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ORIGINAL RESEARCH

Escherichia coli from Human Wounds: Analysis of Resistance to β-Lactams and Expression of RND Efflux Pumps

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Purpose: Resistance of pathogenic strains of *Escherichia coli* to β-lactams, particularly to ampicillin, is on the rise and it is attributed to intrinsic and acquired mechanisms. One important factor contributing to resistance, together with primarily resistance mechanisms, is a mutation and/or an over-expression of the intrinsic efflux pumps in the resistance-nodulation-division (RND) superfamily. Among these efflux pumps, AcrA, AcrB, TolC, and AcrD play an important role in antimicrobial co-resistance, including resistance to β-lactams.

Materials and Methods: Twelve *E. coli* isolates obtained from patients' wounds and the control strain of *E. coli* ATCC 25922 were analyzed. The phenotypic resistance of these isolates to selected β-lactams was assessed by determination of the minimal inhibitory concentration. Additionally, the prevalence of β-lactamase genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}, and bla_{AmpC}) was screened by PCR. Real-time qPCR was used to determine the expression of the selected efflux pumps *acrA, acrB, tolC*, and *acrD* and the repressor *acrR* after the exposure of *E. coli* to ampicillin.

Results: Phenotypic resistance to β-lactams was detected in seven isolates, mainly to ampicillin and piperacillin. This was corroborated by the presence of at least one acquired *bla* gene in each of these isolates. Although *E. coli* strains varied in the expression of RND-family efflux pumps after the ampicillin exposure, their gene expression indicated that these pumps did not play a major role in the phenotypic resistance to ampicillin.

Conclusion: Each *E. coli* isolate displayed unique characteristics, differing in minimum inhibitory concentration (MIC) values, prevalence of acquired *blaTEM* and *blaCTX-M* genes, and expression of the RND-family pumps. This together demonstrates that these clinical isolates employed distinct intrinsic or acquired resistance pathways for their defense against ampicillin. The prevalence and spread of ampicillin resistant *E. coli* has to be monitored and the search for ampicillin alternatives is needed.

Keywords: clinical isolates, ampicillin resistance, resistance mechanisms, antibiotics

Introduction

The emergence of resistant pathogenic bacterial strains is a significant concern worldwide. Antibiotics in the β-lactams family are among the most commonly prescribed drugs with many clinical FDA (Food and Drug Administration)-approved indications and off-label clinical uses.[1] They have highly reactive β-lactam ring inhibiting penicillin-binding proteins responsible for the cell wall synthesis.[2] Among these, ampicillin (AMP) is commonly used as a drug of the first choice for treatments of respiratory, gastrointestinal, and urinary tract infections as well as meningitis caused by *Escherichia coli, Proteus mirabilis, Salmonella species*, enterococci, staphylococci, and other bacterial taxa. It is also used as prophylaxis in neonatal group B *streptococcal* infections and in surgeries.[3,4] However, an extensive use of AMP has led to the development of bacterial resistance in many bacteria, including *E. coli*,[5] resulting in higher antibiotic dosages for an effective treatment.[6]

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Mechanisms of resistance to β-lactams, including ampicillin, are numerous and complex. Resistance to β-lactams is primarily mediated by β-lactamases that hydrolyze the β-lactam ring in the periplasmic space, rendering the antibiotic inactive.[7] This effect can be achieved through the production of an intrinsic cephalosporinase AmpC, encoded by the bla_{AmpC} gene and activated by a variety of β-lactams.[8] In addition to the intrinsic resistance, many acquired β-lactamases have been described. The most common plasmid-mediated β-lactamases in Gram-negative bacteria are TEM and SHV enzymes, encoded by bla_{TEM} and bla_{SHV} with different protein variants, respectively.[2] Moreover, in *E. coli*, CTX-M β-lactamase is also commonly present.[9] Other resistance factors to β-lactams include a mutation of β-lactam targets and overexpression of efflux pumps.[10,11] Efflux pumps play an important role in resistance as they export antibiotics, including β-lactams, out of bacterial cells,[12,13] which leads to multidrug resistance (MDR).[11,14–17] Generally, bacterial MDR efflux pumps are categorized into five families.[18,19] Clinically significant in antibiotic transport in Gram-negative bacteria are efflux pumps from the resistance-nodulation-cell division (RND) superfamily,[20] that includes AcrA, AcrB, TolC, and AcrD.[21] So far, only the pump AcrB has been directly linked to AMP resistance.[22]

This study focused on the phenotypic and genotypic characterization of the clinical isolates of *E. coli* from the infected wounds with emphasis on the resistance to β-lactams. Furthermore, the gene expression of RND family efflux pumps (*acrA, acrB, acrD, tolC*) of *E. coli* under AMP pressure was investigated to elucidate their role in AMP resistance.

Materials and Methods

Sample Collection

Escherichia coli isolates were obtained in 2018–2019 from the long-lasting purulent wounds at different stages and with varying levels of infection severity. The collection was carried out with the agreement of 91 patients attending the Outpatient Department of Chronic Wounds at the Trauma Hospital in Brno, Czech Republic, and approved by the Ethics Committee of the hospital. After the bacterial collection by a sterile swab, swabs were immediately placed in the Amies transport medium with charcoal (Med-Lab trade, Vranovice, Czech Republic) and transferred to a laboratory. From identified isolates, the study focused only on identified *E. coli* isolates.

Cultivation and Identification of Isolates

For bacterial identification, the swabs were streaked on Endo agar (St. Anne´s Hospital, Brno) and incubated at 37°C overnight. After 24 h, morphologically different individual colonies were harvested from each plate and stored in 0.5 mL of 80% glycerol at −80°C. From individual colonies, bacterial strains were identified by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany). The identification was also confirmed by the 16S rRNA gene amplification and sequencing. The total DNA from each bacterial isolate was extracted with the NucleoSpin Microbial DNA kit (Macherey Nagel, Germany). The 16S rRNA gene was amplified by PCR in the 25 µL reaction mixture as follows: 0.5 µL of dNTP, 0.2 µL of *Taq* DNA polymerase (5000 units/mL), 2.5 µL of 10X standard *Taq* reaction buffer (New England Biolabs, Ipswich, MA, USA), 0.5 µL of the forward primer (10 µM) 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.5 µL of the reverse primer (10 µM) 1492R (5'-GGTTACCTTGTTACGACTT-3') [23], 15.8 µL of deionized water and 5.0 µL of the template DNA (10 µg/mL~50 ng). Amplification was done on the Mastercycler nexus (Eppendorf, Germany) with cycling conditions: 90°C for 4 min., 30 cycles of 90°C for 30 sec., 58°C for 30 sec., 68°C for 105 sec., followed by 68°C extension for 10 min. and 4°C hold. For sequencing, the amplified fragments of DNA were purified by the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and sent for sequencing with amplification primers for commercial sequencing service (SeqME, Dobris, Czech Republic). Obtained sequences were assembled and compared against the GenBank database at NCBI.

Antibiotic Susceptibility Testing and Screening for the Resistance Genes

Phenotype

Antibiotic susceptibility testing (AST) was performed using the BD Phoenix™ M50 instrument (Franklin Lakes, NJ, USA). Five concentrations of each antibiotic including the breakpoint were measured. Bacterial samples were prepared according to the manufacturer guidelines. Tested β-lactams and their combinations with β-lactamase inhibitors with concentrations are listed in Table 1.

Table 1 Selected β-Lactams and Their Concentration Range Used for Determination of the Minimal Inhibitory Concentration

Group of ATB	Antibiotics		Concentrations [μg/mL]
β-lactams	Cephalosporins	Cefuroxime	2–8
		Ceftazidime	0.5–8
		Cefotaxime	1–4
		Cefepime	1–8
	Carbapenems	Imipenem	0.25–8
		Ertapenem	0.25–1
		Meropenem	0.125–8
	Penicillins	Ampicillin	2–8
		Amoxicillin-clavulanate (f)	2/2 – 32/2
		Piperacillin	4–16
		Piperacillin-tazobactam	4/4 – 16/4

Phenotype of Ampicillin Susceptibility by Minimum Inhibitory Concentration Determination

All *E. coli* isolates and the negative control strain ATCC 25922 were cultivated overnight on 5% Columbia blood agar (37°C). Overnight cultures were diluted in double concentrated Mueller-Hinton broth to OD_{600} 0.1 AU, 100× dilution was used for MIC determination using serial dilution of ampicillin in the 96-well plates. Plates were incubated for 24 hrs at 37°C and with shaking. The final absorbance was measured (λ = 600 nm) by the MultiScan EX Microplate Photometer (Thermo Fisher, Germany). Minimum inhibitory concentration (MIC) was measured in technical duplicates.

Genotype

Preparation of the Lysates

Isolates of *E. coli* were grown on 5% Columbia blood agar overnight at 37°C. Five bacterial colonies of each isolate were placed in 200 μL of sterile water (LC-MS grade, LiChrosolv®, Sigma-Aldrich), re-suspended and vortexed. Bacterial solution was incubated for 10 min at 100°C and then immediately put on ice for 5 min to cause heat shock. The lysate was centrifuged (35,000 rcf, 2 min, 4°C) and supernatant containing DNA was stored at −20°C until further use.

Screening for Resistance Genes

Based on the phenotype determined by the BD Phoenix™ M50 instrument, four *bla* genes (Table 2), together with *acrA, acrB, tolC, acrD* and *acrR* were selected for PCR identification. The primers for *acrD* and *acrR* were designed based on the sequence of *E. coli* strain K-12 (NC_000913.3) using the PrimerQuest online tool of Integrated DNA Technologies (www.eu.idtdna.com) (Table 3). *Escherichia coli* ATCC 25922 was used as a negative control and *E. coli* ATCC 35218 was used as a positive control. 10 μL of the Hot Start *Taq* 2× Master Mix (New England BioLabs) was mixed with 0.5 μL of each primer (10 μM), 3 μL of bacterial lysate and filled up to 20 μL with sterile water. The following conditions were used for

Table 2 Primers and the Expected Size of PCR Products for Detection of Genes Encoding β-Lactamases

Gene	Primer Sequences	Amplicon Size (bp)	References
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGTGCCATA	544	[23]
bla_{SHV}	F: TCAGCGAAAAACACCTTG R: TCCCGCAGATAAATCACC	472	[24]
bla_{TEM}	F: TACGATACGGGAGGGCTTAC R: TTCCTGTTTTTGCTCACCCA	716	[25]
bla_{AmpC}	F: CCCCGCTTATAGAGCAACAA R: TCAATGGTCGACTTCACACC	631	[26]

Table 3 Primers and the Expected Size of PCR Products of Genes Encoding the Efflux Pumps and the Repressor from RND Family Superfamily and the Reference Gene *rpsL*

Genes of Interest	Primer Sequences	Amplicon Size (bp)	References
acrA	F: CTCTCAGGCAGCTTAGCCCTAA R: GCAGAGGTTCAGTTTTGACTGTT	106	[27]
acrB	F: GGTCGATTCCGTTCTCCGTTA R: CTACCTGGAAGTAAACGTCATTGGT	105	[27]
acrD	F: CACTGCTCTGAGACGACATATAC R: CTCATTGCCCGTTGAACAATAC	167	This study
acrR	F: CTGGGAACTGTCAGAATCCAATA R: TAATCGACGCCGTTCTTCTG	139	This study
tolC	F: AAGCCGAAAAACGCAACCT R: CAGAGTCGGTAAGTGACCATC	101	[27]
rpsL	F: GCAAAAACGTGGCGTATGTACTC R: TTCGAAACCGTTAGTCAGACGAA	103	[27]

amplification: 95°C for 3 min followed by 30 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 60 sec with final extension 72°C for 5 min. PCR products were analyzed on 1% agarose gel, stained with MIDORI^{GREEN} (0.06 μg/mL) and visualized by the Azure Biosystems C600 Multifunction Imaging System (Azure Biosystems, USA) under the UV light.

Expression of RND Efflux Pumps After Ampicillin Treatment

Escherichia coli was cultivated overnight on 5% Columbia blood agar at 37°C. Based on the ampicillin MIC results, one concentration of ampicillin (1.875 μg/mL) was selected to treat all isolates. The next day, three colonies from each isolate (biological triplicate) were put in Luria-Bertani broth adjusted to pH 7 with and without ampicillin in 15 mL sterile falcon tubes and incubated at 37°C for 16 h with shaking. Bacteria in the stationary phase were harvested and washed once with phosphate-buffered saline (6440 rcf, 10 min, 4°C).

RNA was isolated by the TRIzol Reagent® according to manufacturer instructions (TRIzol Reagent, Invitrogen, Carlsbad, CA) with the modification of the lysis step after homogenization, when the samples were incubated at −20°C for 10 min. After the isolation, RNA was purified by the ethanol precipitation protocol according to manufacturer instructions[28] with modification of incubation time and temperature. Instead of −20°C for overnight, we used −80°C for one hour and the remaining DNA was removed by the TURBO DNA-*free*™ Kit (Ambion®, Life Technologies Europe BV) using 5.0 μg of the total RNA. The concentration and purity of RNA was determined by the NanoDrop™ OneC Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). RNA integrity was verified by gel electrophoresis in 2% agarose gel (90V/30min) supplemented with 1% of bleach and stained by MIDORI^{GREEN} (0.06 μg/mL). The gel was visualized by Azure c600 from Azure Biosystems (Dublin, California, USA). The RNA samples were aliquoted and stored in −80°C until further analyses.

cDNA of bacterial isolates was prepared by the first strand cDNA synthesis kit for RT-PCR (Roche, Germany, Mannheim), using random hexamers as primers, according to manufacturer instructions. Five hundred nanograms of RNA after the TURBO DNase treatment were used as a template for reverse transcription. Prepared cDNA (20 μL) was diluted in UltraPure™ Dnase/Rnase-Free Distilled Water to a total volume 200 μL.

All bacterial isolates were tested for the presence of MDR efflux pumps genes *acrA, acrB, acrD, tolC*, repressor *acrR* and the reference gene *rpsL* (Table 3) by PCR. If the genes were detected in *E. coli* isolates, transcriptional expression of these pumps by real-time qPCR was determined. Analysis was performed using the qTOWER^3 system (Analytik Jena, Jena, Germany). For each reaction, 10 μL of diluted reverse-transcribed cDNA (25 ng/rxn) was subjected to PCR amplification in a 20 μL final volume containing 9 μL of Luna® Universal qPCR Master Mix (New England Biolabs) and 0.5 μL (10 μM) of each primer. The following conditions were used for amplification: 95°C for 3 min, 30 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec with final extension at 72°C for 2 min. To ensure the specificity of the amplified PCR products, a melting curve analysis was performed.

The relative quantities of mRNA of each gene of interest were calculated by ΔΔCT method. First of all, the expression of each gene of interest (GOI) was normalized to the reference gene (REF) *rpsL* ($\Delta Ct = Ct_{REF} - Ct_{GOI}$), then the differences in relative gene expression between treated and untreated isolates were calculated ($\Delta\Delta Ct = \Delta Ct_{Treated} - \Delta Ct_{Untreated}$). Results were presented as mean of ΔΔCt (~Log2 Fold Change) from three biological replicates and ± SEM calculated by GraphPad Prism 8.0.1. (GraphPad Software, CA, USA). This program was also used to perform an unpaired *t*-test between untreated and treated isolate from ΔCt values of biological triplicate to determine the significance ($p < 0.05$) of the obtained differences.[27]

Results

Isolates Quantification

A total of 219 bacterial isolates were collected from 91 clinical patients with different types of infected wounds. In this study, we specifically focused on up to four *E. coli* isolates from each patient (twelve in total) (Table S1).

Phenotypic Determination of *E. coli* Resistance to β-Lactams

The MIC of all tested β-lactams and their combination with inhibitors is shown in Table S2. Four out of twelve isolates were susceptible to all tested β-lactams. One isolate (38B1a) showed resistance only to ertapenem and remaining seven isolates were resistant to two or more of tested β-lactams (Table S2). From all *E. coli* isolates, 58.3% showed resistance to ampicillin and piperacillin respectively, and 33.3% to amoxicillin-clavulanate (Table 4). Based on these findings, we focused on ampicillin resistance exclusively. In this study, the MIC of ampicillin was assessed for all isolates, including the control strain *E. coli* ATCC 25922. The categorization of isolates as either sensitive or resistant to AMP was based on the MIC values according to the EUCAST guidelines for the year 2022[29] (Table 4). EUCAST considers an MIC value above 8.0 μg/mL as indicative of resistance to AMP.[23–26]

Genotypic Determination of *bla* Genes

Bacterial isolates were examined for the presence of four *bla* genes. *Escherichia coli* isolates sensitive to AMP did not carry any acquired *bla* genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}) and only the intrinsic bla_{AmpC} gene was detected. In contrast, in strains resistant to AMP, in addition to $bla_{AmpC,}$ at least one acquired *bla* gene was found. The bla_{TEM} gene was present in 6 out 7 strains and the strain without bla_{TEM} and one more isolate were positive for bla_{CTX-M} (Table 5).

Table 4 Classification of *E. coli* Isolates and the Control Strain ATCC 25922 Based on the Minimal Inhibitory Concentration (MIC) to Ampicillin (AMP). The BD Phoenix System Classification of Isolates as Susceptible (S) and Resistant (R) to Ampicillin (AMP), Piperacillin (PIP) and Amoxicillin-Clavulanic Acid (AMC)

Classification	*E. coli* Isolates	MIC [μg/mL] on AMP	Resistance from BD Phoenix		
			AMP	PIP	AMC
Sensitive to AMP	ATCC 25922	7.5	S	S	S
	8B1	3.75	S	S	S
	8B2	3.75	S	S	S
	8C1	3.75	S	S	S
	38B1 a	3.75	S	S	S
	48C1	7.5	S	S	S
Resistant to AMP	13B1	>1000	R	R	R
	60C1	>500	R	R	R
	8C2	500	R	R	S
	37B1	>500	R	R	S
	45B1	>500	R	R	S
	63C1	>500	R	R	R
	70C2	>500	R	R	R

Table 5 Prevalence of β-Lactam Resistance Genes in *E. coli* Isolates

Classification	*E. coli* Isolates	β-Lactamase Genes			
		bla_{TEM}	bla_{CTX-M}	bla_{SHV}	bla_{AmpC}
Sensitive to AMP	ATCC 25922	-	-	-	+
	8B1	-	-	-	+
	8B2	-	-	-	+
	8C1	-	-	-	+
	38B1 a	-	-	-	+
	48C1	-	-	-	+
Resistant to AMP	13B1	+	+	-	+
	60C1	-	+	-	+
	8C2	+	-	-	+
	37B1	+	-	-	+
	45B1	+	-	-	+
	63C1	+	-	-	+
	70C2	+	-	-	+

Notes: abbreviation "+" means a presence of the tested gene and "-" means an absence of the tested gene.

Effect of Ampicillin on Expression of RND-Family Efflux Pumps Genes in *E. coli*

Before conducting the expression analyses, the presence of MDR efflux pump genes (*acrA, acrB, acrD, tolC*), as well as the *acrR* gene coding for the transcriptional regulatory protein, was confirmed in all isolates using PCR.

Tested *E. coli* differed in the expression of tested genes after ampicillin exposure (Figure 1). Among susceptible strains with no acquired *bla* genes, a significant up-regulation of *acrB* was found in the control strain (2.1× higher) and the isolate 48C1, where the expression level was 5.6× higher. In contrast, a significant down-regulation of *acrA* (2.8× decreased in 8B1 and 3.7× decreased for 8C1) and *acrD* (15.9× decreased for 8B1 and 18.2× decreased for 8C1) was detected in isolates 8B1 and 8C1. In the control strain, down-regulation of *acrD* (15.3× decreased) and *acrR* (4.6× decreased) was found as well and no significant changes in the expression of these genes were observed in all other susceptible isolates (Figure 1). In resistant isolates, the significant changes in expression were seen in 13B1, 37B1, 63C1 and 70C2 isolates. Among them, 13B1 and 37B1 isolates with the bla_{TEM} gene, had up-regulated *acrA*. A significant down-regulation of *acrB* (2.7× decreased for 63C1 and 3.4× decreased for 70C2) was observed in 70C2 and 63C1 isolates, containing the bla_{TEM} gene. Furthermore, in the isolate 70C2, the *acrD* gene was also down-regulated (20.7× decreased). No significant changes in expression of *tolC* were detected in any of our isolates (Figure 1).

Discussion

Resistance of clinical isolates of *E. coli* to β-lactams, especially to ampicillin, is on a rise.[30–34] About twenty years ago, the prevalence of ampicillin resistance in the human clinical isolates was only around 8.2%.[35,36] Even when the resistance occurred, the MIC was slightly above the FDA breakpoint (>8 μg/mL) only, with rare exceptions, when the MIC to ampicillin reached >236 μg/mL.[37,38] Nowadays the ampicillin resistance in *E. coli* isolates from human infections has increased to nearly 85% with MIC values commonly exceeding >250 μg/mL.[39–44] In our study, MIC values of *E. coli* resistant isolates have been even much higher and exceeded 500 μg/mL.[5,41,42] The staggering ampicillin MIC is likely due to combination of several β-lactam resistance mechanisms.[5,41–43]

Resistance to β-lactams is primarily mediated by β-lactamases.[7] Presence of the AmpC cephalosporinase in all *E. coli* results in resistance to most penicillins.[44] In the presence of extended spectrum of β-lactamases (ESBLs) from the acquired plasmids, the expression of bla_{AmpC} gene is either poorly expressed or not expressed at all.[8] In our study, both susceptible and resistant *E. coli* strains carried the bla_{AmpC} gene. Within susceptible isolates, this gene ostensibly contributes to a marginally elevated ampicillin MIC that does not surpass the FDA breakpoint.[8] Conversely, in resistant strains featuring ESBLs, the presence of this gene appears to have no influence on the heightened MIC against ampicillin. Notably, the elevated MIC levels towards ampicillin are likely attributed to the presence of acquired *bla* genes.

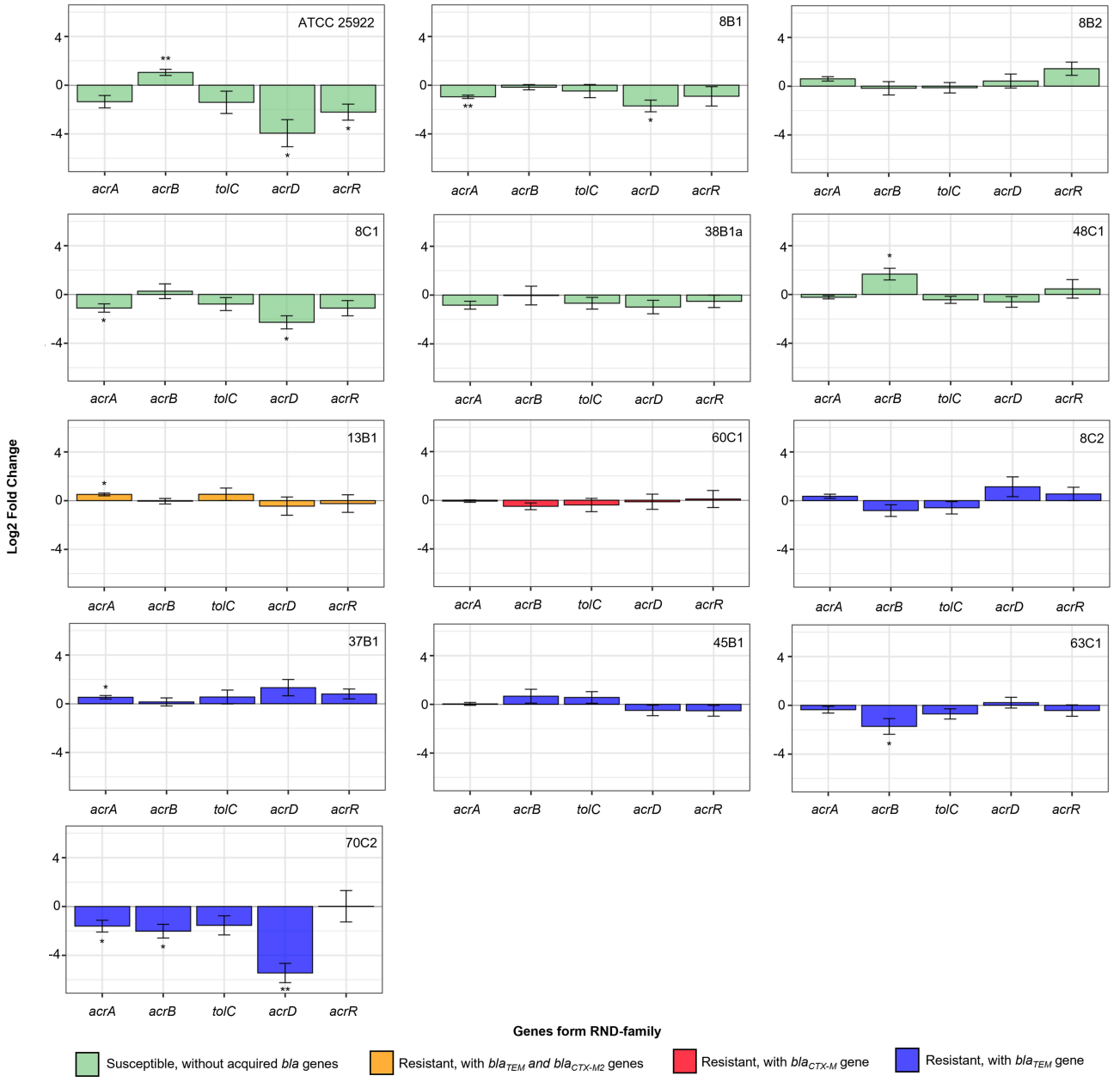

Figure 1 Expression of tested genes *acrA, acrB, tolC, acrD* and *acrR* from RND family in *E. coli* isolates: ATCC 25922, 8B1, 8B2, 8C1, 38B1a, 48C1, 13B1, 60C1, 8C2, 37B1, 45B1, 63C1, 70C2 after AMP treatment normalized to gene *rpsL*. Untreated and treated isolate after AMP exposure were compared and significance was calculated by unpaired *t*-test from ΔCt values. The significance is represented as stars according to the p-value (* *p*-value ≤ 0.05, ** *p*-value ≤ 0.01) above or below the column. Green coloured columns represent susceptible isolates, Orange isolates containing two acquired *bla* genes, blue and red isolates containing only one type of *bla* gene.

Noteworthy, among the β-lactamases in *E. coli* are TEM, CTX-M, and SHV, with the lion's share of ampicillin resistance being attributed to TEM.[45] While CTX-M and SHV β-lactamases have been linked to ampicillin resistance in *Klebsiella pneumoniae* clinical isolates,[46–48] their link to ampicillin resistance of *E. coli* has not been established.[49,50] In our study, as in other clinical studies, bla_{TEM} was found to be the most common gene encoding β-lactamases followed by $bla_{\text{CTX-M}}$ and bla_{SHV}.[5,51–54] In the presence of both bla_{TEM} and $bla_{\text{CTX-M}}$, the MIC for ampicillin in *E. coli* clinical isolates was in range 32.0 to 256.0 μg/mL.[54,55] Comparing to the upper MIC levels in the previous studies, in our study, *E. coli* isolates with bla_{TEM} or $bla_{\text{CTX-M}}$ had the MIC for ampicillin four times higher (>1000 μg/mL), and two times higher (>500 μg/mL). Overall, ESBLs aside, *E. coli* probably employs a variety of resistance mechanisms against β-lactams, which are needed to elevate the MIC for ampicillin.

Escherichia coli commonly employs a diverse set of efflux pumps from the RND-superfamily resulting in resistance to antibiotics. Among these pumps, only the AcrB has been directly linked to ampicillin resistance.[22] Additionally, the AcrA pump, working together with AcrB, is responsible for intrinsic resistance to fluoroquinolones and aminoglycosides.[27] Both their genes, *acrA* and *acrB*, are located on the same operon, therefore their expression levels should correlate[56] as confirmed in several studies;[27,57] however, other studies reported an opposite regulation.[57–59] Both, the same and opposite, expressions were found in our *E. coli* isolates after ampicillin treatment as well. Expression of *acrA* and *acrB* genes is influenced by the repressor protein AcrR,[60] thus the overexpression of *acrAB* would correlate with down-regulation of the *acrR* gene. In our study, this trend was seen in the control strain ATCC 25922. In other isolates, the expression of *acrR* remained unchanged, even when *acrA* or *acrB* showed different expressions. This might be due to a mutation in the *acrR* gene or the involvement of other *acrAB* repressors with higher affinity to *acrAB* protomor, such as AcrS. Furthermore, the binding affinity of AcrR in the *acrAB* promotor is lower than that of AcrS.[61] The pump AcrA and AcrB can assemble with other pumps from the RND-family and create a tripartite system spanning both the inner and the outer membrane, leading to multidrug resistance.[62,63] While the primary tripartite system is composed of AcrAB and the TolC pump, when the AcrD pump is overexpressed, it can replace AcrB and this newly formed system can confer resistance to antibiotics as well.[64,65] Significant down-regulation for the *acrD* gene was observed in several *E. coli* isolates after ampicillin treatment but no significant changes were detected in *tolC*. Based on these results, it is likely that the assembly of the tripartite system does not take place under ampicillin pressure, as the AcrD pump is primarily linked to aminoglycosides resistance and may not be necessary for survival in the presence of ampicillin. Resistance to ampicillin in *E. coli* is commonly caused by a combination of several resistance mechanisms. In our study, we primarily detected the presence of ESBLs genes. The up-regulation of the *acrB* gene was only observed in two out of six sensitive *E. coli* strains, suggesting it may have some protective effect against ampicillin. Overall, the resistance mechanisms in clinical isolates of *E. coli* to ampicillin are complex and numerous and further studies analysing more isolates are needed to better understand all mechanisms *E. coli* employs for protection to ampicillin and other β-lactams.

Conclusions

This pilot study aimed to investigate ampicillin resistance and its mechanisms in *E. coli* isolates from human wounds. Phenotype and genotype screening for β-lactam resistance genes and the expression of efflux pumps from the RND-family under ampicillin stress were performed. Remarkably, each *E. coli* isolate displayed unique characteristics, differing in the MIC values to ampicillin as well as in the prevalence of acquired bla_{TEM} and $bla_{\text{CTX-M}}$ genes and in the expression level of the RND-family pumps. This diversity indicates that these clinical isolates employed distinct intrinsic or acquired resistance pathways for their defence against ampicillin. The prevalence and spread of ampicillin resistance in clinical as well as non-clinical isolates should therefore be monitored and new antimicrobial compounds developed to replace ampicillin in the future.

Abbreviations

AMP, ampicillin; MDR, multi-drug resistance; MIC, minimum inhibitory concentration; RND, resistance-nodulation-division.

Ethics Statement

The study was approved by the Ethics Committee of the Trauma Hospital in Brno. The relevant guidelines and regulations provided in the Declaration of Helsinki were followed for handling human samples. Informed consent was waived by the Ethics Committee of the Trauma Hospital in Brno since this study did not involve any interventions. Patients were treated anonymously.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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