ertapenem, colistin, meropenem, and imipenem.

3.2. Genome Characterization. *E. coli* EC-IMP153 genome consisted of 5,504,170 bp and a G+C content of 50.5% in 146 contigs, with 5,384 coding sequences (CDS) and a total of 116 predicted RNAs. RAST also distinguished genes encoding carbohydrate metabolism (777), amino acids and derivatives (410), cofactors, vitamins, prosthetic groups, pigments (286), cell wall and capsule (284), and virulence disease and defense (116) (Figure 1)

3.3. Isolate Typing. *In silico* (MLST1) based analysis using seven house-keeping genes (*adk, fumC, gyrB, icd, mdh, purA,* and *recA*) classified EC-IMP153 as belonging to ST-405 based on Achtman scheme and ST-44 based on the Pasteur scheme. The serotype was predicted to be O102:H6.

3.4. Mobile Genetic Elements. EC-IMP153 was positive for the plasmids with the following incompatibility groups: IncF (IncFII, IncFIA, and IncFIB), IncI1-α, and Col (BS512) *in silico* pMLST analysis using the FAB (FII:FIA:FIB) typing scheme for IncF plasmids, showing that the replicons belonged to the F31:A4:B1 type. Plasmid profiling by PBRT confirmed

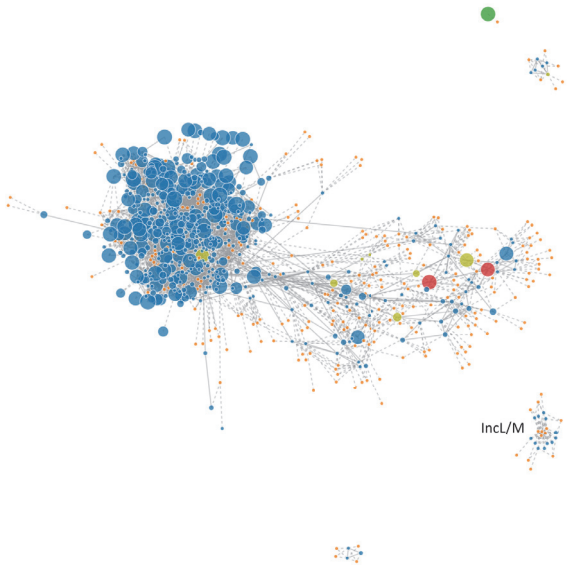

Figure 2: PLACNETw caption of plasmid IncL/M. Separate representation of chromosomal genome and IncL/M plasmid in *E. coli* EC-IMP153.

the presence of IncFIA, IncFII, IncI1-α, IncL, and IncW type plasmids. PLACNETw network (Figure 2) revealed the presence of fragmented, short read contigs corresponding to the IncL/M plasmid that specifically carried the $bla_{\text{OXA-48}}$ gene.

IS-Finder identified 158 insertion sequences (ISs) and 180 open-reading frames (ORFs) related to ISs. Important ISs families included IS*1* family (IS*1A*, IS*1B*, IS*1D*, IS*1G*, IS*1H*, IS*1R*, IS*1S*, IS*1SD*, IS*1X2*, and IS*1X4*), IS*1380* family (IS*Ecp1* and IS*Ec9*) with IS*Ecp1* detected upstream of $bla_{\text{CTX-M-3}}$ gene, IS*3* family (IS*103*, IS*1203*, IS*1397*, IS*150*, IS*2*, IS*911*, IS*Ec27*, IS*Ec52*, IS*Kpn8*, IS*Sd1*, and IS*Fl10*), and IS*5* family (IS*5*, IS*5D*, and IS*Kpn26*).

3.5. Identification of Antibiotic Resistance Genes. $bla_{\text{TEM-1B}}$, $bla_{\text{CTXM-3}}$, and $bla_{\text{OXA-1}}$, in addition to the gene encoding a tetracycline efflux protein *tet(A)* and *tet(R)*, coexisted on the same plasmid IncFII. $bla_{\text{CTX-M-3}}$ was associated with a Tn*2*/Tn*3* hybrid with an upstream IS*Ecp1*. The downstream end of Tn*2* was truncated by IS*2I*. This multiresistance region (MRR) also included *tet(A), tet(R)* genes and $bla_{\text{OXA-1}}$ (Figure 3).

$bla_{\text{OXA-48}}$ gene specific PCR assay was additionally used on a plasmid extract which confirmed that the isolate was $bla_{\text{OXA-48}}$ carrier. Sequence analysis was done and the sequence matched with the publically available NCBI sequences for the $bla_{\text{OXA-48}}$ gene. $bla_{\text{OXA-48}}$ was the only resistance determinant found on IncL plasmid associated with Tn*1999* transposon. Only one copy of IS*1R* was found upstream of the gene and *lysR* being located downstream.

Aminoglycoside (*aac(3)-IId)*, macrolide (*mphA)*, and tetracycline (*tet(A)* and *tet(R))* resistance genes were detected. CARD Resistance Gene Identifier (RGI) further revealed the presence of other resistance determinants including $bla_{\text{CMY-70}}$ (β-lactam resistance) and *mfd*, *gyrA,* and *parC* (fluroquinolone resistance). Mutations in *gyrA* and *parC* genes leading to a single amino acid substitution in *parC* (S80I) and double substitutions in *gyrA* (S83L and D87N) were also detected.

3.6. Genomic Comparison. Circular visualization and comparison of the genomic sequences were generated using CGViewer server. *E. coli* EC-IMP153 was compared with 50579417 and *E. coli* K-12 MG1665 (Figure 4) and with LAU-EC4 and LAU-EC5 (Figure 5).

3.7. SNPs Based Phylogenetic Analysis. wgSNPs-based phylogenetic analysis of EC-IMP153 with 36 reference genomes downloaded from NCBI separated the isolates into three major clades (I, II, and III). Clade II was further subdivided into four subclades. Four ST-405 isolates including EC-IMP153, two CTX-M-15, and OXA-1 producing isolates (LAU-EC4 and LAU-EC5) [26], *E. coli* 50579417 harboring CTX-M-1 and OXA-48 genes isolated from Norway [24], and *E. coli* Z1002 belonging to ST-11 all clustered together. The distribution of the isolates correlated mainly with the area of isolation and ST (Figure 6).

4. Discussion

This study, to the best of our knowledge, is the first in-depth comparative genomic analysis of ST-405 OXA-48 producing MDR *E. coli*, linked to bacteremia isolated from a patient in Lebanon. Bloodstream infections (BSIs) caused by *E. coli* have been associated with prolonged hospital stay [25]. ST-405 is classified as one of the important ExPEC lineages [27] and has been involved in the spread of genes encoding ESBLs, cephamycinases, and carbapenemases [28, 29]. Previous surveillance studies conducted across European countries and North and South America have shown that around 20 to 45% of ExPEC were resistant to the first line of antibiotics including cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole [30]. EC-IMP153 was of serotype O102:H6, previously linked to *E. coli* ST-405 strains in France [31], and to ST964 (O102:H6) strains in Norway [32].

EC-IMP153 was positive for the $bla_{\text{OXA-48}}$, an important carbapenem-resistant determinant. The OXA carbapenemases are presented by different class D OXA enzymes such as OXA-23-like, OXA-24-like, OXA-48, OXA-51-like, and OXA-58-like [33]. Poirel et al. (2011) [34] linked the distribution of OXA-48 producers, particularly in the Mediterranean region and in Western Europe, to the spread of the IncL/M-type plasmid, which carries the $bla_{\text{OXA-48}}$ gene as the only resistant determinant. This was in harmony with our results, and EC-IMP153 was positive for $bla_{\text{OXA-48}}$ and carried the IncL plasmid type as confirmed by WGS, PBRT testing, and PLACNETw. Earlier studies indicated that plasmids from different countries around the world carrying the OXA-48 gene shared related features; being self-conjugative, similar in size and not having other resistance determinants [22, 35]. Sequence analysis of IncL/M plasmid showed that the $bla_{\text{OXA-48}}$ gene is bracketed by two copies of IS*1999*, giving rise to a composite transposon Tn*1999* [4].

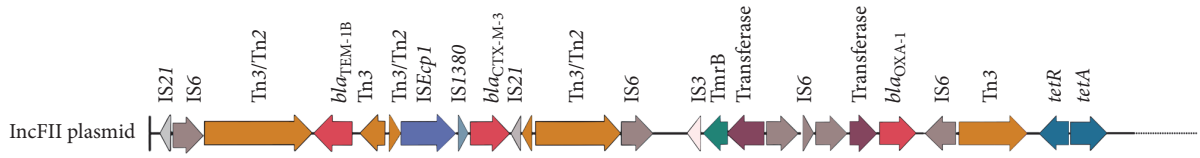

Figure 3: IncFII plasmid genomic environment. Structure of the $bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{OXA-1}}$ genes on IncFII plasmid in *E. coli* EC-IMP153.

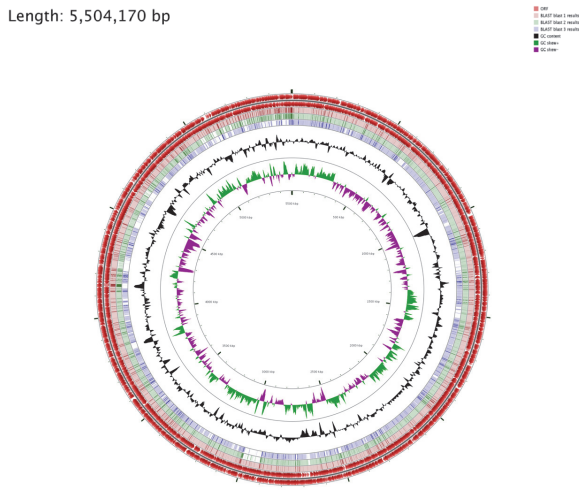

Figure 4: Circular genome representation of EC-IMP153 compared with *E. coli* 50579417 and *E. coli* K-12 MG1665. The outermost ring: EC-IMP153 open-reading frames (ORF) on both forward and reverse strands (red), *E. coli* blast 1 results for EC-IMP153 (light pink), *E. coli* blast 2 results 50579417 (green), *E. coli* blast 3 results K-12 MG1665 (purple) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green), and G+C negative skew (purple). Image created using CGview Server.

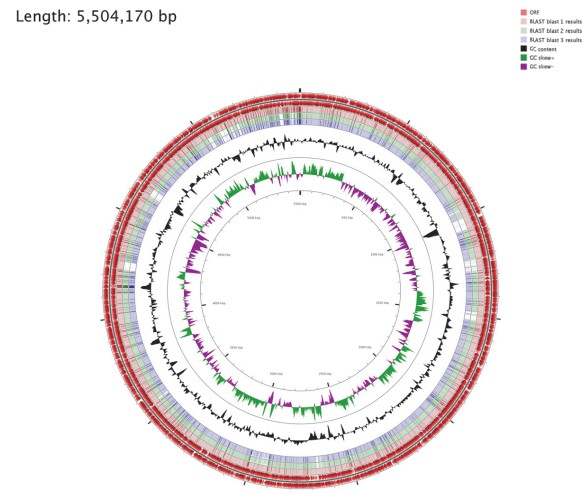

Figure 5: Circular genome representation of EC-IMP153 compared with *E. coli* LAU-EC4 and *E. coli* LAU-EC5. The outermost ring: EC-IMP153 open-reading frames (ORF) on both forward and reverse strands (red), *E. coli* blast 1 results for EC-IMP153 (light pink), *E. coli* blast 2 results LAU-EC4 (green), *E. coli* blast 3 results LAU-EC5 (purple) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green), and G+C negative skew (purple). Image created using CGview Server.

Furthermore, detailed analysis of resistance determinants revealed that EC-IMP153 was positive for $bla_{\text{CTX-M-3}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{CMY-70}}$, *tet(A)*, *tet(R)*, and *aac(3)-lld*. The CTX-M enzymes have been the most predominant among *Enterobacteriaceae* [36]. CTX-M group 1 is represented by $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-3}}$, and $bla_{\text{CTX-M-15}}$, with $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ being associated with plasmids belonging to incompatibility groups IncI1, IncFII, and IncN [37], most of which were detected in this study. $bla_{\text{CTX-M-3}}$ and $bla_{\text{OXA-1}}$ were found coexisting on IncFII plasmid in EC-IMP153. IncFII originated from *Kluyvera ascorbata* through IS*Ecp1* that functions as a promoter for the expression of adjacent genes [38, 39]. Although IS*Ecp1* is bracketed by two 14-bp inverted repeats (IRL and IRR), it mobilizes adjacent regions through IRL and alternative similar sequences forming 5-bp direct repeats (DR) [38]. Studying the genetic content of IncF plasmid in EC-IM1P53 revealed that the transposition unit carrying $bla_{\text{CTX-M-3}}$ is inserted in the Tn*2*/Tn*3* hybrid, which also carries $bla_{\text{TEM-1B}}$ and was in harmony with previous reports [40, 41]. This model was also detected in $bla_{\text{CTX-M-15}}$ positive *E. coli* isolates; $bla_{\text{CTX-M-3}}$ is the progenitor of $bla_{\text{CTX-M-15}}$ differing by a single amino acid substitution Asp-240 to Gly [38] and based on Ambler numbering [42]. Additionally, as previously reported, the downstream end of Tn*2* was truncated by IS*21* [43]. The association of Tn*2* with ISs may also be important for the spread of $bla_{\text{CTX-M}}$, especially as complete or partial copies of Tn*2* are frequently found in MRRs and on plasmids [40].

The $bla_{\text{CMY-2}}$ gene was first detected in 1990 [44]. Plasmid-mediated AmpC genes express resistance to broad-spectrum cephalosporins [45]. Latest reports highlighted new variants of CMY, CMY-70, which was detected in this study and was registered in Lahey database (http://www.lahey.org/Studies/other.asp#table1) [46]. On the other hand, sequence comparison at the amino acid level for *gyrA* and *parC* genes detected in EC-IMP153 revealed the presence of the single substitution S80I in ParC [47] and (S83L and D87N) in gyrA, conferring resistance to fluoroquinolones [48].

Replicon sequence typing of the detected IncF plasmid in EC-IMP153 revealed that it belonged to pMLST F31:A4:B1. This ST was found to be a common pMLST in *E. coli* ST617, ST131, and ST44. *E. coli* EC-IMP153 had other different virulence determinants, including genes linked to different STs

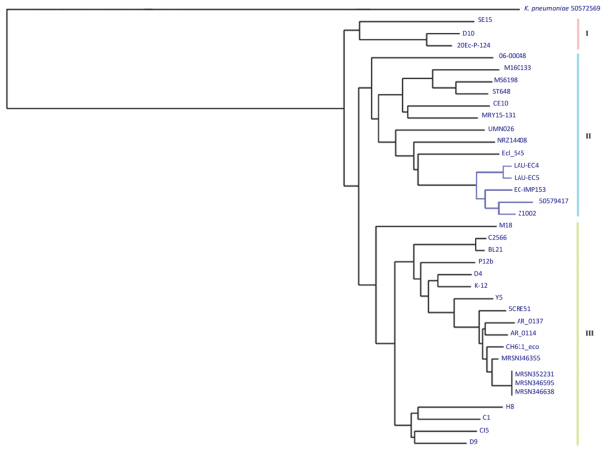

Figure 6: SNPs based phylogenetic tree. EC-IMP153 (LJOJ01000000), 50579417 (NZ_LNHL00000000), OXA-48 producing *E. coli* isolated from Norway, LAU-EC4 (AYOP0100000000), LAU-EC5 (AYOG0100000000) isolated from Lebanon, and Z1002 (AE005174) clustering in one clade.

(ST69, ST393, and ST405), associated with biofilm formation (*fimH, papC and papG, fyuA or kpsMT II*), which could favor persistence [49]. In addition, Col (BS512) plasmid detected in EC-IMP153 is known as the *Shigella boydii* plasmid pBS512 with replicon type FIIA and being categorized as invasive plasmid with relation to type three secretion system (T3SS) [50].

SNPs were considered to investigate the phylogeny and detect possible clonal links between EC-IMP153 and 36 other isolates. The wgSNPs-based phylogenetic analysis distributed the isolates depending on both the isolation site and STs. Similar to other reports, isolates clustering together on the same clade were of the same phylogenetic group [51] but with different resistance profiles [52]. Visual representation of circular genomes showed the presence of unique genes found in EC-IMP153 but not in 50579417, LAU-EC4, LAU-EC5, and K-12 MG1665.

5. Conclusion

In this study, we identified the resistome of IncF plasmids and the genetic environments surrounding $bla_{\text{OXA-48}}$ producing *E. coli* isolated from Lebanon. The isolate was a MDR that coproduced ESBLs and other plasmid-mediated resistance determinants. Our findings suggest that many IncF as well other plasmids have incorporated into the OXA-48 positive isolate. Because of limited therapeutic options and higher mortality caused by these carbapenem-resistant *Enterobacteriaceae*, continuous surveillance and molecular characterization of OXA-48 producers are needed to shed light upon all of the transmission pathways.

Data Availability

Whole Genome Shotgun project of *Escherichia coli* isolate EC-IMP153 has been deposited at DDBJ/EMBL/GenBank under the accession number LJOJ00000000. The version described in this paper is version LJOJ00000000.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Reference genomes accession numbers retrieved from GenBank Nucleotide database. *(Supplementary Materials)*

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Review Article

Resistant Gram-Negative Bacteria and Diagnostic Point-of-Care Options for the Field Setting during Military Operations

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The spread of multidrug-resistant bacteria in resource-poor settings affects the military medical service in case of deployments of soldiers to war and crisis zones. Patients with war injuries are prone to colonization or infection with multidrug-resistant bacteria. Resistant Gram-negative bacteria play a dominant role in military wound infections. Problematic hygiene conditions on deployment facilitate exposition of soldiers with subsequent colonization. Although colonizing strains are frequently cleared from their hosts after returning from deployment, transmission to close contacts of the soldiers in the home country cannot be excluded and therapeutic options are reduced if colonization progresses to invasive infection. Since sophisticated culture-based diagnostic approaches are typically not available in the field setting on deployment, molecular rapid diagnostic test systems are an option for transmission control if the locally prevalent molecular resistance mechanisms are known. Efforts for global resistance surveillance can contribute to better understanding of resistance distribution and spread at deployment sites. This review summarizes experience of the military medical services with multidrug resistance on deployment and with the influx of resistant strains to the home country and discusses potential use of available molecular rapid test systems as an option for the field setting.

1. Introduction

Traveling to resource-limited areas is associated with reversible but substantial changes of the human gut microbiome [1]. If this phenomenon is potentiated by the influences of traveler's diarrhea and consumption of antibiotic drugs, the enteric selection risk for multidrug-resistant bacteria increases tremendously [2]. Accordingly, multidrug resistant pathogens and especially Gram-negative bacteria are frequent colonizers of the gut of travelers returning from the tropics [2–5]. Treatment in sub-Saharan African healthcare facilities was shown to increase the colonization risk to more than 90% [6]. However, specific exposure risks beyond the healthcare setting are widely unknown so far [7].

Concomitant to the reversion of the gut microbiome to the pretravel status [1], enteric colonization with multidrug-resistant bacteria is usually reduced in the course of several months, with asymptotic dynamics of pathogen clearance [8]. However, 11.3% returned travelers remained colonized even 12 months after their return in a recent assessment [9] and the probability of ESBL transmission to household members was 12.0%.

As screening for multidrug-resistant colonizers is still poorly standardized [10], it remains questionable what a negative screening result after previous colonization with resistant bacteria really means: either definite vanishing of the resistant pathogen or just a shift to a concentration below the detection limit. As preanalytic conditions like swabbing techniques [11] and the use of enrichment broths [12] were shown to relevantly affect the reliability of enteric screening approaches, it is highly likely that a proportion of individuals with apparently cleared colonization remains colonized with multidrug-resistant bacteria on a level below the diagnostic threshold. For these persons, there is a risk of selective

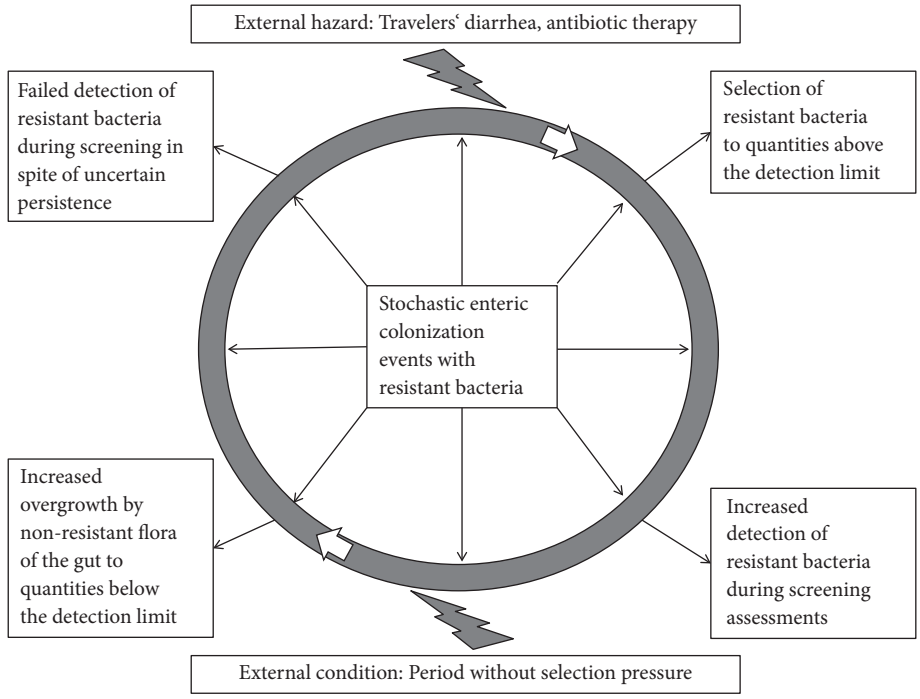

Figure 1: Examples of the influence of external conditions on the detection of enteric colonization with resistant bacteria by culture-based screening approaches [2, 8].

enrichment of the multidrug-resistant bacteria under antibiotic pressure (Figure 1).

Similar to civilian travelers, soldiers deployed to resource-limited settings are exposed to a relevant acquisition risk of colonization or infections with multidrug-resistant bacteria [13]. In spite of a high likelihood of exposure, scientific literature on multidrug-resistant bacteria in deployed or returned soldiers is only scarcely available compared with our knowledge from civilian settings. Most of the assessments are published by US American or British authors. Recognizing the urgency of the issue, the US military early established a multidrug-resistance surveillance network [14]. The "Antimicrobial Resistance Monitoring and Research Program" was designed to allow for large-scale antimicrobial resistance surveillance [15].

But even prior to systematic surveillance approaches, military medicine had to deal with resistance problems from the beginning of modern antimicrobial therapy as occasionally documented in scientific literature. While early experience of the medical armed forces with antibiotic resistance in the last century was primarily focused on *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, and Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) [16–21], multidrug resistance in Gram-negative *Enterobacteriaceae* and Gram-negative rod-shaped bacteria is presently realized as an increasing menace.

In this narrative review, published experience of military medical services with Gram-negative multidrug-resistant bacteria is summarized and mobile, field compatible diagnostic systems are introduced.

2. Mode of Literature Review

Literature search was performed using the data bases NCBI PubMed https://www.ncbi.nlm.nih.gov/pubmed/, last accessed 4th May 2018) and Google Scholar (https://scholar.google.de, last accessed 4th May 2018) using the key words "military medicine", "multidrug-resistance", "molecular rapid testing", "Gram-negative", "rapid diagnostic test", "ESBL", "carbapenemase", "Xpert", "BioFire", and "Amplex" in various combinations. Assessment of suitability for this narrative review was based upon the subjective impression of the authors.

3. Experience from Military Medical Facilities in Theater

Military conflicts are associated with an increased risk of distribution and spreading of multidrug-resistant bacteria. Influx of newly detectable strains of multidrug-resistant bacteria into crisis and war zones has been described. In the course of the Euromaidan riots in the Ukraine, a bla_{NDM-1}-producing *Klebsiella pneumoniae* strain of the clonal complex ST11 was isolated for the very first time from a wound of an injured individual in this country [22].

However, traditional culture-based microbiology is difficult to maintain in deployment settings and thus rarely available in theater in crisis and war zones, especially in resource-limited settings. Therefore, data on microbial resistance of isolates from the deployment site are particularly scarce. Again, the resource-rich US American armed forces are

an exemption. In order to identify extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* and other resistant bacteria, identification systems are provided in deployed laboratories by US forces [23].

The associated effort has led to providing a considerable set of data by the US military. In a study period between 2005 and 2007, 2,242 US casualties from Operation Iraqi Freedom and Operation Enduring Freedom were screened for multidrug-resistant bacteria. The three most frequently isolated pathogens comprised Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) but also Gram-negative *Klebsiella pneumoniae* and *Acinetobacter* spp., each leading to nosocomial infection rates between 2% and 4% [24]. Interestingly, the overall detection rates for Gram-negative pathogens were much higher in locals in a role 3 medical facility (field hospital) in Iraq than in US forces irrespective of the sample material, while for Gram-positive bacteria, a prevalence inverse to the previously described was recorded [25].

High rates of multidrug resistance, in particular among Gram-negative organisms, were reported from war injuries during the recent conflicts in Iraq and Afghanistan [26]. In a recent point prevalence assessment from the European Union Training Mission in Mali (EUTM MLI), an enteric colonization rate of 27.1% (13 / 48) ESBL-positive *Enterobacteriaceae* could be demonstrated for European soldiers with traveler's diarrhea [27, 28].

Acinetobacter spp. are feared due to their complex resistance patterns, resulting in complex therapeutic regimens in the case of systemic infections, e.g., in primarily sterile compartments [29]. As early as during the 2003-2005 military operations of the US military in Iraq, predominantly osteomyelitis but also burn and deep wound infections with *Acinetobacter* spp. required complex antibiotic treatments for 6 weeks [30]. Carbapenem-resistance, which was frequently caused by $bla_{Oxa\text{-}23}$ expression in *Acinetobacter* spp., was shown to be associated with prolonged stays in hospital and on intensive care units (ICU) of military treatment facilities [31]. In resource-limited deployment settings with restricted numbers of ICU beds, this can be problematic and even more so in the case of outbreak situations due to nosocomial transmission.

Factors affecting the risk of postsurgical wound infections of soldiers on deployment including those due to multidrug-resistant pathogens comprise a variety of elements including the presence of devitalized tissue, foreign bodies, blood clots, seroma, and contamination of wounds with bacteria from the casualties' skin, the environment, and the hospital itself [32]. Of note, the very early wound stages directly after infection are predominantly associated with susceptible strains as shown in a study with casualties in Iraq with only two out of 49 cases with MRSA detection and no proof of resistant Gram-negative flora [33]. This suggests transmission of multidrug-resistant strains in later stages of wound management in the military field medical care setting.

Due to the resistance-associated difficulties in antibiotic treatment, wound infections with carbapenem-resistant bacteria are particularly feared. To quantify the dimension of this problem, the US military medical service conducted an assessment of carbapenem-resistant *Enterobacteriaceae* prevalence in wounds of military personnel within a 6-years-period from 2009 to 2015. Fortunately, as few as 0.4% (16 out of 4090 strains) collected strains were carbapenem-resistant. The isolates most frequently comprised *Enterobacter aerogenes* (44%), *Klebsiella pneumoniae* (37%), and *Escherichia coli* (19%). In five strains from two patients, the responsible carbapenemase genes (4x $bla_{KPC\text{-}3}$, 1x $bla_{NDM\text{-}1}$) were successfully identified [34].

Caring for patients with multidrug-resistant pathogens is a risk of getting colonized and of further spreading these pathogens. An intensive patient contact of less than 30 minutes including endotracheal suctioning from a wounded US soldier without use of a surgical mask was shown to be sufficient to allow for transmission of multidrug-resistant *A. baumannii* to a healthcare worker as confirmed by molecular typing [35]. Such examples are suitable to underline the necessity for patient care in protective equipment if multidrug-resistant bacteria have to be expected, making the management of patients more complex and expensive.

4. Experience from Military Medical Healthcare Facilities in the Home Country

Acquisition of colonization with multidrug-resistant bacteria by soldiers on deployment consequently leads to an influx of resistant strains into military hospitals in the home countries where soldiers are treated in case of repatriation due to severe diseases or injuries. Next to this, healthy returnees from deployments are at risk of spreading colonizing multidrug-resistant bacteria among their families as previously shown for civilian travelers [9]. Fortunately, the earlier described phenomenon of spontaneous loss of ESBL-positive *Enterobacteriaceae* from the gut of civilian travelers after returning from the tropics [8] could be confirmed for soldiers. In a recent assessment of 828 German soldiers returning from deployments between 2007 and 2015, the average colonization rate with *Enterobacteriaceae* with resistance against third-generation cephalosporins was only 4.7% (39 / 828) 3 months after returning [36] while during tropical deployment colonization rates of up to 27% were observed in European soldiers with diarrhea [27, 28]. All isolates were *Escherichia coli* and ESBL was the most frequently detected resistance mechanism (37 ESBL, 1 ESBL + ampC, and 1 uncertain mechanism) [36]. In comparison, prevalence of *E. coli* with resistance against third-generation cephalosporins in the German population ranged between 5% to <10% in 2007 and 10% to <25% in 2015 in the assessment period as suggested by the European Centre for Disease Prevention and Control (ECDC, https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc, last accessed 4th May 2018). The distribution of ESBL-positive colonizing bacteria in the returned German soldiers differed by deployment site. In returnees from an UN-observer mission, where soldiers purchased their food on local markets and were exposed to the local hygiene conditions in this way, colonization rates up to 20% were observed [36]. In contrast, no *Enterobacteriaceae* with resistance against third-generation cephalosporins were isolated from samples from

soldiers returning from nontropical deployment sites like Kosovo in the same assessment [36]. On average, however, colonization rates were similarly low and on the level of the home country 3 months after returning.

The asymptotic decolonization curve as suggested by Ruppé et al. [8], however, does not exclude transmission risks in the immediate term after returning from abroad. This topic was addressed by several studies which focused on prevalence in local military medical facilities in both the home countries or partner countries and abroad.

From a US military hospital in the home country, a longitudinal observation on the development of the spread of ESBL-positive *E. coli* and *K. pneumoniae* over a 7-years-period from 2003 to 2011 was described. From 2005 to 2010, ESBL incidence was moderately increased from low baseline levels for *E. coli* from 0.13% to 1.0% and for *K. pneumoniae* from 1.0% to 2.55% with predominance in females with urinary tract infections. Nearly half of the infections with ESBL-positive strains were not associated with comorbidities [37].

Between 2007 and 2011, the distribution of clonal lineages of ESBL-producing *E. coli* in US military service members in the home country was shown to resemble the distribution in other North American populations with dominance of ST10 (24%), ST131 (16%), and ST648 (8%). Clonal identity was also shown to be suitable to predict the most likely resistance pattern [38].

As shown in a report from 2015, the incidence of the particularly problematic *Enterobacteriaceae* with carbapenem-resistance in US military medical facilities was fortunately still as low as 1 per 100,000 patient years, although proportions differed among years, geographical regions, and bacterial species. Consumption of fluoroquinolones was shown to trigger the detection of carbapenem-resistant *E. coli* while no other significant associations could be demonstrated [39].

During a three-years period from 2009 to 2012, active screening-based surveillance for colonization with multidrug-resistant bacteria was conducted by US military at Landstuhl Regional Medical Center (Landstuhl RMC), Germany, and at three other regional treatment facilities. Colonization rate in Landstuhl was 6.6% and thus comparable with the local population in Germany. In comparison, it was nearly double as high (12.4%) at the three other facilities. Multidrug-resistant *E. coli* was most frequently identified, followed by *A. calcoaceticus-baumannii* complex and *K. pneumoniae*, without relevant quantitative changes over the assessment period [40].

Presently, the US screening efforts for multidrug-resistant bacteria were intensified and amended by whole genome next-generation sequencing. With multiple global sampling sites, the resulting strain collection comprises several 10,000 isolates [41].

The Trauma Infectious Disease Outcome Study [42] on deployment-related trauma in the period from 2009 to 2014 classified Gram-negative rod-shaped bacteria to be multidrug-resistant if resistance to ≥3 antibiotic classes or, alternatively, expression of extended spectrum β-lactamases (ESBL) or carbapenemases were observed. Based on this definition, a total of 26% (n= 245) military trauma patients with infections were affected by multidrug-resistant bacteria. The most commonly isolated species comprised *E. coli* (48.3%, n=262), *Acinetobacter* spp. (38.6%, n=210), and *K. pneumoniae* (8.4%, n=46). Risk factors for colonization with multidrug-resistant Gram-negative bacteria were severe trauma, comprising blast injuries and traumatic amputations. These data confirm that the association of war-related trauma and colonization or infection by multidrug-resistant bacteria is considerable.

The influx of multidrug-resistant bacteria with wounded soldiers also leads to secondary phenomena. As the risk of resistance has to be considered in case of calculated antibiotic therapy of severely ill patients, broad-spectrum antibiotic drugs have to be used [43] with the risk of additional selection of resistant pathogens.

Military conflicts also lead to an influx of multidrug-resistant bacteria to civilian hospitals of countries where care for transferred war-injured patients or refugees from crisis zones is provided. E.g., the prevalence of multidrug-resistant bacteria in war casualties from Libya transferred to a civilian tertiary hospital in Germany was assessed. In total, multidrug-resistant pathogens were detected in 60% of the patients. Carbapenem-resistant Gram-negative bacteria predominated (37%), but also Gram-positive MRSA (16%) was observed. Carbapenem-resistance was detected in *K. pneumoniae*, *A. baumannii*, *E. coli*, *Enterobacter cloacae*, and *Serratia marcescens* with bla_{NDM} (n = 17), $bla_{OXA\text{-}48}$ (n = 15), and $bla_{OXA\text{-}23}$ (n = 9) being the most frequently detected carbapenemase genes [44]. Multiple other studies with war-injured patients from the recent conflicts in Libya and Syria [45–53] showed comparable results.

In German military hospitals, intense colonization of patients with war injuries from crisis and war zones in Libya, Syria, and the Ukraine has been observed [54, 55]. Molecular analyses by rep-PCR and NGS suggested that nosocomial transmission within the military hospitals could be reduced to very low rates by the enforcement of strict hygiene precautions. Clonal identity of nonnosocomial strains, however, suggested transmission events either in medical facilities in the countries of origin or during evacuation flights under narrow spatial conditions. In spite of considerable efforts to achieve local decolonization by disinfectant washing, the results were only moderately better than the spontaneous decolonization rates. In addition, the effects depended on the compliance of the patients [56].

5. Diagnostic Point-of-Care Solutions for Potential Use on Deployment

Diagnosis of bacterial resistance in military deployment settings is challenging. Biochemical approaches like Microscan (Siemens AG, Munich, Germany) panels have been used to identify ESBL-positive *Enterobacteriaceae* in deployed laboratories of the US military [23]. However, culture-based resistance testing is laborious and difficult to provide on small missions in resource-limited settings.

In recent years, various molecular rapid diagnostic test (RDT) systems have been introduced for the identification of a number of quantitatively important resistant genes. The

most frequently described molecular RDT systems for such purposes comprise PCR-based tools like the Xpert system (Cepheid, Sunnyvale, CA, USA) and the FilmArray system (BioFire Diagnostics, Inc., Salt Lake City, UT, USA), as well as loop-mediated-amplification-(LAMP)-based tools like the eazyplex system (AmplexDiagnostics GmbH, Gars Bahnhof, Germany) which will be described in more detail below.

While such molecular RDT tools are usually rapid and easy-to-apply, so the demand of skilled and highly qualified laboratory personnel can be reduced, they still require electrical power, maintenance, and appropriate transport logistics in the field. Another disadvantage is the fact that only the targeted resistance genes are detected. Therefore, the interpretability of their results largely depends on precise knowledge of the local resistance patterns and the underlying molecular mechanisms. In the case of multidrug-resistant Gram-negative pathogens, numerous resistance mechanisms may play a role while molecular RDT systems only detect the more frequent resistance genes. Accordingly, they are suitable for tracking a defined outbreak strain with a targeted resistance gene. However, a reliable exclusion of phenotypic resistance is not feasible in this way.

In detail, Public Health England recently compared three molecular systems for the detection of carbapenemases, i.e., the Check-Direct CPE kit (Check-Points BV, Wageningen, The Netherlands), the molecular RDT systems eazyplex SuperBug complete A kit (AmplexDiagnostics GmbH), and the Xpert Carba-R kit (Cepheid). All assays including the two RDT correctly identified all assessed strains with bla_{KPC}, bla_{VIM}, bla_{NDM}, and classic $bla_{\text{OXA-48}}$ carbapenemase genes while the coverage of other carbapenemase genes varied. The authors concluded that, among other factors, the preferred choice of gene coverage will be relevant for purchase decisions [57]. Several Xpert (Cepheid) systems were evaluated in various studies. While the Xpert MDRO (Cepheid) assay targets the carbapenemase genes bla_{KPC}, bla_{NDM}, and bla_{VIM} [58], the Xpert Carba-R assay (Cepheid) detects the carbapenemase genes $bla_{IMP\text{-}1}$, bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48}$, and bla_{VIM} [59]. In 2015, however, French investigators had shown weakness of the Xpert Carba-R approach regarding the identification of $bla_{OXA\text{-}48}$-like carbapenemase genes [60]. Consequently, the Xpert Carba-R v2 (Cepheid) was designed to allow the additional detection of $bla_{OXA\text{-}181}$ and $bla_{OXA\text{-}232}$ in addition to the spectrum of the Xpert Carba-R system [61].

However, a point of concern is the fact that the most systems are evaluated either with colonies which require prior culture-based growth or with mere screening materials like swabs from hygiene screenings. Accordingly, they are of uncertain reliability if a diagnosis directly from clinical sample material is desired and prior culture-based growth shall be avoided.

5.1. Evaluation of Molecular RDT Systems with Agar Cultures and Hygiene Swabs. While hygiene swabs can be used for surveillance purposes, the results of testing of such swabs showing mere colonization are not useful for the management of an individual patient. Nevertheless, such studies provide a first overview on performance characteristics of molecular RDT systems and are thus summarized in the following.

As an example of such evaluations from agar cultures, the eazyplex system (AmplexDiagnostics GmbH) correctly identified bla_{OXA} and bla_{MBL} carbapenemase genes in 82 non-related *Acinetobacter* spp. within less than 30 minutes per reaction [62]. Again, the importance of precise knowledge on prevalent local resistance mechanisms by active surveillance in the area of deployment has to be stressed for the interpretation of respective results.

Other studies were focused on hygiene swabs or stool samples, which provide epidemiological surveillance information but are not of use for clinical diagnosis. In 2013, an evaluation of the Xpert MDRO (Cepheid) assay targeting the carbapenemase genes bla_{KPC}, bla_{NDM}, and bla_{VIM} was published in comparison with culture with and without broth enrichment for rectal, perirectal, and stool samples. Sensitivity, specificity, and positive and negative predictive value were 100%, 99.0%, 93.0%, and 100% for bla_{KPC}, respectively, and 100%, 99.4%, 81.8%, and 100% for bla_{VIM}, respectively. No such statement could be made for bla_{NDM} due to lacking samples. In a serial dilution of stool samples spiked with a bla_{NDM}-positive *K. pneumoniae* strain, 100% positivity at dilutions from 300 to 1,800 colony forming units (cfu) / ml and 93.3% at 150 cfu / ml were observed [58].

The Xpert Carba-R assay (Cepheid) targeting carbapenemase genes $bla_{IMP\text{-}1}$, bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48}$, and bla_{VIM} showed a positive and negative agreement with culture and DNA sequencing as well as a positive and negative predictive value of 60%-100%, 98.9%-99.9%, 95%-100%, and 100%, respectively, when directly applied on rectal swabs [59]. Other authors suggested good performance of the system as well [63]. In a small Korean assessment, the Xpert Carba-R assay was more sensitive for the detection of carbapenemase-positive enteric colonization than culture [64].

In a small study with screening swabs from assumed high risk patients for carbapenemase-positive bacteria, 100% sensitivity, 99.13% specificity, 85.71% positive predictive value, and 100% negative predictive value were suggested for the Xpert Carba-R v2 system in comparison with selective culture [65].

5.2. Evaluation of Molecular RDT Systems with Clinical Sample Materials. Little data is available for the application of the Xpert systems with clinical sample materials. A recent Italian study stressed the importance of local epidemiology for the reliability of the Xpert Carba-R assay in a study with rectal/stoma swabs but also with swabs with abdominal drainage fluid from patients with abdominal sepsis, a material which is of potential interest for the management of severly ill patients. If only carbapenem-resistant bacteria carrying the targeted resistance genes were considered, sensitivity, specificity, and positive and negative predictive value of the Xpert Carba-R system were 100% (95% CI 69.1-100), 94.2% (95% CI 80.8-99.3), 83.3% (95% CI 59.6-97.9), and 100% (95% CI 89.4-100), respectively. If all carbapenem-resistant bacteria were considered, however, these values dropped to 50% (95% CI 24.6-75.3), 93.1% (95% CI 77.2-99.1), 80% (95% CI 44.4-97.5), and 77.1% (95% CI 56.9-89.6), respectively [66]. In another study using the Xpert Carba-R assay at least with spiked bronchial fluids, LOD was calculated to be $< 10^4$ cfu/ml [67],

providing some hints on analytical sensitivity with this important kind of clinical sample material.

Another device for potential use as an RDT in the field is the FilmArray system (BioFire Diagnostics, Inc., Salt Lake City, UT, USA). The FilmArray blood culture identification panel which was designed for rapid identification from positive blood culture materials also comprises three resistance genes (*mecA*, *vanA/B*, and bla_{KPC}), including one (bla_{KPC}) which occurs in Gram-negative pathogens [68–71]. In an eight-center trial with 2,207 positive aerobic blood culture samples in the USA, sensitivity and specificity were 100% for *vanA/B* and bla_{KPC} gene detection each and 98.4% and 98.3% for *mecA* gene detection, respectively [71]. In a South African study, consistency with the reference methods was even 100% for all tested resistance genes [70]. Although blood culture bottles are closed systems which are easy to handle, the assessment with the FilmArray blood culture identification panel nevertheless requires a cultural incubation step and is thus poorly suited as a real point-of-care approach.

Again, it has to be stressed that such molecular RDT approaches can detect the targeted resistance genes only, so results have to be interpreted with care regarding phenotypic resistance since other mechanisms cannot be excluded. Further, it is a major limitation that evaluation data of the introduced systems with clinically important primary sample materials are widely lacking, not allowing definite conclusions on the use of such systems as molecular RDT systems directly from clinical sample materials. So it remains widely unclear whether these assays show reliable results also directly from more complex sample matrices like blood, urine, or sputum or whether they will still require an initial culture-step before testing. If an initial culture-step is required, then the RDT platforms become less useful in the field environment. Suitable studies should be conducted either with spiked samples or with real clinical materials apart from just hygiene swabs with such systems to decide on their suitability for potential future use as stand-alone point-of-care solutions without the necessity of prior steps of culture-based growth.

6. Conclusions

Colonization and infection risks with multidrug-resistant bacteria are relevant issues for soldiers on deployment in high prevalence settings. This is particularly true for war injuries as shown for soldiers [42] and war-injured patients from different countries [54, 55].

Considering the fact that colonization frequently precedes infection, the observation by Yun et al. [25] that local forces in Iraq showed high colonization rates of skin and mucous membranes with Gram-negative bacteria is of particular importance. Similar data on Gram-negative colonization of skin and mucous membranes with Gram-negative *Enterobacteriaceae* were recently described for patients, students, and healthcare workers in the highlands of Madagascar [72]. The reasons are unclear, although high temperatures and humidity which are frequent in subtropical and tropical settings were described to facilitate Gram-negative bacterial growth on human skin [73].

Other sources of exposition include fecal contamination of food. As recently shown for the hotel canteen of the headquarters of the EUTM MLI mission in Western Africa, food contamination with ESBL-positive *Enterobacteriaceae* on deployment can occur when local hygiene conditions are poor [74].

If colonization with multidrug-resistant bacteria leads to infections on deployment, antimicrobial therapeutic options are scarce and prolonged cycles of combined antibiotic therapy become necessary [30]. Being aware of the fact that sophisticated resistance diagnostics are hardly achievable in remote conflict settings, nonspecific therapeutic approaches like silver-nylon dressings which are not prone to clinically relevant resistance selection are discussed [75]. Another option repeatedly discussed in the military medical service [76] is phage therapy as a potential alternative or at least an add-on to traditional antibiotic treatment.

The hygiene management of injured patients is also complicated by colonization or infection with multidrug-resistant bacteria, although nosocomial transmission in military medical facilities can be widely prevented if strict hygiene precautions are enforced [54, 55]. The effects of local skin or mucous membrane disinfection on decolonization of such sites are moderate in comparison to spontaneous decolonization rates and depend on the compliance of the patients [56].

If underlying resistance mechanisms are known, e.g., in the case of a local outbreak, molecular RDT systems might support the outbreak management in the field. However, such RDT systems target a restricted spectrum of resistance genes. Accordingly, they show poor sensitivity in case of nonspecific screening for phenotypic resistance as recently demonstrated [66].

If sophisticated diagnostic approaches in the field are not available in case of small deployments, knowledge of local prevalence and distribution of bacterial resistance is helpful for calculated antibiotic therapy in case of severe infections. To facilitate the efforts towards a global surveillance of multidrug-resistant bacteria, the US military service provides next-generation sequencing (NGS) capacities to provide a public database of collected strains from all over the world [41, 77]. As previously shown [78], this technology is suitable not only to show nosocomial transmission of strains but also on-site transmission of resistance genes between different bacterial species in wounds. Also, novel resistance-associated plasmids from remote war zones can be characterized [79]. In a similar way, the whole genome assessment also characterizes the distribution and spread of resistance genes and, thus, allows for association studies.

Multinational cooperation in the field of global resistance surveillance seems desirable to provide information on prevalence and spread of antimicrobial resistance worldwide, both for civilian and for military medical purposes.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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