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Research article

Exploring anti-quorum sensing potential and biofilm inhibitory effect of azithromycin against urinary *Escherichia coli* isolated from a teaching hospital in Alexandria

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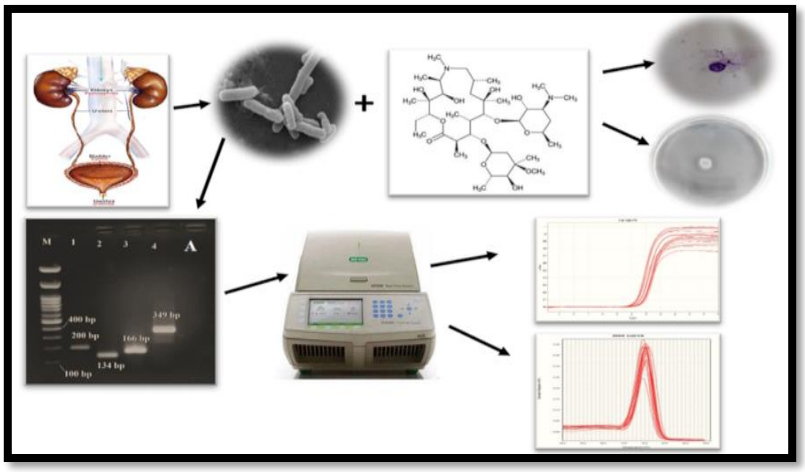
Abstract:



Urinary tract infections (UTIs) are the most prevalent infectious diseases in community and clinical settings. The global emergence of multidrug-resistant (MDR) *E. coli* causing these UTIs necessitates exploring novel approaches. The use of compounds that attenuate bacterial virulence by acting through inhibition of quorum sensing (QS) is gathering momentum nowadays. This research was undertaken to investigate the anti-QS prospects of azithromycin (AZ) against 67 *E. coli* isolated from urine cultures of patients admitted to Alexandria Main University Hospital. Antibiotic susceptibility testing showed that 94% of these isolates were MDR. Swimming, twitching, and biofilm formation abilities were detected in 60%, 52.5%, and 55.2%, of the isolates, respectively. At its sub-inhibitory concentration, AZ hindered swimming and twitching motilities in 75% and 74.3% of the tested isolates, respectively. It reduced the biofilm formation by percentages ranging from 27% to 73%. Azithromycin downregulated the differential gene expression of *luxS*, a QS-regulating gene, and the genes encoding motility, thus proving its anti-QS ability. Further, the combinatory activity of AZ with ceftriaxone, ciprofloxacin, colistin, doxycycline, and tigecycline generated 95% of successful combinations.

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In conclusion, AZ effectively attenuated the QS in urinary *E. coli* isolates leading to a regression in the production of QS-associated virulence factors and hindrance of biofilm formation, thus delivering an avirulent pathogen. In addition, the synergistic combinations succeeded in circumventing the antibiotic resistance of *E. coli* isolates. Hence, AZ could be regarded as an anti-QS agent that might aid the design of innovative treatment alternatives for the management of UTIs caused by MDR *E. coli*.

Keywords: Azithromycin; *E. coli*; Quorum sensing; Urinary tract infections; Differential gene expression; Checkerboard assay

1. Introduction

Urinary tract infections (UTIs) are considered the most common infectious diseases in both clinical and community settings [(1)]. Despite extensive attempts, UTIs still afflict 150 million people annually. This high incidence imposes immense morbidity and substantial medical expenditures. *Escherichia coli* is considered the main culprit for the majority of community-acquired UTIs, accounting for 70–80% of cases [(2)]. The problem has been made worse by the emergence of multidrug-resistant (MDR) *E. coli* in urine isolates [(3)]. This rising antimicrobial resistance (AMR) is becoming one of the most severe public health threats that has been challenging healthcare management. The most significant contributing factor to this phenomenon is the excessive selective pressure brought on by the abuse of antibiotics, their improper prescribing, and their widespread use in agriculture [(4)]. This led Egypt to be categorized as the Arab League nation with the highest resistance levels among urinary *E. coli* isolates in comparison to its neighbors [(5,6)].

To establish a UTI, *E. coli* uses its arsenal of virulence factors which promote colonization and extraintestinal survival in the nutritionally deficient hostile bladder environment.

Using a sophisticated network of cell-to-cell communication called quorum sensing (QS), most uropathogens depend on the production of signaling molecules called autoinducers which have a role in regulating their virulence responses [(7)]. The LuxS/autoinducer-2 (AI-2), indole signaling, SdiA, AI-3/epinephrine/norepinephrine, and extracellular death factor are the five main QS systems engaged in coordinating these traits in *E. coli*. Among these, the AI-2 system is the most common one [(8)].

Nitrofurantoin, trimethoprim-sulfamethoxazole, pivmecillinam, and fosfomycin have been recommended by The European Association of Urology to treat uncomplicated UTIs. Irrational antibiotic policies and the initiation of empirical broad-spectrum antibiotic therapy without waiting for the results of urine cultures have led to elevated rates of AMR, highlighting the urge for alternative novel approaches in the treatment of UTIs [(9)]. In this context, the anti-QS approach has evolved as a promising paradigm with a capacity to attenuate bacterial virulence without exerting the classical selective pressure. The interest in repurposing compounds from natural and semi-synthetic origin as QS inhibitors (QSIs) for the management of numerous infections has surfaced in the literature, with a great diversity of QSIs being reported [(10)]. Among those naturally derived QSIs, curcumin [(11)], cinnamaldehyde, eugenol, and carvacrol [(12)] have been researched as potential alternatives to treat UTIs caused by MDR *E.coli*. Azithromycin (AZ) is a semi-synthetic antibiotic, belonging to the macrolide class, that is active against both Gram-positive and Gram-negative bacteria. At its sub-inhibitory concentrations, AZ was reported to disrupt QS in *Pseudomonas aeruginosa* by inhibiting

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the formation of biofilm and the production of several QS-mediated virulence factors in this organism [13]. However, there is a lack of data concerning the anti-QS effect of AZ in *E. coli*. Hence, we aimed in this study to investigate the anti-QS potential of AZ in urinary *E. coli* isolates by exploring its ability to attenuate the virulence factors mediated by QS including motility and biofilm production. Moreover, an evaluation of the combinatory effect of AZ with certain antibiotics was conducted *in vitro* to find a solution to circumvent the elevated AMR detected among the collected urinary *E. coli* isolates.

2. Materials and methods

2.1. Specimen collection

Sixty-seven *E. coli* urine isolates were obtained from Alexandria University Diagnostic Medical Microbiology laboratory (AUDiMM) affiliated to Alexandria Main University Hospital (AMUH), a tertiary hospital with 1500 beds, between July and October 2018. The isolates which were labeled E1 through E67, were preserved in 20% glycerol-containing Luria-Bertani broth (LB, HiMedia, Mumbai, India) at −80°C. To obtain a fresh culture, each of the tested isolates was cultivated on MacConkey's and eosin methylene blue agar (Oxoid, Hampshire, UK), then incubated at 37°C for 24 h. Gram staining was used for preliminary identification of the isolates while further confirmation was conducted by the in-house prepared biochemical tests: triple-sugar iron, methyl red, indole production, Voges Proskauer, citrate utilization, catalase, and urease tests.

2.2. Determination of minimum inhibitory concentration

The Clinical and Laboratory Standards Institute (CLSI, 2018) [14] recommendations were followed in determining the minimum inhibitory concentrations (MICs) of 10 antibiotics utilizing the broth microdilution (BMD) technique against 67 *E. coli* isolates using Mueller–Hinton broth (MHB, HiMedia, Mumbai, India). The isolates, prepared to match the turbidity of 0.5 McFarland, were tested for their resistance against amikacin, AZ, ceftriaxone, cefepime, colistin, tigecycline, meropenem, ciprofloxacin, doxycycline, and levofloxacin. The antibiotics were purchased from pharmaceutical markets. Each antibiotic was 2-fold serially diluted with sterile distilled water, and aseptically added to 96-well microtiter plates in 100 µL aliquots. Each tested isolate, at a final concentration of 5X 10^5 CFU/mL in double-strength MHB, was added in a volume of 100 µL to a well of the microtiter plate. The plates were then incubated at 37°C for 24 h. Using an ELISA microtiter plate reader (BioTek 800 TS, USA), the absorbance at an optical density (OD_{600}) was measured and the MIC values were determined. Results were interpreted using the CLSI 2018 breakpoints, except for colistin and tigecycline, for which The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2018) [15] guidelines were used. The multiple antibiotic resistance (MAR) indices were determined for *E. coli* isolates according to Mohamed *et al.* [16].

2.3. Phenotypic detection of QS-regulated virulence factors

2.3.1. Motility assays

While twitching motility was tested using LB broth containing 1% agar (B&V Srl, Italy), swimming motility was measured using 0.3% agar plates containing 1% tryptone (Oxoid, Hampshire, UK) and 0.25% NaCl as previously described [17,18]. In the swimming experiment, sterile micropipette tips were used to inoculate aliquots of 1 µL of *E. coli* isolates grown to an OD_{600} of 1.0 at the center of the swimming motility plates. After being incubated at 37°C for 24 h, the diameters of the resulting swimming halos were measured. Using a sterile toothpick, a colony of each isolate was deeply inserted into the

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prepared plate in the twitching assay to touch the agar–dish interface. Following a 48-hour incubation period at 37°C, the agar was removed, and 1% (w/v) crystal violet solution was used to stain the adhered cells on the plate. Next, precise measurements of the violet-stained diameters were made. The twitching motility of the isolates was interpreted according to Vijayakumar *et al*. [19], where a twitching diameter of <5 mm was considered a negative result, a zone diameter of 5-20 mm was interpreted as an intermediate result, and a zone diameter of >20 mm was regarded as a highly positive result. As quality control strains, *E. coli* ATCC 8739 and PAO1 were used in the experiments.

2.3.2. Biofilm formation using crystal-violet assay

The test was performed as previously mentioned with slight alterations [20]. Briefly, a 96-well microtiter plate with a flat bottom (Sigma-Aldrich, USA) was inoculated with 200 µL of an overnight subculture of each of the tested strains in nutrient broth (NB, Oxoid, Hampshire, UK) grown at an OD_{600} of 0.05. The plates were then incubated at 37ºC for 48 h. The produced biofilms were dyed with crystal violet 0.2% (w/v) for 10 min, washed three times with distilled water, and then extracted with 30% glacial acetic acid. The averages of three replicate wells were used to express the results of the absorbance measurements, which were made at 600 nm. The optical density of each strain (ODs) was compared to that of the negative control (ODnc) which contained 100 µl of saline in place of the bacterial inoculum. The formation of the biofilm was classified as strong when 4 ODnc < ODs, moderate when 2 ODnc < ODs ≤ 4 ODnc, weak when ODnc < ODs ≤ 2 ODnc, or no biofilm formation when ODs ≤ ODnc [21].

2.4. Inhibition of QS-mediated virulence factors by AZ

2.4.1. Swimming and twitching motility inhibition assays

Swimming or twitching agar plates were prepared as previously explained with AZ added at a concentration equal to 0.5XMIC. This concentration was estimated according to each isolate's MIC. Isolates displaying a swimming halo zone diameter of ≥40 mm (for swimming motility inhibition assay) or producing a twitching zone diameter of ≥5 mm (for twitching motility inhibition assay) were selected for motility inhibition assays. The experiments were subsequently carried out as described above and the percentage of inhibition exerted by AZ was calculated.

2.4.2. Biofilm inhibition assay

For this assay, isolates with an OD_{600} greater than 0.1 (2X ODnc) suggesting moderate to strong biofilm production were selected. The procedure was followed as explained above. Briefly, 100 µL of AZ in NB at a concentration equal to 0.5XMIC was combined with a volume of 100 µL of an overnight culture of each isolate. After 48 h of incubation at 37°C, the microtiter plates were analyzed for absorbance at OD_{600} using an ELISA microtiter plate reader. The experiment was performed in triplicate.

2.5. Quantitative real-time PCR assay

Using qRT-PCR, the effect of AZ was investigated on the expression levels of *luxS* which is the QS gene, and the virulence genes (*csgA, fimA,* and *fliC*) that are known to be controlled by QS. Selected isolates were cultured in LB broth for 48 h with or without AZ at a concentration equal to 0.5XMIC. Centrifugation at 6000 rpm for 5 min at 4ºC was used to extract bacterial pellets. Using Biozol reagent™ (Bioflux, China) and the manufacturer's guidelines, total RNA was extracted from both treated and untreated cells. The concentration of the obtained RNA was then adjusted using nanodrop (Thermo Fisher, USA). cDNA was generated using TOPscript™ RT DryMix (Enzynomics, South Korea) and quantified by qPCR using

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TOPreal™ SYBR® Green Premix (Enzynomics, South Korea) in a Rotor-Gene Q Real-Time PCR system (Qiagen, Germany) using the primer pairs listed in Additional file 1: **Table S1**. One µL of cDNA, 10 µL of SYBR® Green Premix, 1 µL of 10 µM of each primer, and 7 µL of nuclease-free water were used in each reaction mixture. *gapA* was utilized as an internal control to normalize the target genes' expression levels, and the Pfaffl method ($2^{-\Delta\Delta Ct}$) [22] was employed to determine the relative gene expression. Melting curve analysis of every qRT-PCR was conducted after each cycle to make sure that primer dimers and other artifacts were absent. The experiment was performed using three replicates and fold change was calculated.

2.6. Checkerboard assay

Checkerboard assay was used to analyze the combined efficacy of AZ with ceftriaxone, ciprofloxacin, colistin, doxycycline, and tigecycline against four *E. coli* isolates in 96-well microtiter plates. Each well in each row received a volume of 50 µL of two-fold serially diluted AZ, whereas each well of each column received serial dilutions of the antibiotics. An overnight bacterial subculture that matched the turbidity of 0.5 McFarland was 100-fold diluted in MHB and then introduced in a volume of 100 µL into the checkerboard plates. The plates were incubated at 37ºC for 24 h [23]. Using the following formula, the fractional inhibitory concentration (FIC) index was computed to examine the combined effect of AZ and the studied antibiotics: FIC index = FIC of tested antibiotic + FIC of AZ; FIC of AZ = MIC of AZ in combination divided by MIC of AZ alone. An FIC index of less than 0.5 indicated synergy; 0.5< FIC index ≤0.75 suggested partial synergy; 0.76< FIC index ≤1 indicated additive effect; 1< FIC index ≤4 denoted an indifferent effect; and an FIC index of more than 4 indicated antagonism [24].

2.7. Statistical analysis

SPSS version 25.0 (SPSS, Chicago, IL) was used to perform statistical analysis. To assess the qRT-PCR data, Pearson's correlation coefficient was used. A one-way ANOVA was performed, and then the Bonferroni test was used to compare the variables. In this study, differences were deemed significant at $p \leq 0.05$.

3. Results

3.1. Antimicrobial susceptibility and MAR index

Through the use of biochemical testing, the identity of the collected isolates was verified as *E. coli* as shown in Additional file 2: **Fig. S1.** The susceptibility of the tested isolates to ten antibiotics, including AZ, was determined by BMD technique. Ninety-four percent of the isolates exhibited an MDR phenotype, where they were resistant to more than 3 classes of antibiotics. An elevated resistance rate exceeding 80% was detected among the tested isolates towards ceftriaxone, cefepime, ciprofloxacin, and levofloxacin **Fig. 1**.

Additionally, ceftriaxone, cefepime, and ciprofloxacin displayed the most elevated minimum inhibitory concentration that inhibited 90% of the isolates (MIC_{90}) values reaching >4096, 2048, and 500 µg/mL, respectively as demonstrated in **Table 1**.

Meanwhile, the percentage of susceptibility to amikacin was greatest at 94%, then meropenem at 85% and colistin at 80.5%. No resistant isolates were detected against tigecycline which showed the narrowest MIC range (0.5 – 2 µg/mL) as compared to the other investigated antibiotics. However, 22.4% of the isolates showed intermediate resistance to this glycylcycline antibiotic. Additional file 1: **Table S2** shows the MIC values of the 10 antibiotics against 67 *E. coli* isolates along with the corresponding MAR index, which is a ratio between the number of antibiotics to which an isolate is resistant and the total number of antibiotics to which the isolate is exposed.

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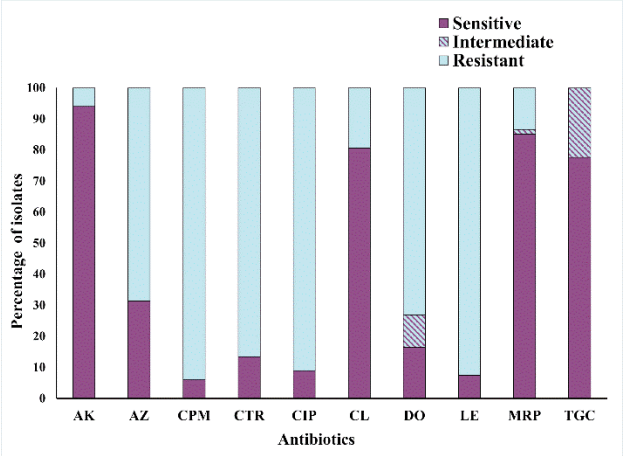

Fig. 1: Antibiotic resistance profiles of 67 *E. coli* isolates. AK refers to amikacin, AZ: azithromycin, CPM: cefepime, CTR: ceftriaxone, CIP: ciprofloxacin, CL: colistin, DO: doxycycline, LE: levofloxacin, MRP: meropenem, and TGC: tigecycline.

Table 1: *In vitro* activity of the investigated antimicrobial agents against 67 *E. coli* isolates.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
Amikacin	4	16	0.125 − 512
Azithromycin	64	256	2 − 1024
Cefepime	512	2048	<0.125 − 4096
Ceftriaxone	>4096	>4096	<0.125 − >4096
Ciprofloxacin	125	500	<0.125 − >1000
Colistin	1	4	0.25 − 16
Doxycycline	16	64	2 − >128
Levofloxacin	31.25	62.5	<0.125 − 500
Meropenem	<0.125	64	<0.125 − 512
Tigecycline	1	2	0.5 − 2

***** MIC_{50} and MIC_{90} are the values of MIC (µg/ml) of the antibiotic that cause inhibition of 50 and 90% of the tested isolates, respectively.

MAR index values greater than 0.2 were calculated for 94% of the isolates, suggesting that the isolates were obtained from an area where infections caused by MDR pathogens were more prevalent.

3.2. Assessment of the virulence attributes regulated by QS

Swimming motility was detected in 60% (*n*=40) of the tested isolates which displayed halo zones of ≥40 mm in diameter **Fig. 2a**. A representative example of swimming motility in E8 isolate displaying a halo zone of 90 mm in diameter is depicted in **Fig. 2b**. Twenty-seven isolates produced halo zones of < 10 mm in diameter on the swimming agar plates and were considered as swimming-negative isolates. Intermediate twitching motility, ranging from 6 to 20 mm in diameter, was observed in 35 of the tested isolates as compared to 32 twitching-negative isolates **Fig. 2a**. A representative example of twitching motility in E8 isolate displaying a twitching motility zone of 18 mm in diameter is shown in **Fig. 2c**. Weak biofilm production ability was detected among the majority of the isolates (*n*=33), while a single isolate showed strong biofilm-producing ability and three isolates were moderate biofilm formers **Fig. 2a**.

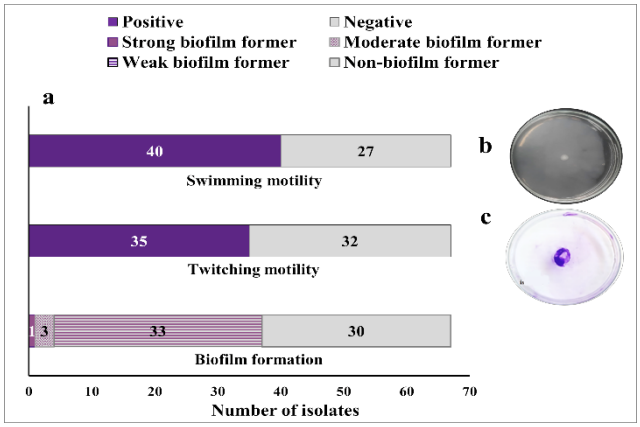

Fig. 2: a Prevalence of swimming motility, twitching motility, and biofilm formation among 67 urinary *E. coli* isolates; **b** E8 isolate showing a swimming halo zone of 90 mm in diameter; **c** E8 isolate demonstrating an intermediate twitching motility zone of 18 mm in diameter.

To establish a relationship between the resistance profiles of the studied isolates and their virulence factors, the mean MAR index was calculated and represented in relation to the swimming, twitching, and biofilm-forming abilities of these isolates **Fig. 3**.

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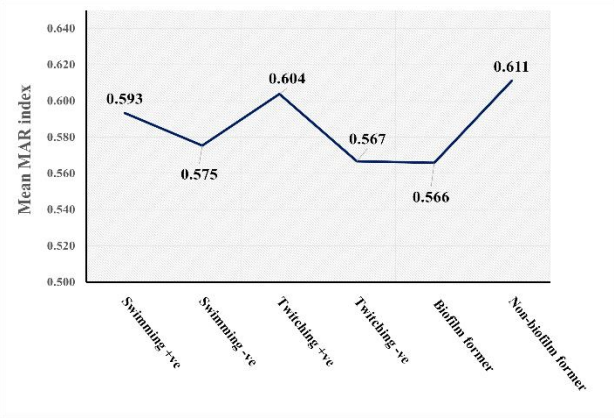

Fig. 3: Distribution of the mean MAR index values among different virulence patterns observed in the 67 tested *E. coli* isolates.

The calculated mean MAR index was observed to be higher in isolates possessing twitching motility as compared to those lacking this type of motility. No major difference in the mean MAR index was detected among the swimming-positive and the swimming-negative isolates. Meanwhile, non-biofilm-forming isolates were found to possess a higher mean MAR index than the biofilm-producer ones.

3.3. Assays for QS inhibition

3.3.1. Suppression of swimming and twitching motilities

Forty isolates with an initial swimming halo zone diameter of at least 40 mm were used to study the swimming motility inhibitory effect of 0.5XMIC of AZ. The isolates were categorized into three subgroups: group 1 consisted of isolates that showed no suppression by AZ, group 2 included isolates experiencing inhibition ranging from 1% to 49%, and group 3 comprised isolates whose swimming motility was impaired by AZ by a value of ≥50%. Eighteen isolates (45%) had their swimming motility decreased by a value of ≥50, indicating that AZ was effective in suppressing this type of motility in 75% of the isolates **Fig. 4a**. Selecting intermediate-motile isolates with twitching zone sizes of 5–20 mm allowed us to examine the inhibitory effect of 0.5XMIC AZ on twitching motility. Azithromycin inhibited the twitching motility of 26/35 isolates by different percentages, where 49% of the isolates had their twitching motility suppressed by a range of 1 to 49%, while the twitching motility of 26% of the isolates was inhibited in the presence of AZ by a value of ≥50% **Fig. 4a**.

3.3.2. Hindering the formation of the biofilm

The potential for 0.5XMIC of AZ to suppress the formation of biofilms was evaluated in isolates showing an OD_{630} more than 0.1 (2X ODnc). Accordingly, four isolates, E51, E52, E58, and E63, producing moderate to strong biofilms were selected for this assay. E51 isolate, which was a strong biofilm-forming isolate initially, had its biofilm formation inhibited by 54% by AZ, with a significant inhibition value at $p \leq 0.05$ **Fig.4b**. The biofilm formation was reduced in E52 and E63 isolates (moderate biofilm-forming isolates) by approximately 1.5-fold, while as compared to its untreated control, the biofilm-forming capacity of E58 isolate was suppressed by 73% when exposed to AZ with a highly statistically significant difference ($p \leq 0.01$) between the two treatments **Fig. 4b**.

3.4. Differential gene expression levels

The expression levels of *luxS* (QS gene) and those of the QS-regulated virulence genes in the presence of 0.5XMIC of AZ were evaluated by qRT-PCR. Among these were the *csgA*, a gene that codes for the curli fimbriae, an essential gene for biofilm formation, *fimA*, a gene which codes for fimbrial adhesin, and the *fliC* gene, a flagellar gene required for motility. E13, E35, E51, E52, E58, and E63 isolates were chosen for the transcriptional investigation. The isolates' evident phenotypic manifestation of motility and biofilm production served as the basis of selection.

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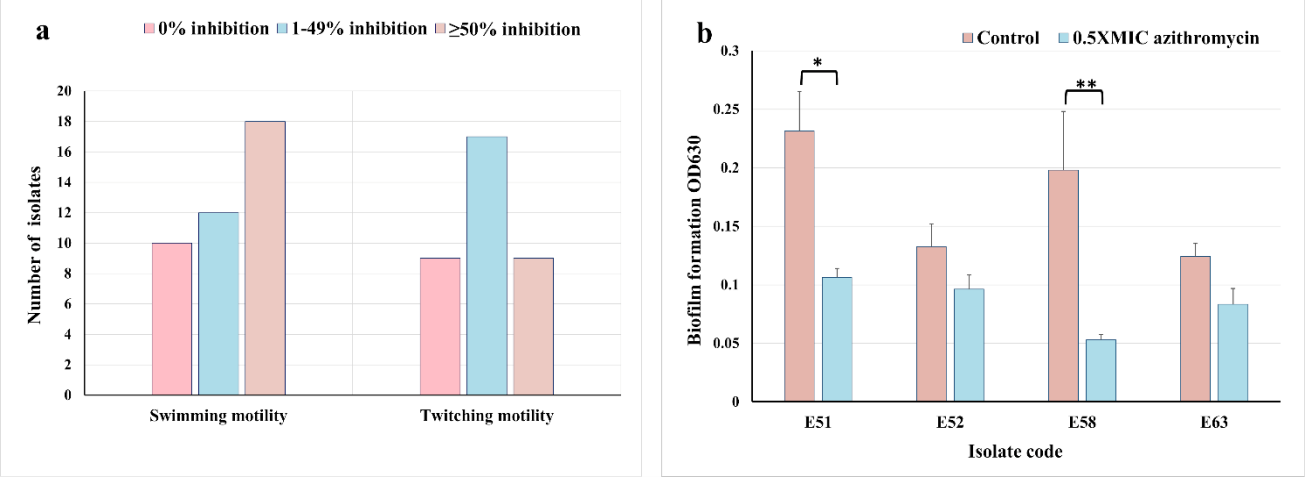

Fig. 4: Impact of azithromycin at 0.5XMIC on the tested *E. coli* isolates' **(a)** Quorum sensing-regulated swimming and twitching motilities; **(b)** E51, E52, E58, and E63 isolates' ability to form biofilms. The data in **Fig. 4b** are presented as the average of three independent experiments with standard deviations denoted by error bars. In comparison to the untreated samples, *p*-values reflect significance at $*p \leq 0.05$ and $**p \leq 0.01$.

Before performing qRT-PCR, screening of the genes of interest was conducted by conventional PCR amplification using the primer pairs and annealing temperature conditions specified in Additional file 1: **Table S1** and a representative example of the agarose gel electrophoresis is shown in Additional file 2: **Fig. S2**. Melting curve analysis of qRT-PCR was conducted after each cycle and representative melting curve analyses showing the expression levels of gapA and *luxS* are depicted in Additional file 2: **Fig. S3.** In 67% of the tested isolates, AZ was able to downregulate the primary QS gene, *luxS*, and the flagellar gene, *fliC*, by varying folds. The expression level of *luxS* was reduced by an average of 1.6 folds, while the downregulation of *fliC* ranged from 1.3 to 3.3 folds and was statistically significant, at $p \leq 0.05$, in the isolates E35 and E51. With a fold reduction ranging from 1.1 to 1.5, the macrolide antibiotic suppressed the *csgA* expression levels in 50% of the isolates. A single isolate, E13, showed a significant upregulation of *csgA* gene. The level of the transcript of *fimA* gene was reduced in E58 isolate by 33%, while in E13, E35, E51, E52, and E63 isolates, treatment with AZ caused an unanticipated overexpression of the *fimA* gene and this upregulation was highly significant in these isolates **(**Additional file 1: **Table S3** and **Fig. 5**).

Following a 48-h treatment with 0.5XMIC AZ in the six selected isolates, the Pearson correlation coefficient was computed to investigate the correlation between the expression levels of *luxS* gene and those of the virulence genes, *csgA*, *fimA*, and *fliC* as shown in **Table 2**. Positive correlations were detected between the levels of expression of *luxS* and those of the investigated virulence genes with significance at $p \leq 0.05$ being displayed upon correlating *luxS* to *csgA*.

3.5. Evaluation of the combinatory efficacy of AZ and selected antibiotics using the checkerboard technique

The synergistic efficacy of AZ combined with ceftriaxone, doxycycline, ciprofloxacin, colistin, and tigecycline was studied in E18, E35, E58, and E63 isolates. These isolates

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had been selected to represent the research collection, where E18, E35, and E63 were MDR, while E58 was a non-MDR isolate **(**Additional File 1: **Table S2)**.

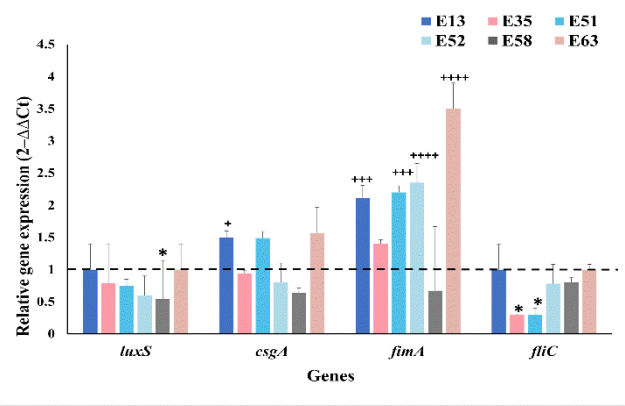

Fig. 5: Relative expression of *luxS* and QS-mediated virulence genes (*csgA*, *fimA*, and *fliC*) in selected *E. coli* isolates after exposure to 0.5XMIC of azithromycin. Utilizing *gapA*, gene expression was normalized and displayed in relation to the expression of genes in the untreated control. Standard deviations are shown by the error bars. The significance of fold decrease at **p* ≤0.05 and fold increase at $^{+}$ *p* ≤ 0.05, $^{+++}$ *p* ≤ 0.001, and $^{++++}$ *p* ≤0.0001 is indicated by the *p*-values.

Table 2: Correlation between *luxS* expression levels and those of the virulence genes following treatment with azithromycin in selected *E. coli* isolates.

Treatment		*csgA*	*fimA*	*fliC*
Azithromycin	*P*	0.862	0.648	0.326
	S	0.027*	0.164	0.528

*Correlation is significant at the two-tailed 0.05 level. *p* refers to the value of the Pearson correlation coefficient, while S indicates significance. Based on the expression levels assessed in E13, E35, E51, E52, E58, and E63 isolates, the correlations were calculated.

The FIC index was used to express the results in **Table 3**, and combinations that were synergistic or partially synergistic were deemed successful. When AZ was added at 0.5XMIC to ceftriaxone, colistin, doxycycline, and tigecycline, 100% successful combinations were obtained. As illustrated in **Fig. 6**, the concentrations of these antibiotics decreased by various folds, ranging from 2 to 256 folds, in the presence of AZ. In 75% of the isolates, AZ exhibited partial synergy when combined with ciprofloxacin; however, testing against E58 yielded an indifferent result.

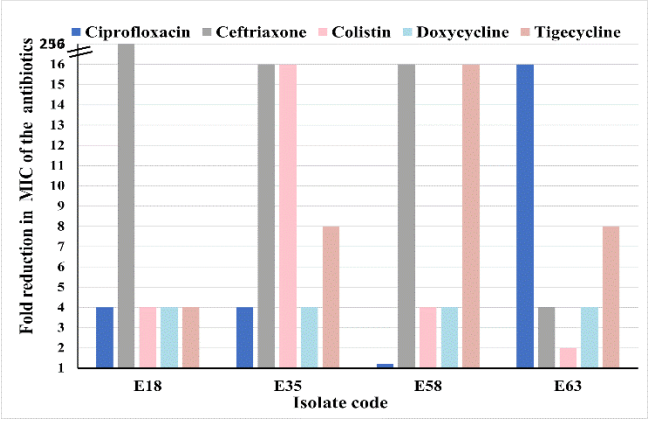

Fig. 6: Fold reduction in the MICs of the tested antibiotics when combined with 0.5XMIC azithromycin against selected *E. coli* isolates.

4. Discussion

Although different pathogens can cause UTIs, the predominant organism is documented to be *E. coli* representing 80-90% and 40-50% of UTI cases encountered in community and hospital settings, respectively [25]. In hospital settings, UTIs constitute a major public health problem as these infections are usually associated with an increasing resistance rate to antimicrobials, posing a real challenge to clinicians while treating them. In the current study, 94% of urinary *E. coli* isolates were MDR. A number of Egyptian governorates have lately reported similar high incidences of MDR *E. coli*. According to studies by Abdelaziz *et al.* and Abdelkhalik *et al.*, the prevalence of MDR *E. coli* in the capital, Cairo, reached 60% and 80%, respectively [26,27].

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Table 3: FIC index for azithromycin's combined activity with selected antibiotics against tested *E. coli* isolates.

	FIC index (Interpretation)				
	Azithromycin (0.5XMIC)+				
	CIP	**CTR**	**CL**	**DO**	**TGC**
E18	0.75 (PSyn)	0.5 (Syn)	0.375 (Syn)	0.5 (Syn)	0.375 (Syn)
E35	0.75 (PSyn)	0.56 (PSyn)	0.375 (Syn)	0.5 (Syn)	0.625 (PSyn)
E58	1.1 (Ind)	0.375 (Syn)	0.75 (PSyn)	0.5 (Syn)	0.25 (Syn)
E63	0.56 (PSyn)	0.75 (PSyn)	0.75 (PSyn)	0.5 (Syn)	0.625 (PSyn)

CIP: ciprofloxacin, CTR: ceftriaxone, CL: colistin, DO: doxycycline, TGC: tigecycline. Ind, PSyn, and Syn indicate indifference, partial synergy, and synergy, respectively.

Masoud *et al*. reported a 73% incidence in the Upper Egypt sector [28]. El-Sokkary *et al.* identified an MDR pattern in 87.5% of *E. coli* isolates in Zagazig, in the northeastern part of Egypt [29]. This high rate of AMR, along with the findings from our study in Alexandria, in northern Egypt, reflects an alarming national scenario that calls for special attention to halt the propagation of these MDR *E. coli* in our community. Our collection of isolates scored an average MAR index of 0.545, an apparently high value that was consistent with the previous report conducted by Masoud *et al* in Egypt [30]. This high MAR index could be a cause for concern, as it indicates the presence of antibiotic-resistant pathogens in the collection area. Self-medication, blind diagnosis, and excessive antibiotic usage have been condemned for those elevated MAR index values [31]. Cephalosporin resistance was detected in over 85% of the isolates in our collection. Numerous studies carried out in Egypt stated that *E. coli* isolates displayed very high resistance against ceftriaxone making the use of this widely prescribed antibiotic quite questionable [32-34]. These high levels of resistance may be explained by the ease of access, comparatively low cost of cephalosporins, and regular prescription in Egyptian medical institutions. A notable increase in fluoroquinolone resistance among urinary *E. coli* isolates has been reported universally in hospital settings. This has led the Infectious Diseases Society of America (IDSA) to release guidelines that reserve the administration of fluoroquinolones in severe cases, or when the implication of the first-line drugs is not possible [35]. In this study, 91% and 92.5% of the isolates demonstrated resistance to ciprofloxacin and levofloxacin, respectively. These results are in concordance with those reported by Said *et al*.,[36] in 2021, who detected high resistance rates to levofloxacin (56.3%) and ciprofloxacin (69.1%) among urinary *E. coli* isolated in Egypt. Meropenem showed 85% susceptibility with an MIC_{50} of less than 0.125 µg/mL. In Egyptian hospitals, this carbapenem is only used as a last-resort antibiotic and is rarely provided in outpatient departments [37]. However, the worldwide documentation of an upsurge in *E. coli* resistant to carbapenems indicates that the use of this category of antibiotics should be exercised with caution [38]. The study's findings indicate that apart from meropenem, amikacin, colistin, and tigecycline are viable therapeutic alternatives for treating resistant urinary *E. coli* isolates, where these antibiotics achieved susceptibility rates reaching 94%, 80%, and 78%, respectively.

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The results of the current investigation showed that 55.2% of the isolates produced biofilm, which is consistent with a number of prior reports originating from India [39], Egypt [40], and Nepal [41].

The motility of *E. coli* has been extensively studied due to its involvement in virulence, biofilm formation, colonization, and tropism of this pathogen [42]. In addition, motility provides a significant competitive fitness enabling the strains possessing this feature to outcompete those lacking it. Swimming motility is flagella-driven while twitching motility is a flagella-independent motility that is controlled by type-IV fimbriae [43]. It was found that 60% and 52.2% of the tested isolates in the current study exhibited swimming and twitching phenotypes, respectively. In a study carried out by Mihaylova *et al.* [44], a comparable percentage of swimming motility, 75%, was reported, however, the authors did not detect any twitching motility among the investigated urinary *E. coli* isolates.

The escalating AMR witnessed nowadays in conjunction with decreased rates of novel agent discovery highlights the necessity of examining alternate treatment approaches to ensure that physicians have access to a stable supply of efficacious therapeutics [45]. In this setting, researchers' focus has shifted to targeting the bacterial QS system to hinder virulence opening the door for new anti-virulence tactics [12]. Azithromycin is a semi-synthetic antibiotic belonging to the macrolide class. In Egypt, this antibiotic is readily available and within the affordable limit of most of the population, hence, the use of AZ for the treatment of UTIs is a common practice. Apart from its antimicrobial activity, AZ, at sub-MICs, was found to influence the QS system of *P. aeruginosa*, where it was reported to suppress the motility, biofilm formation, and virulence of this pathogen [46,47]. Unfortunately, little information is known about the effect of AZ on the virulence of *E. coli* or how well this macrolide can attenuate the QS system in this isolate. Therefore, the goal of the current investigation was to assess AZ's anti-virulent potential and anti-QS efficacy against urine *E. coli* isolates. The results of Goyal *et al*. [48] were consistent with the BMD approach used in this study, which showed that 68.7% of the isolates were resistant to AZ. Nevertheless, sub-inhibitory concentrations (0.5XMIC) of AZ possessed an anti-motility effect on the tested isolates, where 75% and 74.3% of the isolates had their swimming and twitching motilities, respectively, hindered by AZ. As far as we know, this study is the first to offer light on the inhibitory potential of AZ on the motility phenotypes in *E. coli* clinical isolates. The most relevant studies that we could retrieve reported an inhibitory effect of AZ, at its sub-MIC levels, on the swimming motility in *Salmonella enterica* and *P. aeruginosa* [46,47,49].

We further examined the ability of AZ to impede the biofilm-forming capacity of the tested isolates. Biofilm formation in *E. coli* has been considered an important virulence hallmark that provides resistance against penetration of antimicrobials, as well as against the host immune defenses such as phagocytosis, oxidative stress, and nutrient deprivation [50]. Since *E. coli 's* QS systems are believed to control the transition between the various stages of biofilm formation, there is a chance to intervene using an anti-QS strategy [51]. At sub-MIC, AZ was found to cause a significant reduction in the biofilm-forming ability of the tested isolates and this decrease ranged from 27% to 73%. In their work, Ahsan *et al*. [52] showed that sub-MICs of AZ suppressed the initial stages of biofilm formation investigated in *E. coli* isolated from UTI patients, where AZ markedly reduced the viable count of planktonic cells. Yet, the authors noted that these sub-MICs were unable to eradicate the already-formed biofilms in these isolates [52]. Nevertheless,

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the anti-biofilm-forming ability of AZ was consolidated by other researchers who demonstrated the effectiveness of AZ, at concentrations equivalent to 0.25XMIC, to inhibit the formation of biofilms in *P. aeruginosa* [53,54].

Intrigued by the anti-QS phenotypic outcomes, we endeavored to explore the molecular basis of this anti-QS impact by identifying the differential expression of the virulence genes *(fimA, csgA, and fliC*) and *luxS* using qRT-PCR. At sub-MIC, AZ downregulated *luxS* gene in 67% of the isolates that were examined, suggesting that the hypothesized mechanism for QS suppression is inhibition of the AI-2 system via the *luxS* pathway. The expression levels of *luxS* in two isolates, E13 and E63, were not changed upon treatment with AZ. In this particular case, it is possible that the downregulation of additional genes involved in the AI-2 QS system, such as *pfs*, but not covered in this investigation, happened [55]. A maximum of 3.3-fold reduction was observed in 67% of the isolates for the expression levels of the flagellar-mediated motility gene *fliC*. Similarly, 50% of the isolates exhibited an average reduction fold of 1.3 for the curli fimbriae gene *csgA*, which is an essential component of biofilm formation. Studies assessing the genetic causes of anti-QS mechanisms of AZ are very scarce in the literature with no research investigating these mechanisms in *E. coli* isolates. Two studies corroborated our results, where Nalca *et al.* [56] demonstrated that AZ was able to decrease the expression of the gene encoding the flagellar protein, *fliC*, and Swatton *et al.* [46] reported that at sub-MIC, AZ downregulated the expression of *fliC* and *fliD*. However, both studies investigated the differential expression of these genes in *P. aeruginosa* rather than *E. coli* isolates. Surprisingly, an upregulation of *fimA*, and *csgA* genes in 5/6 and 3/6 of the isolates, respectively, was detected upon treatment with AZ. This unexpected overexpression may have been caused by the fact that, in response to adverse environmental conditions, bacterial cells tend to enhance the expression of specific genes, in this case, the *fimA* and *csgA* genes, in an attempt to compensate for the suppression imposed on other related genes [12].

The relationship between the expression levels of *luxS* and those of *fimA*, *csgA*, and *fliC* genes was statistically analyzed using the Pearson correlation coefficient. Positive correlations were depicted between the levels of expression of *luxS* and those of the investigated virulence genes after treatment with AZ, with significance displayed upon correlating *luxS* and *csgA*. These positive correlations suggest that AZ, through the attenuation of the virulence attributes regulated by the QS circuit, exerted an anti-QS ability. This suggestion is supported by the research of Yang *et al*. [57] which found that the downregulation of *luxS* gene imposed impaired motility, hampered biofilm formation, and downregulated the flagellar productions in *E. coli* isolates.

The combinatory effect of AZ with five antibiotics among the ones usually prescribed in UTI management, generated 95% of successful combinations as confirmed by the checkerboard technique. The results of these combinations are not well documented in the literature, particularly with regard to the urine *E. coli* isolates. Azithromycin acts by inhibiting protein synthesis while exerting an additional anti-inflammatory effect, an effect that is believed to lead to synergy when combined with other antibiotics [58]. The results of the time-kill assay published by Gómara-Lomero *et al.* [59], which examined AZ/tigecycline in *Klebsiella pneumoniae*, corroborated the observations of the current investigation. The synergy detected upon combining AZ and tigecycline is believed to be due to the dual inhibition of protein synthesis targeting 30S and 50S ribosomal

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subunits, respectively [59]. Azithromycin enhanced the effect of colistin in AZ/colistin combination, and this was in accordance with previous results reporting the synergistic effect of this combination in resistant isolates of *K. pneumoniae* and *E. coli* [60, 61]. The obtained values of FICI corresponding to AZ/colistin were in accordance as well with another study investigating the effect of the combination in *A. baumannii* [62]. Saini *et al*. [63] detected synergy upon combining AZ with ciprofloxacin when tested in *P. aeruginosa.* The inhibition of the cell wall synthesis by ceftriaxone is thought to be enhanced by the bacteriostatic action of AZ which acts on another bacterial target, the protein synthesis, resulting in the display of synergy. Bishr *et al*. [64] found that 44.7% of AZ/ceftriaxone provided synergy in methicillin-resistant *Staphylococcus aureus*. The combined effect of doxycycline, a tetracycline member, with AZ was synergistic. A similar finding was reported by Dillon *et al.* [65] where the authors detected synergy between minocycline, another member of tetracycline, with AZ when tested *in Acinetobacter baumannii, P. aeruginosa, and K. pneumoniae*.

In the ongoing battle against antibiotic resistance, the successful combinations detected in this study, some of which had not been previously tested against resistant *E. coli* isolates, may offer a fresh viewpoint on how to combat the negative effects of high antibiotic doses.

We are aware of and accept the study's limitations. The statistical significance of synergistic combinations was constrained due to their investigation on a limited number of isolates. Additionally, further *in vivo* evaluation is necessary to elucidate the precise mechanism underlying AZ's anti-QS effect and its anti-virulent potential so that its effectiveness can be leveraged in future clinical practice.

5. Conclusions

This study provides insights into the mechanisms behind the anti-QS potential of AZ. We conclude that sub-MIC of AZ effectively attenuates the QS systems in *E. coli* by downregulating the QS gene, luxS, leading to a regression in the production of QS-associated virulence factors and hindrance of biofilm formation, thus delivering an avirulent pathogen. Additionally, the AMR of *E. coli* isolates was successfully mitigated by using AZ in combination with the studied antibiotics. Therefore, it appears that AZ has the ability to function as an anti-QS agent, which may aid in the development of new therapeutic alternatives for the management of UTIs caused by MDR *E. coli*.

Abbreviations

AI-2: autoinducer-2, AMUH: Alexandria Main University Hospital, AMR: antimicrobial resistance, AZ: azithromycin, BMD: broth microdilution, CLSI: Clinical and Laboratory Standards Institute, FIC: fractional inhibitory concentration, LB: Luria-Bertani broth, MAR: multiple antibiotic resistance, MDR: multidrug-resistant, MHB: Mueller–Hinton broth, MIC: minimum inhibitory concentration, NB: nutrient broth, OD: optical density, qRT-PCR: quantitative real-time polymerase chain reaction, QS: quorum sensing, QSIs: quorum sensing inhibitors, and UTIs: urinary tract infections.

Supplementary materials:

The supplementary materials include Additional File 1 (Tables) and Additional File 2 (Figures). The references [66-70] are cited in the supplementary materials: Additional File 1 (Tables).

Declarations

Consent for publication

All authors have read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary additional files.

Ethical approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. The urine samples were collected by AUDiMM laboratory of Alexandria Main University Hospital, *E. coli* isolates were provided anonymously and used in this study. No specimens were collected from patients under the age of 16. The authors did not have any contact with the patients. The informed consent was waived by the Ethics Committee of Alexandria University, Faculty of Medicine which approved the study under IRB number: 00012098 and the Federal Wide Assurance FWA number: 00018699(Date:9/4/2023) (http://www.hhs.gov/ohrp/assurances/index.htm)

Authors' contributions

HAM: Methodology, validation, data curation, and writing the original draft. **HMO:** Conceptualization, reviewing, and editing. **ASZ**.: Study design, validation, data curation, reviewing, and editing. **NMM**: Study design, validation, data curation, writing, reviewing, and editing. All authors commented on previous versions of the manuscript.

Highlights

- Compounds attenuating bacterial virulence through inhibition of quorum sensing are gathering momentum nowadays.
- Anti-quorum sensing potential of azithromycin against multidrug-resistant *E. coli* isolated from urine was investigated.
- Azithromycin hindered the swimming and twitching motilities and reduced the biofilm formation in tested isolates.
- It downregulated the expression of the quorum-sensing genes and genes encoding motility, thus proving its anti-quorum-sensing ability.
- The combinatory activity of azithromycin with selected antibiotics generated successful combinations that could be beneficial for the treatment of urinary tract infections caused by *E. coli*.

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