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Biofilm formation by ESBL-producing strains of *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Objectives: Biofilm production in extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae provides a favourable environment for the exchange of antibiotic-resistance genes and could facilitate widespread dissemination. We aimed to assess biofilm development in ESBL-producing *E. coli* and *K. pneumoniae* isolates and determine how development relates to microbiological characteristics and clinical outcomes.

Methods: 147 ESBL-producing *E. coli* and 82 *K. pneumoniae* were genetically characterized. Biofilm formation was measured at 1.5, 4, 6, and 24 h during culture in blood heart infusion using a microbead immobilization assay (BioFilm Ring test®). Results were given as biofilm formation index (BFI) with lower values indicating increased presence of biofilm (range = 0–21).

Results: In total, 57.1% of strains were strong producers of biofilm (BFI < 2), whereas 13.4% lacked biofilm production (BFI > 18). Standard biofilm production (BFI < 7) was common in *E. coli* isolates (61.9%). For *E. coli*, biofilm production was less frequently observed in ST131 clones ($p = 0.03$) but more frequently in strains harbouring toxin ($p = 0.008$) or adhesin ($p = 0.008$) virulence factor genes. Despite almost all *K. pneumoniae* having standard biofilm production (90.2%), there was a 2.4-times higher odds of observing biofilm in ST29/147/323 versus other ST-types ($p = 0.13$). Patients with standard biofilm producing isolates were not at increased risk of transfer to intensive-care (odds-ratio = 2.80, 95%CI = 0.59–13.21) or death within 12-months (odds-ratio = 1.61, 95%CI = 0.75–3.43).

Conclusion: In these ESBL-producing strains, biofilm production is linked to certain virulence factors in *E. coli* and is common in *K. pneumoniae*. Further exploration of whether biofilm production increases dissemination and risk of severe clinical outcomes is needed in larger collections of isolates.

1. Introduction

Bacteria are planktonic or exist as aggregates of cells, the latter of which is referred to as biofilm. Biofilms are heterogeneous structures consisting of bacterial populations in an extra-cellular matrix, are present in the environment, and have the capacity to colonize diverse surfaces (Donlan and Costerton, 2002). Bacteria with biofilms have certain advantages when compared to planktonic bacteria: increased interspecific metabolic cooperation (Shapiro, 1998), quorum-sensing (De Araujo et al., 2010), increased tolerance to host immune responses, requiring higher concentrations of antibiotic agents (Ceri et al., 1999), and increased capacity for bacterial conjugation (Björklöf et al., 2000; Hennequin et al., 2012).

In parallel, the prevalence of multi-resistant Enterobacteriaceae, namely strains able to produce extended spectrum β-lactamase (ESBL), have been drastically increasing over the past decade and thus have become a worldwide public health concern (Boucher et al., 2009). Their spread depends on bacterial conjugation whereby plasmids carrying ESBL genes are transferred. The proximity of bacteria in biofilms creates a favourable environment for the exchange of genetic material, especially by conjugative transfer (Björklöf et al., 2000). Coupled with the heightened survival of bacteria in biofilm, its formation might be an important factor explaining widespread distribution of multi-resistant plasmids, such as CTX-M.

The two most notable Enterobacteriaceae with increasing prevalence of antibiotic resistance are *Escherichia coli* and *Klebsiella pneumoniae*. *E. coli* is responsible for 130–175 million urinary tract infections worldwide and 127,500 cases of sepsis in the United States each

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year, resulting in high rates of morbidity and mortality and extensive burden in health care costs (Russo and Johnson, 2003). *K. pneumoniae* is the third most prevalent cause of bacteraemia associated with Gram-negative bacillus in France (www.onerba.fr). Since it was one of the first microorganisms to develop resistance to aminosides (Christensen and Korner, 1972), it has played a central role in the spread of antibiotic resistance. *K. pneumoniae* has also gained particular notoriety with the emergence of hypervirulent strains including multiresistant ones (Turton et al., 2018; Paczosa and Mecsas, 2016; Surgers et al., 2016). Despite the major therapeutic and clinical concerns with these microorganisms, few studies have examined their ability to produce biofilm and hence their potential for further dissemination.

The objective of the study herein was to determine the host and bacterial factors associated with biofilm in ESBL-producing *E. coli* and *K. pneumoniae* isolates from a large university hospital. We also intended to quantify the kinetic profiles of biofilm development, including its determinants, within these microorganisms.

2. Materials and methods

2.1. Study design and patients

Isolates were obtained from 213 patients enrolled in a cross-sectional study with the aim of determining the epidemiological characteristics of ESBL-producing Enterobacteriaceae at a single university teaching hospital (Saint-Antoine Hospital, Paris, France) from April 2012 to April 2013 (Surgers et al., 2017). Patients presenting with an ESBL-positive sample were identified and their samples were further tested for biofilm formation.

Characteristics of the study population and isolates have been summarized in a previous publication (Surgers et al., 2017). In brief, roughly half of patients were female (53.6%) and median age was 70 years (IQR = 56–85). At least one comorbidity was present in 134 (62.9%) patients and 159 (74.7%) had at-risk exposure for ESBL carrier status (i.e., antibiotic therapy, previous hospitalization, or travel abroad) 3 months prior to infection. Of these patients, 229 strains including 147 *E. coli* et 82 *K. pneumoniae* were isolated from the following samples: urine ($n = 150$; $n = 105$ *E. coli* and $n = 45$ *K. pneumoniae*), pus ($n = 39$; $n = 21$ *E. coli* and $n = 18$ *K. pneumoniae*), blood culture ($n = 21$; $n = 14$ *E. coli* and $n = 7$ *K. pneumoniae*), broncho-alveolar lavage ($n = 15$; $n = 6$ *E. coli* and $n = 9$ *K. pneumoniae*), and from an intravascular device ($n = 4$; $n = 1$ *E. coli* and $n = 3$ *K. pneumoniae*).

2.2. Assessing clinical characteristics

A patient was defined as “infected” if their referent physician decided to treat with antibiotics or as “colonization” otherwise. Portal of entry was classified as lung, urinary tract, digestive tract, or unknown according to the referent physician. Acquisition of ESBL-producing Enterobacteriaceae was characterized as follows: hospital-acquired, if the first positive sample was detected > 48 h (h) after admission; healthcare-associated, if the first positive sample was detected ≤48 h after admission and the patient underwent hospitalization within 3 months prior; or community-acquired, if the first positive sample was detected ≤48 h of admission without any recent hospitalization. Data on transfer to the intensive care unit (ICU) and all-cause deaths within 12 months after entry into care were obtained from electronic patient medical records. Only “infected” patients had available data on ICU transfer.

2.3. Genotyping of strains

Data on bacterial characterization were obtained as previously described (Surgers et al., 2017). Different multiplex PCR systems were used to determine β-lactamase genes (Dallenne et al., 2010), phylogenetic groups for *E. coli* (Clermont et al., 2013), multilocus sequence typing (MLST) for *K. pneumoniae* (Diancourt et al., 2005), as well as plasmids carrying resistance ESBL genes (Compain et al., 2014a, 2014b; Carattoli et al., 2005). Using a PCR-based method, strains of *E. coli* were screened for 12 genes encoding putative virulence factors, which included adhesins [*ibeA* putative invasion, *sfa/foc* S or F1 C fimbriae, *papC* genes of P fimbrial operon, *papG* (II and III alleles), *flu* and Dr-binding *draBC*], toxins (*hlyC* hemolysin and *cnf1* cytotoxic necrotizing factor) and iron capture systems (*iucC*, *fyuA* iron uptake, *iroN*, salmochelin receptor) (Bonacorsi et al., 2006; Ulett et al., 2007; Johnson and Stell, 2000). For *K. pneumoniae*, genes encoding seven separate virulence factors were detected using multiplex PCR: *allS*, *rmpA*, type 3 fimbriae (*mrKD*), siderophores (*entB*, *kfu*, *ybtS*, *iutA*) and the two capsular serotypes K1 (*magA*) and K2 (*wzi*) (Compain et al., 2014a, 2014b).

2.4. Assessing biofilm formation

Biofilm formation was assessed by a microbead immobilization assay [BioFilm Ring test® (BRT), Biofilm control, St Beauzire, France] as previously described (Chavant et al., 2007; Olivares et al., 2016). The BRT offers a reproducible and quantifiable measure of biofilm production and has shown strong correlation with crystal violet staining (Crémet et al., 2013). Since this assay is automated, any non-standardized washing or staining techniques are removed. Briefly, microorganisms were cultured overnight in blood heart infusion (BHI). Medium standardized bacterial cultures were incubated at 37 °C in 96-well microtiter plates with magnetic beads. After different times of incubation (1h30 min, 4 h, 6 h, 24 h), microplates were placed onto a magnetic block and read by a BRT device. For each experiment, we used a positive control (strong biofilm producer strain, personal collection) and two negative controls (one without any strains and the other with a strain incapable of producing biofilm after multiple BRT runs). The plates were analysed using the Biofilm Control software, which gives a quantitative Biofilm Formation Index (BFI) ranging from 0 to 21. Higher BFI values correspond to high mobility of beads under magnetic action due to lack of biofilm, while lower values correspond to full immobilization of beads due to the presence of biofilm. Each strain was tested in two independent experiments and the average of the two experiments was used as the final BFI value.

2.5. Statistical analysis

Unless otherwise stated, all comparisons were made using Kruskal-Wallis rank test for continuous variables and Pearson's χ^2 or Fisher's Exact test for categorical variables. All analysis was carried out by STATA statistical software (v12.1, College Station, TX, USA) and a p-value < 0.05 was considered significant.

Initially, we modelled the kinetics of the BFI during the first 6 h of incubation. If isolates were able to achieve levels < 2 prior to 6 h, they were assumed to have maintained this level until the 6 h time-point. Change from baseline BFI was modelled over incubation time using mixed-effect linear regression in which a random-intercept was incorporated to account for within-isolate variability. Average BFI levels and their 95% confidence intervals (CI) were directly calculated from this model using the delta-method. In order to test overall differences in kinetics between factor levels, the interaction between factor and interval time was tested while including its individual effects.

In subsequent analysis, biofilm production was represented as follows: (i) presence or absence of standard biofilm defined as achieving a level < 7 or ≥7 within 24 h, respectively; and (ii) rapid, slow, and no standard biofilm development defined as achieving a cut-off < 7 within ≤4 h, > 4 h, and never, respectively. We based this cut-off on the distribution of BFI values at the last time point (median levels), which also corresponded to group-trajectory projections from preliminary analysis. Nevertheless, it should be noted that lack of biofilm production is generally considered at a BFI > 18 and no standardized cut-off is recommended for confirmation of biofilm production. In order to

understand the role of bacterial or clinical determinants on biofilm production, both biofilm end-points were examined in a two-part, univariable analysis. First, genetic characteristics were compared between biofilm production groups while stratified on either *E. coli* or *K. pneumoniae* species. Second, host factors and outcomes observed during/after infection were compared between biofilm production groups for all isolates.

3. Results

3.1. Genetic characteristics of ESBL-producing strains

Among the 147 *E. coli* isolates, the most common ESBL sequence was CTX-M-15 ($n = 65$, 44.2%), followed by CTX-M-14 ($n = 34$, 23.1%) and CTX-M-1 ($n = 21$, 14.3%). Virulent phylogenetic groups constituted 60.5% of *E. coli* isolates (B2, $n = 74$; D, $n = 15$), while all others were less virulent (A, $n = 21$; B1, $n = 12$; C, $n = 12$; E, $n = 7$; F, $n = 6$). Of the 82 (35.8%) *K. pneumoniae* isolates, the large majority produced CTX-M-15 ($n = 74$, 90.2%), with much lower prevalence of CTX-M-14 ($n = 5$, 6.1%) and CTX-M-1 ($n = 2$, 2.4%). A wide range of MLST types were identified, while some ST types were more frequently observed: ST29, $n = 19$ (23.2%); ST147, $n = 11$ (13.4%); ST323, $n = 16$ (19.5%); and ST405, $n = 5$ (6.1%). Only 4 (4.9%) strains were identified with K2 and none with K1 capsular genes.

3.2. Kinetics of biofilm production during incubation

The average difference (± standard deviation) between the two BFI measures within isolates was constant over time: 1.0 ± 0.1 at 1.5 h, 1.2 ± 0.1 at 4 h, and 1.2 ± 0.1 at 6 h. Overall, average BFI levels dropped from 21 to 18.9 (95%CI = 18.1–19.6) at 1.5 h, 8.4 (95%CI = 7.7–9.2) at 4 h, and finally to 6.2 (95%CI = 5.4–6.9) at 6 h (Fig. 1A) of incubation. The rate of BFI decline was significantly faster for *K. pneumoniae* than *E. coli* ($p < 0.001$), particularly at 4h and 6h (Fig. 1B).

Faster unadjusted kinetics were also identified for the following characteristics: overall – source of episode (in three groups: nosocomial, health-care associated, community acquired; $p < 0.001$); *E. coli* –toxin ($p < 0.001$) or adhesin ($p = 0.003$) virulence factors; *K. pneumoniae* MLST type ($p = 0.045$) (Supplementary Table 1).

The distribution of BFI levels at each time-point of incubation is given in Fig. 1C. Almost one-third of strains had a BFI level < 2 at 4 h. Most strains were able to strongly produce biofilm with a BFI < 2 (57.1%) by the end of 24 h, while a minority did not indicate any biofilm production (BFI > 18, 13.4%). A significantly higher proportion of *K. pneumoniae* strains produced BFI levels < 2 within 24 h compared to *E. coli* (78.1% versus 45.6%, $p < 0.001$).

3.3. Bacteriological and clinical determinants of biofilm development

For *E. coli* bacteria (Table 1), biofilm development was significantly less likely to be observed in ST131 strains and those with the *fyuA* virulence factor ($p = 0.03$ and $p = 0.01$, respectively), whereas it was more likely for strains with *hly* and *papC* virulence factors ($p = 0.009$ and $p = 0.006$, respectively). In particular, rapid biofilm development was observed more frequently in strains with toxin (*hly*, $p = 0.009$; *cnf1*, $p = 0.04$) and adhesin (*papC*, $p = 0.005$; *papGIII*, $p = 0.04$; *papGII*, $p = 0.05$) virulence factors (Table 1). Any multivariable analysis was precluded by the high collinearity between these genetic components. Nevertheless, the virulence factors less likely to be observed on non-

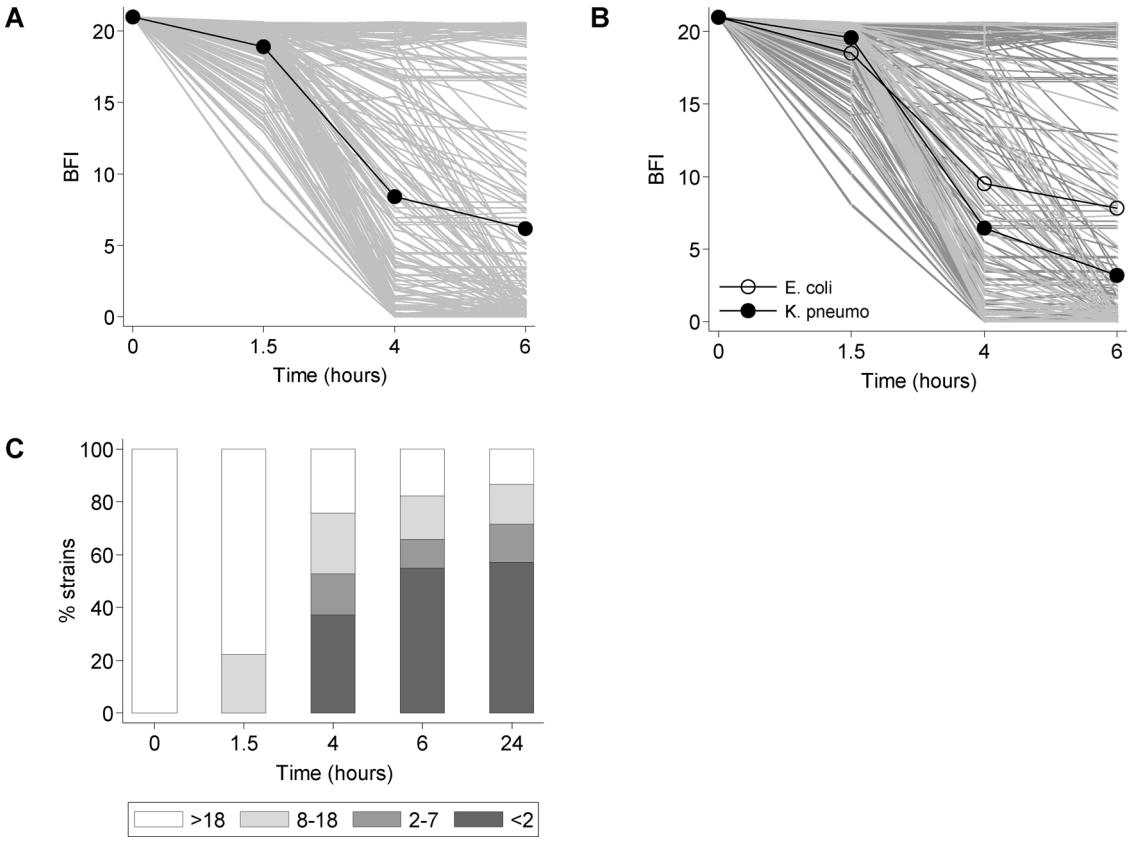

Fig. 1. Levels of biofilm formation index (BFI) over time.
In (**A**), individual trajectories of BFI over time are represented as grey lines and average BFI at each time point as a connected line. This figure is also stratified on the bacterial species *E. coli* (dark grey lines) and *K. pneumoniae* (light grey lines) (**B**). The distribution of BFI at each time point, presented as cumulative percent of strains achieving at or below a given threshold, is provided in (**C**).

Table 1
E. coli bacterial determinants of biofilm development.

	Developed biofilm*				
	Rapid (*n* = 71)	Slow (*n* = 20)	Total (*n* = 91)	None (*n* = 56)	*p*†
Genetic characteristics					
CTX-M-15 BLSE sequence	32 (45.1)	5 (25.0)	37 (40.7)	28 (50.0)	0.3
ST131	23 (32.4)	3 (15.0)	26 (28.6)	26 (46.4)	0.03
Phylogenetic group B2/D	46 (64.8)	4 (20.0)	50 (55.0)	39 (69.6)	0.08
Virulence factors					
Siderophores	60 (84.5)	15 (75.0)	75 (82.4)	52 (92.9)	0.09
fyuA	49 (69.0)	10 (50.0)	59 (64.8)	47 (83.9)	0.01
iucC	46 (64.8)	13 (65.0)	59 (64.8)	43 (76.8)	0.13
iroN	17 (23.9)	7 (35.0)	24 (26.4)	16 (28.6)	0.8
Toxins	22 (31.0)	1 (5.0)	23 (25.3)	4 (7.1)	0.008
hly	20 (28.2)	0 (0)	20 (22.0)	3 (5.4)	0.009
cnf1	17 (23.9)	1 (5.0)	18 (19.8)	4 (7.1)	0.06
Adhesins	41 (57.8)	7 (35.0)	48 (52.8)	17 (30.4)	0.008
sfa	7 (9.9)	0 (0)	7 (5.4)	3 (5.4)	0.7
papC	26 (36.6)	4 (20.0)	30 (33.0)	7 (12.5)	0.006
papGIII	10 (14.1)	0 (0)	10 (11.0)	1 (1.8)	0.05
papGII	15 (21.1)	2 (10.0)	17 (18.7)	4 (7.1)	0.06
ibeA	8 (11.3)	3 (15.0)	11 (12.1)	3 (5.4)	0.3
flu	12 (16.9)	1 (5.0)	13 (14.3)	4 (7.1)	0.3
draBC	3 (4.2)	2 (10.0)	5 (5.5)	2 (3.6)	0.6
Total nb of virulence factors	3 (1-5)	2 (1-3)	2 (1-5)	2 (2-3)	0.5

All statistics are *n* (%) with the exception of total number of virulence factors, presented in median (IQR).

* Rapid and slow biofilm development defined as achieving a Biofilm Formation Index below seven within ≤4 and > 4 h, respectively.

† Comparisons were made between strains that produced versus did not produce biofilm after 24 h. Significance between groups determined using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables.

Table 2
K. pneumoniae bacterial determinants of biofilm development.

	Developed biofilm*				
	Fast (*n* = 51)	Slow (*n* = 23)	Total (*n* = 74)	None (*n* = 8)	*p*†
Genetic characteristics					
CTX-M-15 BLSE sequence	44 (86.3)	22 (95.7)	66 (89.2)	8 (100)	0.9
MLST (29/147/323)	31 (60.8)	13 (56.5)	44 (59.5)	2 (25.0)	0.13
Virulence factors					
Serotype K2	3 (5.9)	1 (4.4)	4 (5.4)	0 (0)	0.9
Siderophores	25 (49.0)	13 (56.5)	38 (51.4)	4 (50.0)	0.9
iutA	1 (2.0)	1 (4.4)	2 (2.7)	0 (0)	0.9
ybtS	22 (43.1)	10 (43.5)	32 (43.2)	5 (50.0)	0.7
kfu	9 (17.7)	4 (17.4)	13 (17.6)	2 (25.0)	0.6
Allantoin metabolism (*allS*)	51 (100)	23 (100)	74 (100)	8 (100)	*ntp*
Mucoid regulator (*rmpA*)	1 (2.0)	0 (0)	1 (1.4)	0 (0)	0.9
Adhesin (*mrkD*)	50 (98.0)	23 (100)	73 (98.7)	8 (100)	0.9
Total nb of virulence factors	1 (1–2)	2 (1–2)	2 (1–2)	2 (1–3)	0.9

All statistics are *n* (%) with the exception of total number of virulence factors, presented in median (IQR).

* Rapid and slow biofilm development defined as achieving a Biofilm Formation Index below seven within ≤4 and > 4 h, respectively.

† Comparisons were made between strains that produced versus did not produce biofilm after 24 h. Significance between groups determined using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables. *ntp*, no test performed.

ST131 versus ST131 strains, respectively, were *fyuA* (0% vs 49.1%, $p < 0.001$) and *papGII* (30.2% vs 66.6%, $p = 0.002$), which indicates some grouping in these genetic factors with respect to biofilm formation. There were no genetic determinants for *K. pneumoniae* bacteria (Table 2), likely owing to the few strains unable to produce biofilm (*n* = 8, 9.8%).

Table 3
Clinical characteristics associated with biofilm development.

	Developed biofilm*				
	Fast (*n* = 122)	Slow (*n* = 43)	Total (*n* = 165)	None (*n* = 64)	*p*†
Bacterial species					< 0.001
E. coli	71 (58.2)	20 (46.5)	91 (55.2)	56 (87.5)	
K. pneumoniae	51 (41.8)	23 (53.5)	74 (44.9)	8 (12.5)	
Source of infection					0.048
Nosocomial	81 (66.4)	25 (58.1)	106 (64.2)	32 (50.0)	
Health-care associated/ Community-acquired	41 (33.6)	18 (41.9)	59 (35.8)	32 (50.0)	
Location of infection [*N* = 97]					0.01
Urine	25 (43.1)	9 (52.9)	34 (45.3)	17 (77.3)	
Lungs	8 (13.8)	2 (11.8)	10 (13.3)	2 (9.1)	
Digestive tract	22 (37.9)	4 (23.5)	26 (34.7)	1 (4.6)	
Skin	3 (5.2)	2 (11.8)	5 (6.7)	2 (9.1)	
Associated bacteremia	19 (15.6)	6 (14.0)	25 (15.2)	6 (9.4)	
Type of episode					0.5
Colonization	60 (49.2)	24 (55.8)	84 (50.9)	38 (59.4)	
Palliative infection	3 (2.5)	0	3 (1.8)	1 (5.7)	
Treated infection	59 (48.4)	19 (44.2)	78 (47.3)	25 (39.1)	

All statistics are *n* (%).

* Rapid and slow biofilm development defined as achieving a Biofilm Formation Index below seven within ≤4 and > 4 h, respectively.

† Comparisons were made between strains that produced versus did not produce biofilm after 24 h. Significance between groups determined using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables.

The frequency of biofilm development between various clinical factors is given for all isolates in Table 3. There was a significant difference in biofilm development between specific sources of infection (nosocomial versus health-care associated/community-acquired) ($p = 0.048$). Infections observed in urine were less likely to exhibit biofilm development ($p = 0.008$), while the opposite was true for digestive tract infections ($p = 0.006$). The proportion harbouring biofilm-producing strains was not significantly different between patients with colonized versus treated/palliative infection ($p = 0.5$).

3.4. Serious clinical outcomes and biofilm development

In the 99 patients with established infection, 16/18 of patients (88.9%) transferred to the ICU had biofilm producing isolates versus 60/81 of patients (74.1%) who were not. This difference was not significant ($p = 0.2$), even when stratifying on *E. coli* ($p = 0.3$) or *K. pneumoniae* ($p = 0.9$) isolates. If we assume that all colonized individuals were not admitted to the ICU, 88.9% ($n = 2/18$) and 70.9% ($n = 59/144$) transferred and non-transferred patients, respectively, had strains with biofilm ($p = 0.17$). There was a slight trend in increasing ICU transfer rates with increasing rate of biofilm development: none, $n = 2/23$ (8.7%); slow, $n = 3/17$ (17.7%); fast, $n = 13/59$ (22.0%) ($\tau_b = 0.13$); however, this was not significant (p for non-parametric trend = 0.17).

In the 158 patients with data on all-cause mortality, 13/66 of patients (80.3%) who died had strains with biofilm, compared to 66/92 of patients (71.7%) who remained alive ($p = 0.2$). These proportions were, respectively, 68.4% and 63.6% for *E. coli* ($p = 0.6$) and 96.4% and 83.8% for *K. pneumoniae* ($p = 0.13$). There were no significant differences in biofilm presence between dead versus alive patients, respectively, with colonization (88.0% versus 71.7%, $p = 0.16$) or infection (71.9% versus 75.6%, $p = 0.7$). There was again a slight trend in increasing death with higher rates of biofilm development: none,

n = 13/39 (33.3%); slow, n = 12/32 (37.5%); fast, n = 41/87 (47.1%) (τ_b = 0.12); yet was not significant (p for non-parametric trend = 0.13).

4. Discussion

Biofilm production provides an important pathogenic advantage, allowing protection against host immune responses and reduced susceptibility to antibiotic therapy (Donlan and Costerton, 2002). In this large collection of strains obtained from a single centre, we noticed that biofilm was produced in roughly three-quarters of isolates, demonstrating the troubling extent of this problem in ESBL-producing microorganisms. Furthermore, biofilm was significantly more prevalent in *K. pneumoniae* than *E. coli* species and in digestive tract infections. The extensive genetic characterization of these strains has helped further our understanding of the more common circumstances under which biofilm can be observed.

Interestingly, we found a close relationship with several different virulence factors and the capacity to produce biofilm. Biofilm was much less common among *E. coli* isolates with siderophore virulence factors, with the strongest difference observed in *iucC*. In contrast, a previous study by Hancock et al. (2008) found that the siderophore virulence factor *fyuA* was required for efficient biofilm formation in *E. coli*, but by itself does not account for increased biofilm production. Nevertheless, their study only included urinary tract infections and hence the discrepancy with our study might also be due to infection location. Isolates harbouring *fyuA* require biofilm production in iron-poor environments, such as urine, whereas it might not be necessary in other sites.

In contrast, the presence of toxin or adhesin virulence factors was significantly associated with increased biofilm production. Furthermore, the toxin *hly* and *cnf1* virulence factors, as well as adhesin *papC*, *papGIII*, and to some extent *papGII* factors, were associated with rapid development of biofilm, as defined by our assay. Our findings support the predominant role of these virulence factors in biofilm production, consistently observed across *E. coli* isolates from various settings with differing genetic characteristics (i.e. non-ESBL, colonization, lacking antimicrobial resistance genes, etc.) (Naves et al., 2008a, 2008b). The *hly* virulence factor has also been implicated in biofilm development during prostatitis due to *E. coli* infection (Soto et al., 2007). Given that simultaneous biofilm production across certain virulence factors tends to cluster in pathogenicity islands, which at times results in increased virulence, their prevalence is fairly concerning.

Other genetic factors have been certainly implicated in biofilm production. Clermont et al. described biofilm in CTX-M-15 producing *E. coli* ST131 that was not contained within the CTX-M plasmid (Clermont et al., 2008). In another study, the prevalence of biofilm production was significantly higher among ST131 than non-ST131 isolates regardless ESBL-production and antibiotic resistance (Kudinha et al., 2013). More refined analysis from Pantel et al. has demonstrated largely diverse biofilm activity in *E. coli* ST131 clones (Pantel et al., 2016), as substantiated by others (Olesen et al., 2014; Shin and Ko, 2015). In this study, we also observed a strong relationship between ST131 strains and two virulence factors, *fyuA* and *papGII*, both of which, when absent, are significantly associated with biofilm production. Perhaps the variability of ST131 subclones or types of concomitant virulence factors in our cohort could explain why an inverse association was observed between ST131 and biofilm production.

With regards to *K. pneumoniae*, the pervasiveness of biofilm in ESBL-producing strains has been demonstrated in a previous study (Yang and Zhang, 2008). Type 3 fimbriae are known to serve as appendages mediating the formation of biofilm and are a major determinant of fimbrial binding specificity (Murphy and Clegg, 2012). As all isolates in this study were ESBL-producing and 98.8% of *K. pneumoniae* had *mrkD*, a protein located at the tip of the type 3 fimbriae; it is unsurprising that this bacterial species was able to produce biofilm at such a large extent. Inasmuch as we could determine, biofilm development was more frequent in the widespread *K. pneumoniae* types ST29, ST147, and ST323; while data from biofilm kinetics demonstrated a significantly faster rate of biofilm production in these clones compared to others. How these results relate to the dissemination of *K. pneumoniae* would need further investigation.

One of the more novel aspects of this study was the evaluation of biofilm kinetics during incubation, permitting us to distinguish isolates with rapid versus slow biofilm production. With *E. coli* in particular, isolates with slow biofilm development rarely had characteristics that are classically identified as “pathogenic” (i.e.CTX-M-15, ST131, or the virulent phylogenetic groups B2/D). Rather, the more “pathogenic” bacteria exhibited a bimodal pattern of either rapid or no biofilm development. In contrast, *K. pneumoniae* isolates had much faster kinetics of biofilm production compared to *E. coli*, especially within 4–6 h of incubation, resulting in the vast majority of *K. pneumoniae* bacteria reaching BFI $<$ 2 within 24 h.

We did not notice any relationship between biofilm-producing isolates and ICU transfers or deaths within 12-months. However, the high overall mortality rate due to a study population with multiple comorbidities and lack of sample size might have restricted our capacity to determine a statistically significant difference. To our knowledge, there are conflicting reports on how biofilm production could serve as an underlying cause of morbidity and mortality. Other extrinsic factors are likely implicated. For example, biofilm-producing bacteria are often found on foreign objects (i.e. urinary catheters, intra-vascular devices, etc.), could be cleared when these objects are removed, and thus provide limited effect on clinical outcomes (Guembe et al., 2018). Conversely, biofilm formation appears to play a role in recurrence of some infections, making them difficult to clear (Soto et al., 2006), and are particularly difficult to treat (Høiby et al., 2015). These factors could confound its direct association with mortality. Larger datasets would be needed to clearly establish the relationship with biofilm and more severe clinical outcomes in patients infected with ESBL-producing microorganisms.

Other limitations of our study should be acknowledged. First, there are a variety of techniques available to study biofilm development in vitro (Lebeaux et al., 2013), yet these methods have not been standardized. Biofilm production also varies under certain experimental conditions (i.e. presence of oxygen, culture mediums, etc.) (Hancock et al., 2011; Naves et al., 2008a, 2008b), further complicating between-assay comparisons. Nevertheless, we opted to use a novel method based on the immobilization of magnetic beads by bacteria when biofilm is secreted, which was able to reduce the number of non-standardized steps (Chavant et al., 2007) and has shown strong correlation with the crystal violet method (Crémet et al., 2013). This method has also demonstrated higher sensitivity in detecting biofilm in the early phases of production (Chavant et al., 2007; Lebeaux et al., 2013), making it particularly useful to study its kinetics. Second, considering that there is no official definition for biofilm production with the BRT device, we used a cut-off derived from our study isolates at an index level of 7. Clearer definitions of more biologically and clinically meaningful values of the BFI need to be established. Third, we were unable to characterize plasmids carrying antimicrobial resistance for roughly half of all strains. The link between resistance genes and biofilm production was not assessed due to the lack of available data. Fourth, we did not test for all known virulence factors. Finally, although we present results from a wide range of clinical settings, the increased heterogeneity might have impacted our ability to characterize phenomenon specific to infection site. Studies with greater numbers of isolates would be more adequate in addressing these questions.

In conclusion, biofilm production was more common in ESBL-producing *E. coli* harbouring adhesin and toxin virulence factors, while it was extensively present across most *K. pneumoniae* ESBL-producing isolates. Since there was a slight trend in increasing mortality with more rapid biofilm development, further exploration on the clinical value of biofilm kinetics should be conducted in larger collections of

isolates. Further understanding of biofilm kinetics and development could be improved by comparing these isolates to non-ESBL-producing or wildtype *E. coli* and *K. pneumoniae* infections within similar settings.

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Transparency declarations

None to declare.

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Appendix A. Supplementary data

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References

Björklöf, K., Nurmiaho-Lassila, E.L., Klinger, N., Haahtela, K., Romantschuk, M., 2000. Colonization strategies and conjugal gene transfer of inoculated Pseudomonas syringae on the leaf surface. J. Appl. Microbiol. 89 (3), 423–432.

Bonacorsi, S., Houdouin, V., Mariani-Kurkdjian, P., Mahjoub-Messai, F., Bingen, E., 2006. Comparative prevalence of virulence factors in Escherichia coli causing urinary tract infection in male infants with and without bacteremia. J. Clin. Microbiol. 44 (3), 1156–1158.

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B., Bartlett, J., 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin. Infect. Dis. 48 (1), 1–12.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63 (3), 219–228.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., Buret, A., 1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. 37 (6), 1771–1776.

Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M., Bernardi, T., 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. J. Microbiol. Methods 68 (3), 605–612.

Christensen, S.C., Korner, B., 1972. An endemic caused by multiresistant Klebsiella in an urological unit. Scand. J. Urol. Nephrol. 6, 232–238.

Clermont, O., Lavollay, M., Vimont, S., Deschamps, C., Forestier, C., Branger, C., Denamur, E., Arlet, G., 2008. The CTX-M-15-producing Escherichia coli diffusing clone belongs to a highly virulent B2 phylogenetic subgroup. J. Antimicrob. Chemother. 61, 1024.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups: a new E. coli phylo-typing method. Environ. Microbiol. Rep. 5 (1), 58–65.

Compain, F., Poisson, A., Le Hello, S., Branger, C., Weill, F.-X., Arlet, G., Decré, D., 2014a. Targeting relaxase genes for classification of the predominant plasmids in Enterobacteriaceae. Int. J. Med. Microbiol. 304 (3–4), 236–242.

Compain, F., Babosan, A., Brisse, S., Genel, N., Audo, J., Ailloud, F., Kassis-Chikhani, N., Arlet, G., Decré, D., 2014b. Multiplex PCR for detection of seven virulence factors and K1/K2 capsular serotypes of Klebsiella pneumoniae. J. Clin. Microbiol. 52 (12), 4377–4380.

Crémet, L., Corvec, S., Batard, E., Auger, M., Lopez, I., Pagniez, F., Dauvergne, S., Caroff, N., 2013. Comparison of three methods to study biofilm formation by clinical strains of Escherichia coli. Diagn. Microbiol. Infect. Dis. 75 (3), 252–255.

Dallenne, C., Da Costa, A., Decré, D., Favier, C., Arlet, G., 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother. 65 (3), 490–495.

De Araujo, C., Balestrino, D., Roth, L., Charbonnel, N., Forestier, C., 2010. Quorum sensing affects biofilm formation through lipopolysaccharide synthesis in Klebsiella pneumoniae. Res. Microbiol. 161 (7), 595–603.

Diancourt, L., Passet, V., Verhoef, J., Grimont, P.A., Brisse, S., 2005. Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J. Clin. Microbiol. 43, 4178–4182.

Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15 (2), 167–193.

Guembe, M., Alonso, B., Lucio, J., Pérez-Granda, M.J., Cruces, R., Sánchez-Carrillo, C., Fernández-Cruz, A., Bouza, E., 2018. Biofilm production is not associated with poor clinical outcome in 485 patients with Staphylococcus aureus bacteraemia. Clin. Microbiol. Infect. 24 (June (6)), 659.

Hancock, V., Ferrières, L., Klemm, P., 2008. The ferric yersiniabactin uptake receptor Escherichia coli in human urine. Microbiology 154 (Pt 1), 167–175.

Hancock, V., Witsø, I.L., Klemm, P., 2011. Biofilm formation as a function of adhesin, growth medium, substratum and strain type. Int. J. Med. Microbiol. 301 (7), 570–576.

Hennequin, C., Aumeran, C., Robin, F., Traore, O., Forestier, C., 2012. Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate. J. Antimicrob. Chemother. 67 (9), 2123–2130.

Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G.L., Coenye, T., Donelli, G., Hall-Stoodley, L., Holá, V., Imbert, C., Kirketerp-Møller, K., Lebeaux, D., Oliver, A., Ullmann, A.J., Williams, C., ESCMID Study Group for Biofilms and Consulting External Expert Werner Zimmerli, 2015. ESCMID guideline for the diagnosis and treatment of biofilm infections. Clin. Microbiol. Infect. 21 (Suppl. 1), S1–25.

Kudinha, T., Johnson, J.R., Andrew, S., Kong, F., Anderson, P., Gilbert, G.L., 2013. Escherichia coli sequence type 131 (ST131) as a prominent cause of antibiotic resistance among urinary Escherichia coli isolates from reproductive- age women. J. Clin. Microbiol. 51, 3270–3276.

Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181 (1), 261–272.

Lebeaux, D., Chauhan, A., Rendueles, O., Beloin, C., 2013. From in vitro to in vivo models of bacterial biofilm-related infections. Pathogens 2 (2), 288–356.

Murphy, C.N., Clegg, S., 2012. Klebsiella pneumoniae and type 3 fimbriae: nosocomial infection, regulation and biofilm formation. Future Microbiol. 7 (8), 991–1002 PMID: 22913357.

Naves, P., del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., Dahbi, G., Blanco, M., Ponte Mdel, C., Soriano, F., 2008a. Correlation between virulence factors and in vitro biofilm formation by Escherichia coli strains. Microb. Pathog. 45 (2), 86–91..

Naves, P., del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., Rodríguez-Cerrato, V., Ponte, M.C., Soriano, F., 2008b. Measurement of biofilm formation by clinical isolates of Escherichia coli is method-dependent. J. Appl. Microbiol. 105 (2), 585–590.

Olesen, B., Frimodt-Møller, J., Leihof, R.F., Struve, C., Johnston, B., Hansen, D.S., Scheutz, F., Krogfelt, K.A., Kuskowski, M.A., Clabots, C., Johnson, J.R., 2014. Temporal trends in antimicrobial resistance and virulence associated traits within the Escherichia coli sequence type 131 clonal group and its H30 and H30-Rx subclones, 1968 to 2012. Antimicrob. Agents Chemother. 58, 6886–6895.

Olivares, E., Badel-Berchoux, S., Provot, C., Jaulhac, B., Prévost, G., Bernardi, T., Jehl, F., 2016. The BioFilm ring test: a rapid method for routine analysis of Pseudomonas aeruginosa biofilm formation kinetics. J. Clin. Microbiol. 54 (3), 657–661.

Paczosa, M.K., Mecsas, J., 2016. Klebsiella pneumoniae: going on the offense with a strong defense. Microbiol. Mol. Biol. Rev. 80 (3), 629–661.

Pantel, A., Dunyach-Remy, C., Ngba Essebe, C., Mesureur, J., Sotto, A., Pagès, J.M., Nicolas-Chanoine, M.H., Lavigne, J.P., 2016. Modulation of membrane influx and Efflux in Escherichia coli sequence type 131 has an impact on bacterial motility, biofilm formation, and virulence in a Caenorhabditis elegans model. Antimicrob. Agents Chemother. 60 (5), 2901–2911.

Russo, T.A., Johnson, J.R., 2003. Medical and economic impact of extraintestinal infections due to Escherichia coli: focus on an increasingly important endemic problem. Microbes Infect. Inst. Pasteur. 5 (5), 449–456.

Shapiro, J.A., 1998. Thinking about bacterial populations as multicellular organisms. Annu. Rev. Microbiol. 52, 81–104.

Shin, J., Ko, K.S., 2015. Effect of plasmids harbouring blaCTX-M on the virulence and fitness of Escherichia coli ST131 isolates. Int. J. Antimicrob. Agents 46, 214–218.

Soto, S.M., Smithson, A., Horcajada, J.P., Martinez, J.A., Mensa, J.P., Vila, J., 2006. Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic Escherichia coli. Clin. Microbiol. Infect. 12 (10), 1034–1036.

Soto, S.M., Smithson, A., Martinez, J.A., Horcajada, J.P., Mensa, J., Vila, J., 2007. Biofilm formation in uropathogenic Escherichia coli strains: relationship with prostatitis, urovirulence factors and antimicrobial resistance. J. Urol. 177 (1), 365–368.

Surgers, L., Boyd, A., Girard, P.M., Arlet, G., Decré, D., 2016. ESBL-producing strain of hypervirulent Klebsiella pneumoniae K2. France. Emerg Infect Dis. 1687–1688.

Surgers, L., Boyd, A., Boelle, P.Y., Lalande, V., Jolivot, P.A., Girard, P.M., Arlet, G., Cambier, C., Homor, A., Decre, D., Meynard, J.L., 2017. Clinical and microbiological determinants of severe and fatal outcomes in patients infected with Enterobacteriaceae producing extended-spectrum β-lactamase. Eur. J. Clin. Microbiol. Infect. Dis. 36 (7), 1261–1268.

Turton, J.F., Payne, Z., Coward, A., Hopkins, K.L., Turton, J.A., Doumith, M., Woodford, N., 2018. Virulence genes in isolates of Klebsiella pneumoniae from the UK during 2016, including among carbapenemase gene-positive hypervirulent K1-ST23 and' non-hypervirulent' types ST147, ST15 and ST383. J. Med. Microbiol. 118–128.

Ulett, G.C., Valle, J., Beloin, C., Sherlock, O., Ghigo, J.M., Schembri, M.A., 2007. Functional analysis of antigen 43 in uropathogenic Escherichia coli reveals a role in long-term persistence in the urinary tract. Infect. Immun. 75 (7), 3233–3244.

Yang, D., Zhang, Z., 2008. Biofilm-forming Klebsiella pneumoniae strains have greater likelihood of producing extended-spectrum beta-lactamases. J. Hosp. Infect. 68 (4), 369–371.