AKADÉMIAI KIADÓ

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REVIEW ARTICLE

Association between biofilm-production and antibiotic resistance in *Escherichia coli* isolates: A laboratory-based case study and a literature review

MÁRIÓ GAJDÁCS[1,2*], KRISZTINA KÁRPÁTI[3], ÁDÁM LÁSZLÓ NAGY[4], MÁTÉ GUGOLYA[2], ANETTE STÁJER[5] and KATALIN BURIÁN[6]

[1] Department of Oral Biology and Experimental Dental Research, Faculty of Dentistry, University of Szeged, Tisza Lajos krt 63., 6720 Szeged, Hungary

[2] Institute of Medical Microbiology, Faculty of Medicine, Semmelweis University, Nagyvárad tér 4., 1089 Budapest, Hungary

[3] Department of Orthodontics and Pediatric Dentistry, Faculty of Dentistry, University of Szeged, Tisza Lajos körút 64-66., 6720 Szeged, Hungary

[4] Department of Prosthodontics, Faculty of Dentistry, University of Szeged, Tisza Lajos körút 62–64., 6720 Szeged, Hungary

[5] Department of Periodontology, Faculty of Dentistry, University of Szeged, Tisza Lajos körút 62–64., 6720 Szeged, Hungary

[6] Department of Medical Microbiology, Albert Szent-Györgyi Health Center and Faculty of Medicine, University of Szeged, Semmelweis utca 6., 6725 Szeged, Hungary

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ABSTRACT

Bacteria can enhance their survival by attaching to inanimate surfaces or tissues, and presenting as multicellular communities encased in a protective extracellular matrix called biofilm. There has been pronounced interest in assessing the relationship between the antibiotic resistant phenotype and biofilm-production in clinically-relevant pathogens. The aim of the present paper was to provide additional experimental results on the topic, testing the biofilm-forming capacity of *Escherichia coli* isolates using *in vitro* methods in the context of their antibiotic resistance in the form of a laboratory case study, in addition to provide a comprehensive review of the subject. In our case study, a total of two hundred and fifty (n = 250) *E. coli* isolates, originating from either clean-catch urine samples (n = 125) or invasive samples (n = 125) were included. The colony morphology of isolates were recorded after 24h, while antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Biofilm-formation of the isolates was assessed with the crystal violet tube-adherence method. Altogether 57 isolates (22.8%) isolates were multidrug resistant (MDR), 89 isolates (35.6%) produced large colonies (>3 mm), mucoid variant colonies were produced in 131 cases (52.4%), and 108 (43.2%) were positive for biofilm formation. Biofilm-producers were less common among isolates resistant to third-generation cephalosporins and trimethoprim-sulfamethoxazole (P = 0.043 and P = 0.023, respectively). Biofilms facilitate a protective growth strategy in bacteria, ensuring safety against environmental stressors, components of the immune system and noxious chemical agents. Being an integral part of bacterial physiology, biofilm-formation is interdependent with the expression of other virulence factors (especially adhesins) and quorum sensing signal molecules. More research is required to allow for the full understanding of the interplay between the MDR phenotype and biofilm-production, which will facilitate the development of novel therapeutic strategies.

KEYWORDS

Escherichia coli, antibiotic resistance, biofilm formation, colony morphology, crystal violet, urinary tract infections, literature review, virulence

*Corresponding author. Tel.: +36 62 342 532.
E-mail: mariopharma92@gmail.com, gajdacs.mario@stoma.szote.u-szeged.hu

INTRODUCTION

The emergence of antimicrobial resistance (AMR) is a continuously evolving challenge, which threatens the effective therapy of patients and successful operation of healthcare institutions worldwide [1, 2]. Pathogenic bacteria may become resistant to a wide range of chemically-unrelated antibiotics through a multitude of (either chromosomally-encoded or plasmid-mediated) resistance mechanisms [3]; multidrug resistance (MDR) is defined as non-susceptibility to at least one antimicrobial compounds in three or more antimicrobial categories [4]. Based on the projections of the "Burden of AMR Collaborative Group", in 2015, over 700,000 MDR infections and ~33,110 excess death have been recorded in the European Union (EU) alone, while according to the O'Neill Report (sequestered by the National Health Service in the United Kingdom), MDR infections may lead to 10 million excess deaths by 2050 [5, 6]. At present, the group of "ESKAPE" bacteria (E: *Enterococcus faecium* [7], S: *Staphylococcus aureus* [8], K: *Klebsiella pneumoniae* [9], A: *Acinetobacter baumannii* [10], P: *Pseudomonas aeruginosa* [11] and E: other members of *Enterobacteriaceae* [12]) receive the most attention, due to their overall disease burden, mortality rates and continuously increasing rates of resistance.

During *in vitro* experiments (i.e. when bacteria are cultivated in various culture media), bacteria most often present in their planktonic (or free-living) states. However, in harsh environmental conditions or *in vivo* in the infected host, bacteria enhance their survival by attaching to inanimate surfaces or tissues, and presenting as multicellular communities encased in a protective extracellular matrix, called biofilm [13]. In fact, based on a recent estimation by the National Institute of Health (NIH), >60% on infections *in vivo* are caused by microorganisms embedded in biofilm [14]. Biofilms are composed of exopolysaccharides (EPS), nucleic acids (environmental DNA), proteins, lipids, various ions and water, secreted by multiple bacterial communities, providing a survival advantage for all embedded bacteria [15]. In addition to "classical" resistance-determinants expressed by bacteria, the production of biofilms is another major concern, often leading to recalcitrant, chronic infections (e.g., in catheter-associated infections, skin and soft tissue infections, dental caries) [16–18]. Biofilms protect bacteria against the penetration and accumulation of various noxious substances and antibiotics in effective concentrations (resulting in 10–10,000-times higher minimal inhibitory concentrations); additionally, antibiotics are not effective against metabolically-inactive/dormant bacteria (often called as small-colony variants) [15, 16]. Strong biofilm-formation is an important characteristic of all members of the "ESKAPE" pathogens [19]; thus, in recent years, there has been pronounced interest in assessing the possible relationship between their antibiotic resistant/MDR status and biofilm-production in these bacteria [20, 21]. Despite the large number of published studies available, many of these experiments employed different methodologies, and they often resulted in conflicting findings [22, 23]. The aim of the present paper was to provide additional experimental results on the topic of potential relationships between the biofilm-forming capacity and the antibiotic resistance phenotype in *Escherichia coli* using *in vitro* methods (in the form of a laboratory case study), in addition to a comprehensive review of the subject.

CASE STUDY

Collection and identification of *E. coli* isolates

A total of two hundred and fifty (n = 250) *E. coli* isolates were included in this study, which were kindly provided from the strain collection of the Department of Medical Microbiology, Albert Szent-Györgyi Health Center and Faculty of Medicine, University of Szeged. The study uses a cross-sectional design, with microorganisms that were isolated between 2018.07.01. and 2020.01.31., from two types of different clinical materials, i.e. clean-catch urine samples from patients with laboratory-confirmed urinary tract infections (UTIs) (n = 125) and from invasive infections (n = 125), being randomly selected to be included in our experiments. Identification of the isolates was carried out based on classical phenotypic and biochemical panel-based methods [24]. All isolates included in the study were re-identified as *E. coli* before further assays. During our experiments, *E. coli* ATCC 25298 (pan-susceptible, "wild strain", strong biofilm-producer [25]), *E. coli* ATCC 35218 ($bla_{\text{TEM-1}}$-producer, weak biofilm-producer [26]; obtained from the American Type Culture Collection, Manassas, VI, USA), *E. coli* 15/12569 (resistant to ciprofloxacin; $MIC_{\text{ciprofloxacin}}$ = 2 mg L^{-1}), *E. coli* 17/47012 (resistant to fosfomycin; $MIC_{\text{fosfomycin}}$ = 64 mg L^{-1}) and *E. coli* 16/30098 (resistant to trimethoprim-sulfamethoxazole; $MIC_{\text{trimethoprim-sulfamethoxazole}}$ = 16 mg L^{-1}) were used as control strains [27].

Colony characteristics

The bacterial specimens were cultured using eosine methylene blue (EMB) agar (bioMérieux, Marcy-l'Étoile, France) plates. To record colony morphology of the bacterial isolates, EMB plates were inoculated and incubated at 37 °C for 24 h, in an aerobic atmosphere. After the incubation period, colony morphologies were assessed visually (for size, mucoid nature and lactose-fermentation) and these data were recorded. Colonies were considered small if their side was below ≤3 mm, or large if their size was >3 mm [26].

Antimicrobial susceptibility testing, detection of resistance

Antimicrobial susceptibility testing (AST) for ciprofloxacin (CIP), nitrofurantoin (NIT), fosfomycin (FOS), cefpodoxime (CFP), meropenem (MER), gentamicin (GEN) and trimethoprim-sulfamethoxazole (SXT) was performed with

the disk diffusion method (Liofilchem, Abruzzo, Italy) on Mueller–Hinton agar (MHA) plates. The interpretation of the results was based on EUCAST breakpoints v. 9.0. (http://www.eucast.org), corresponding to the members of Enterobacterales. During AST, *E. coli* ATCC 25298 was used as a quality control strain. Isolates were considered resistant to third-generation cephalosporins (3GCs) if the inhibition zone diameter around cefpodoxime 10 µg disks was <21 mm [28]. Isolates were defined as MDR based on the recommendations of Magiorakos et al. [4].

Crystal violet (CV) tube-adherence biofilm-production assay

Screening for biofilm-formation in the isolates was carried out using the tube-adherence method previously described [29]. Briefly, glass tubes containing 1 mL of sterile tryptic case soy broth (TSB; bioMérieux, Marcy-l'Étoile, France) were inoculated with 1 µL of the overnight culture of the tested bacterial strain. The tubes were then incubated statically for 24 h at 37 °C. Verification of planktonic growth was detected visually. After the incubation period, the supernatant was removed, the adhered cells were rinsed three times with phosphate buffer saline (PBS; Sigma-Aldrich; Budapest, Hungary) and the tubes were patted dry on a paper towel. The contents of the tubes were treated for 3 h at room temperature with a 1 mL solution of 0.1% crystal violet (CV; Sigma-Aldrich; Budapest, Hungary) to stain the adhered biomass. The CV solution was removed and the tubes were again rinsed three times with PBS; subsequently, the tubes were patted dry on a paper towel. Biofilm-formation was observed visually; the appearance of visible biofilm lining at the bottom and on wall of the glass tubes were considered positive for biofilm-production [29].

Statistical analysis

Descriptive statistical analysis (including means and percentages to characterize data) was performed using Microsoft Excel 2013 (Microsoft Corp.; Redmond, WA, USA). Additional statistical analyses were performed with IBM SPSS Statistics for Windows 22.0 (IBM Corp., Armonk, NY, USA), using the chi squared-test. P values <0.05 were considered statistically significant. The agreement between the results of the biofilm-production studies and colony morphology was also calculated [27].

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and national and institutional ethical standards. Ethical approval for the study protocol was obtained from the Human Institutional and Regional Biomedical Research Ethics Committee, University of Szeged (registration number: 140/2021-SZTE [5019]).

Antibiotic resistance of *E. coli* isolates included in the study

The results of the AST studies are summarized in Table 1. Out of the $n = 250$ *E. coli* isolates included in this study, the highest resistance rates overall were observed for CIP (43.6%), followed by SXT (34.4%), FOS (18.8%), GEN (11.6%) and NIT (11.2%); resistance against 3GCs was seen in 19.6% of isolates, while no MER resistant isolate was detected. Higher resistance rates in invasive *E. coli* isolates were seen for CIP ($P < 0.001$; $\chi^2 = 10.51$; degrees of freedom [DOF]: 1), SXT ($P < 0.001$; $\chi^2 = 12.78$; DOF: 1), 3GCs ($P < 0.001$; $\chi^2 = 7.63$; DOF: 1) and GEN ($P = 0.049$; $\chi^2 = 4.62$; DOF: 1), while no such differences were seen for FOS and NIT. The number of MDR isolates was also higher among invasive isolates ($P < 0.001$; $\chi^2 = 9.33$; DOF: 1) (Table 1.)

Association of antibiotic resistance with colony characteristics in *E. coli*

35.6% ($n = 89$) of isolates produced large colonies (>3 mm), including $n = 51$ from urinary samples and $n = 38$ from invasive samples ($P > 0.05$; $\chi^2 = 2.69$; DOF: 1). Isolates growing large colonies were less common in *E. coli* strains resistant to CIP ($P = 0.028$; $\chi^2 = 11.83$; DOF: 1), FOS ($P = 0.043$; $\chi^2 = 8.11$; DOF: 1) and 3GCs ($P = 0.39$; $\chi^2 = 10.02$; DOF: 1). Mucoid variant colonies were produced in 52.4% ($n = 131$) of cases, representing $n = 78$ from urinary samples and $n = 53$ from invasive samples ($P < 0.001$; $\chi^2 = 20.06$; DOF: 1). No association was seen among the resistance traits and the mucoid presentation of the isolates. All tested clinical isolates (100%) were lactose-fermenters. During the testing of the control strains, all strains were positive for lactose-fermentation. *E. coli* ATCC 25298 presented with small, mucoid colonies, *E. coli* 16/30098 showed large, non-mucoid colonies, while *E. coli* ATCC 35218, *E. coli* 15/12569 and *E. coli* 17/47012 presented with small, non-mucoid colonies on EMB agar.

Table 1. Rate of antibiotic resistance among *E. coli* isolates included in the study

	Urinary tract isolates ($n = 125$)	Invasive isolates ($n = 125$)	Overall ($n = 250$)
Ciprofloxacin	32.8% ($n = 41$)	54.4% ($n = 68$)	43.6% ($n = 109$)
Fosfomycin	16.0% ($n = 20$)	21.6% ($n = 27$)	18.8% ($n = 47$)
Gentamicin	7.2% ($n = 9$)	16.0% ($n = 20$)	11.6% ($n = 29$)
Nitrofurantoin	8.8% ($n = 11$)	13.6% ($n = 17$)	11.2% ($n = 28$)
Trimethoprim/sulfamethoxazole	23.2% ($n = 29$)	45.6% ($n = 57$)	34.4% ($n = 86$)
Third-generation cephalosporins	12.0% ($n = 15$)	27.2% ($n = 34$)	19.6% ($n = 49$)
Meropenem	0% ($n = 0$)	0% ($n = 0$)	0% ($n = 0$)
MDR	14.4% ($n = 18$)	31.2% ($n = 39$)	22.8% ($n = 57$)

Association of antibiotic resistance with biofilm-formation in *E. coli*

Out of the tested isolates, $n = 43.2\%$ ($n = 108$) were positive in the CV tube-adherence assay: no relevant differences were shown between the urinary and invasive isolates (59 out of 125 vs. 49 out of 125, respectively; $P > 0.05$). On the other hand biofilm-producing isolates were less common among *E. coli* resistant to 3GCs ($P = 0.043$; $\chi^2 = 6.89$; DOF: 1) and SXT ($P = 0.023$; $\chi^2 = 10.19$; DOF: 1); similar association was not shown for CIP, FOS, NIT and GEN-resistant isolates. The agreement (i.e. predictive value) of the production of mucoid-variant colonies on EBM on the results of the biofilm formation assay were calculated: overall, the agreement was 0.824 or 82.4% (urinary isolates: 0.756 or 75.6%; invasive isolates: 0.925 or 92.5%). When assessing control strains, *E. coli* ATCC 25923 presented as positive, while all other control strains were negative.

DISCUSSION, REVIEW OF THE LITERATURE

The objective of our present study was to evaluate the antibiotic resistance levels in a collection of UTI-causing and invasive *E. coli* isolates (acquired via purposive sampling), in addition to scrutinizing a possible relationship between the resistance phenotypes and the biofilm-forming capacities of the respective isolates. Overall, less than 45% of isolates tested were positive for biofilm production in our *in vitro* assay; colony morphology was useful in predicting the results of the biofilm-assay in 82.4% of cases (this was more reliable for invasive *E. coli* isolates). The relatively high predictive value of mucoid-variant colonies for biofilm-production has been demonstrated by our previous study, where $n = 250$ UPEC isolates were tested; in this study, the predictive value for the same tests was 88.1%, and larger colonies were less common in isolates non-susceptible to any antibiotic. In addition to this, mucoid-colony variants and biofilm-producing isolates were less common among UPEC isolates resistant to 3GCs [27]. In this round of experiments, isolates positive for biofilm-formation were less common among 3GC and SXT non-susceptible strains, while no such association was noted for the formation of mucoid colonies. MDR-levels and resistance rates for many individual antibiotics were higher among invasive isolates. Based on recent studies by Magyar et al. and Gajdács et al., *E. coli* represented the most common causative agent in both community-associated and nosocomial UTIs, being the causative agent in 42–56% (between 2005–2014) and 46–66% (between 2008–2017) of cases, respectively [30, 31]. Resistance rates reported by these studies to NIT, FOS, GEN, SXT, CIP and 3GCs were <2% and 1–9%, 0–5% and ~9%, <7% and 3–8%, 19–31% and 20–28%, 19–25% and 13–26%, and 8% and 8–10%, respectively [30, 31]. On the other hand, resistance-levels in invasive *E. coli* isolates (based on EARS-Net Surveillance Data for Hungary for 2017) were ~20% for 3GCs, ~30% for fluoroquinolones and ~17% for aminoglycosides [32].

The inverse relationship of 3GC-resistance and biofilm-production was also highlighted by Lajhar et al., when testing *E. coli* O26 isolates: while resistance to other antimicrobials had no effect on biofilm-formations, positivity was less common in extended-spectrum β-lactamase (ESBL)-producers [33]. Dumaru et al. studied both members for the Enterobacterales order and non-fermenting Gram-negative bacteria for this purpose: in their report, ~63% of isolates were positive for biofilm-production overall, and a strong association was seen between biofilm-positivity, MDR status, and production of carbapenemase enzymes, while no such association was seen for the production of ESBLs [34]. Similarly, various Gram-negative bacteria were included in the study of Cepas et al., where resistance biofilm-positivity was more commonly seen in isolates resistant to aminoglycosides and 3GCs, while no overall association was proven with the MDR phenotype for *E. coli* [35]. Soto et al. compared the virulence and biofilm-forming capacity of *E. coli* originating from infections of various portions of the genito-urinary tract (i.e. cystitis, pyelonephritis and prostatitis): in their study, prostatitis-associated isolates expressed biofilm and virulence-factors more frequently, while biofilm-positivity was less common in fluoroquinolone-resistant strains [36]. In a similar study to ours, involving $n = 208$ UPEC strains, Neupane et al. concluded that ESBL-positive strains were more common among biofilm-producers [37]. In a Nepalese study by Raya et al., over a thousand clean-catch urine samples were processed from 182 diabetic and 917 non-diabetic patients, respectively: UTIs were more common among diabetic patients (17.4% vs. 42.9%); in addition, biofilm-production was more commonly seen in association with SXT, 3GCs and CIP resistance, and the MDR phenotype [38]. Whelan et al. have also assessed the possible predictive power of colony morphology in relation to biofilm-formation in *E. coli*; however, in their setting, colony presentation on Cysteine Lactose Electrolyte Deficient (CLED) agar had a very low (4%) predictive power, when $n = 50$ strong biofilm-producing strains were assessed [39]. Demonstrating the relevance of this research area, Zhao et al. have recently published a systematic review and meta-analysis regarding the available evidence on the correlation between biofilm formation, virulence factor-expression and antibiotic resistance in UPEC isolates [40]. Their paper reported a pooled rate of 84.6% for biofilm-positivity (out of which, 44.6%, 24.8% and 26.1% were weak, moderate and strong biofilm-producers, respectively), in addition, most of the papers included in the review highlighted a possible association between bacterial resistance, biofilm-positivity and virulence factor-expression in urinary *E. coli* [40]. Even though a growing number of studies are exploring this field with respect to all relevant "ESKAPE" bacteria, no overarching conclusions may be drawn so far on this topic, based on the available data at present time. Among these published reports, a pronounced heterogeneity may be found, when it comes to the origin of the bacterial isolates, their clonality and antimicrobial susceptibility rates, in addition to the methodologies utilized to ascertain biofilm-formation rate (tube or microtiter-plate based assays

with staining, specific culture media [e.g., Congo Red agar], spectrophotometric assays, electron microscopy or flow chambers, complemented with polymerase chain reaction (PCR) assessing the presence and expression-levels of biofilm-associated and virulence genes, and genetic relatedness of the isolates [41–44]. Our study provides additional data, aiming to provide more clarity and evidence to this field. In the future, the meticulous planning of mechanistic studies on biofilm-formation in MDR ESKAPE bacteria of importance is warranted.

E. coli is an important colonizer in the human and animal gastrointestinal tracts – acting as an equalizer in the gut microbiota – providing nutrients and vitamins to the host as a synergistic act, in addition to inhibiting the growth of other, potentially pathogenic bacteria [45]. Nevertheless, it is also one of the most frequently encountered microorganisms (both among the members of the Enterobacterales order and among „ESKAPE" bacteria) in clinical samples sent to diagnostic microbiology laboratories [46]. While *E. coli* is not an obligate pathogen *per se,* due to its relatively large genome (4.5–5.5 Mb) and high genomic plasticity, strains possessing an appropriate combination of virulence determinants (i.e. adhesins, pili, toxins, iron acquision systems and biofilm) may prove to be successful in their survival *in vivo* [47]; in fact, virulence factors of *E. coli* are more commonly encoded on genomic pathogenicity islands (PAIs; e.g., PAI IIJ96, PAI IJ96, PAI IICFT073, PAI I536, PAI II536, PAI III536, PAI IV536, PAI ICFT073), representing large segments of the bacterial chromosome, often characterized by high cytosine and guanine content [48]. As a matter of fact, based on their disease-causing capacity, *E. coli* strains are often differentiated into various pathotypes, including extra-intestinal pathogenic *E. coli* (ExPEC; including uropathogenic strains [UPEC] and strains responsible for meningitis and sepsis [MNEC]) and various pathotypes causing gastrointestinal ailments, like enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [49, 50]. On the other hand – based on the growing number of genomic data available for this species – based on the phylogeny of *E. coli* sensu stricto, eight phylo-groups (A, B1, B2, C, D, E, F and a cyptic clade I) may be distinguished, based on the new Clermont phylogenetic grouping method [51].

Since the recognition of the clinical role biofilms have in the outcome of infectious processes, biofilm-forming capacity has been a research topic garnering significant interest [52]. Biofilms facilitate a protective growth strategy in bacteria, ensuring safety against environmental stressors (sheer forces, heat and drying damage), components of the immune system (phagocytes, complement) and noxious chemical agents (disinfectants and antimicrobials) [53, 54]. The EPS matrix alters the the pharmacokinetic properties of the anatomical niche and the speed by which antibiotics may penetrate to reach the target pathogens [55]. Although species-wise, a principal secreted component in the biofilm-matrix may be identified, even the composition of monospecies biofilms are mostly heterogenous (owing physiological heterogeneity in adaptation and genetic variability), while multi-community biofilms are even more complex [56]. In *E. coli* biofilm, colanic acid (which is a polymer of glucose, galactose, fucose, and glucuronic acid) is a relevant component of mature biofilms, providing protection against temperature and osmotic changes due to its negative surface charge, while cellulose is important for the structural integrity of these biofilms [57, 58]. On the other hand, biofilms produced by *Pseudomonas* spp. have high alignate content, which provides protection from mechanical damage and biocides (e.g., disinfectants), while for *Acinetobacter* spp. and *Burkholderia cepacia* complex, the poly-β-1,6-N-acetylglucosamine (PNAG) polysaccharide is the critical component for the stability of the biofilm produced [59–62]. Bacteria embedded in the deep layers of biofilm are often metabolically inactive (dormant), which may be a result of low oxygen-density and transcriptional changes to adapt to the nutrient-scarce community-based lifestyle. This state of dormancy also poses and important hurdle for therapy, as many drugs (especially bactericidal agents) require the active division of bacterial cells to be effective [63]. As *E. coli* is one of the most common nosocomial pathogen (especially in catheter-associated UTIs, corresponding to >50% of cases), biofilm-production is of critical importance for its persistence, pathogenicity and survival [64, 65]. The origin of many nosocomial infections is associated with the use of implanted devices and biomaterials, including catheters (urinary, central venous), heart valves, dental, hip or cochlear implants, cerebrospinal fluid shunts or even contact lenses, as contaminant bacteria may rapidly colonize these inanimate surfaces and lead to infections *in vivo* [66, 67]. Biofilm-formation is one of the critical factors in the development of chronic infections, especially ones associated with urinary or intravascular catheters and implanted devices [68].

Irrespective of the disease-presentation, the first key step in the pathogenesis of *E. coli* infections is the adherence to mucosal surfaces; subsequently, aggregation of bacteria inside the biofilm protects them from sheer forces and the onslaught of immune cells [52, 69, 70]. The relevant virulence-determinants of *E. coli* include adhesins (representative genes e.g., *afa*, *CSH*, *fimH*, *fimP*, *pap, sfa, traT*), toxins (cytotoxic necrotizing factor: *Cnf1*, haemolysin: *hlyA*, others: *saT*, *vaT*), suppressors of the immune system (*shiA*, *sisA*, *sisB*, *sivH*) and siderophores (aerobactin: *aer*, salmochelin: *iro*, enterobactin: *ent*, yersiniabactin: *irp*, others: *iuD, iutA, yfcv*), which all contribute to their survival and invasiveness in the infected host [71–74]. Surface factors, such as various adhesins have a pivotal role in mediating the adhesion of *E. coli* to biotic and abiotic surfaces, and the subsequent production of EPS. These include flagella, type 1 fimbriae (encoded by the *fim* operon), P fimbriae (encoded by the *papA-K* operon), S fimbriae and F1C fimbriae (encoded by the *sfa/focDE* genes), curli fimbriae (mediated by the *crl* and *csgA* genes), afimbrial adhesins (encoded by the *afa* and *tos* genes), conjugative (F) pili and cell surface hydrophobicity (mediated by the *CSH*) [75–78]. So-called fimbria-associated

regulators (responsible for the interplay between the *pap*, *foc*, and *fim* operons) have an important role in synchronizing the expression of the various adhesins, depending on the external conditions present [79]. Type 1 fimbriae are critical in the initial step of colonization, through mediating the primary adhesion of the bacteria to the surface, this adhesin has proven to be relevant in the resistance to numerous hydrodynamic conditions in UPEC, including the intravesical and intraluminal parts of the urinary catheter [80]. P, S, F1C fimbria and afimbrial adhesins serve by strengthening the adhesion among the bacteria and the surface; in the subsequent steps mediated by curli fimbriae, aggregation of more bacteria, and production of EPS may take place [81, 82]. Many experimental studies have shown that the presence of genes encoding for adhesins, and their increased expression-levels were associated with strong biofilm-formation in Gram-negative bacteria, which may mirror the processes taking place in the initial stages of biofilm-formation [83]. In the paper by Bunduki et al., a systematic review and meta-analysis was performed from the studies assessing the relationship between virulence genes and biofilm-formation in UPEC, which included nine studies utilizing molecular methods: among adhesins, CSH (80%), fimH (75.3%), fimP (35.6%) and pap (30.2%) were the most common, among immune suppressors shiA (92.1%), sisA (72.2%) and sisB (24.7%) were the representatives, while among secreted factors and siderophores, aerobactin (52.4%), haemolysin (22.1%), and the cytotoxic necrotizing factor-1 (13.3% were the most relevant. Overall, this study also concluded that the presence of virulence factors (especially adhesins) positively correlated with biofilm-formation [84]. Similar findings were published by Selasi et al. for *A. baumannii*, where biofilm-formation showed positive correlation with the expression levels of a number of relevant virulence factors (pili, surface proteins) [85].

Another significant factor in the mediation of bacterial physiology and virulence factor-expression is bacterial cell-cell communication or quorum-sensing [QS], which – in the case of *E. coli* and many other members of Enterobacterales – is mediated by the binding of acyl-homoserine lactones (AHL) and autoinducer-2 (AI-2) directly to transcription factors of relevant genes, to modulate their expression [86, 87]. As QS-mediated changes often occur due to reaching a threshold concentration of these signal molecules (which is a proxy for the population density in the given ecological niche), it is not surprising that biofilm-formation and QS-based mechanisms observe a close interdependence, given that the production of EPS is beneficial for the entire population of the bacteria present [88]. Many authors provided hypotheses on the genetic or biochemical link between biofilm-formation and drug resistance. The perturbation of QS-signaling (either by the degradation of signal molecules or by inducing differential gene expression patterns) offers a possible link, and may explain why high-dose antimicrobials may limit biofilm-formation in microorganisms still in the planktonic phase [89]; on the other hand, many studies have observed that non-lethal concentrations of antibiotics induced biofilm-production in various Gram-positive and Gram-negative bacteria, which may be due to the activation of global stress (SOS) responses, leading to the expression of genes with protective functions against external noxa [90]. In *E. coli*, many of the important virulence factors are found on the PAIs, which may easily be removed from the chromosome, leading to isolates with less potential to cause invasive infections [91]. This may occur through an exposure to fluoroquinolone (FQ) antibiotics – which increases the chance of deletions and transposition of DNA in the chromosome – consequently leading to FQ-resistant strains, with reduced potential to cause invasive infection in the urinary tract [92]. Other authors suggested another explanation, with regards to biofilm-formation and the presence of specific β-lactamases: Aziz et al. studied *A. baumannii* carrying the the extended spectrum β-lactamase $bla_{\text{PER-1}}$, and found that these isolates produced a more robust biofilm, compared to non-carriers. They have proven *in vitro*, that $bla_{\text{PER-1}}$-positive isolates adhered to epithelial cells more efficiently, which is a prerequisite for the early stages of biofilm-production [93]. In contrast, in a study involving *P. aeruginosa* isolates, Gallant et al. noted an opposite trend, where isolates carrying the $bla_{\text{TEM-1}}$ β-lactamase had low potential to form biofilm (compared to non-carriers), which was then attributed to the low adhesive potential of these isolates [94]. The interaction between cell surface proteins and biofilm-formation was further verified by Zeighami et al. for *Acinetobacter* spp., where they found that isolates with a deficient biofilm associated protein (Bap; with important roles in the first stages of attachment and aggregation) had lower capacity to form biofilm; it is worth noting that under physiological conditions, Bap interacts with the major porin (OmpA) of *Acinetobacter* spp., therefore porin-deficient mutants (which may show phenotypic resistance to a variety of antibiotics) will be less efficient EPS producers [95].

CONCLUSIONS

Biofilm-forming pathogens are an important clinical concern, as they are an important cause of recalcitrant, difficult-to-treat infections, which often affect hospitalized, co-morbid patients. The removal of catheters and other implanted devices may be an important aspect of managing biofilm-associated infections, these interventions are often invasive, and decrease the quality of life in the affected patients. On the other hand, while there have been progress in the development of synthetic compounds affecting the metabolic processes of bacteria or the structural integrity of EPS, there are currently no licensed agents available to specifically target biofilms. As of now, the association between drug resistance and biofilm-forming capacity is still a matter of debate, which may also be influenced by the origin and phylogroup distribution of the isolates. More research is required to allow for the full understanding of the interplay between the MDR phenotype and biofilm-production, which will facilitate the development of novel therapeutic strategies for ESKAPE pathogens.

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