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ORIGINAL ARTICLE

'Supermutators' found amongst highly levofloxacin-resistant *E. coli* isolates: a rapid protocol for the detection of mutation sites

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Fluoroquinolone resistance is gradually acquired through several mechanisms. In particular, chromosomal mutations in the genes encoding topoisomerases II and IV and increased expression of the multidrug efflux pump AcrAB-TolC are the most common mechanisms. In this study, multiplex polymerase chain reaction (PCR) protocols were designed for high-throughput sequencing of the quinolone resistance determining regions of topoisomerases genes (*gyrA*, *parC* and *parE*) and/or the expression regulation systems of multidrug efflux pump AcrAB (*acrRAB*, *marRAB* and *soxSR*). These protocols were applied to sequence samples from five subpopulations of 103 clinical *Escherichia coli* isolates. These subpopulations were classified according to their levofloxacin susceptibility pattern as follows: highly resistant (HR), resistant (R), intermediate (I), reduced susceptibility (RS) and susceptible (S). All HR isolates had mutations in the six genes surveyed, with two 'supermutator' isolates harboring 13 mutations in these six genes. Strong associations were observed between mutations in *acrR* and HR isolates, *parE* and R/HR isolates and *parC* and I/R/HR isolates, whereas surprisingly, *gyrA* mutations were common in RS/I/R/HR isolates. Further investigation revealed that strong associations were limited to the triple mutations *gyrA*-S83L/D87N/R237H and HR isolates and the double mutations S83L/D87N and I/R/HR isolates, whereas the single mutation S83L was common in RS/I/R/HR isolates. Interestingly, two novel mutations (*gyrA*-R237H and *acrR*-V29G) were located and found to strongly associate with HR isolates. To the best of our knowledge, the *gyrA*-R237H and *acrR*-V29G mutations have never been reported and require further investigation to determine their exact role in resistance or 'fitness' as defined by their ability to compensate for the organismal cost of gaining resistance.
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INTRODUCTION

Fluoroquinolones (FQs) are bactericidal antimicrobial agents that target topoisomerase II (DNA gyrase) and topoisomerase IV enzymes. They inhibit DNA synthesis by binding to the enzyme–DNA complex and stabilizing DNA strand breaks created by the enzyme.[1] The increasing FQ resistance rates in many bacterial species in recent years is a major concern for the healthcare community.[2,3] FQ resistance is acquired through chromosomal mutations, at a rate of 5×10^{-9}–5×10^{-7}, in genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*).[4–6] FQ resistance can also be acquired through increased expression of the multidrug efflux pump AcrAB due to mutations in operons (*acrRAB*, *marRAB* and *soxSR*).[7–9] Other mechanisms include the acquisition of the plasmid-borne genes *qnr* (prevalence of <2%) and *aac (6′)-Ib-cr* (prevalence of 25%) that encode drug-blocking and drug-modifying proteins, respectively, in addition to other unknown mechanisms.[4,5]

Mutations in topoisomerase genes occur in the quinolone-resistance determining region (QRDR). These mutations cause the mutant enzyme–DNA complex to have a lower affinity to the drug, specifically at positions 83 or 87 for the GyrA subunit of DNA gyrase and positions 78, 80 or 84 for the ParC subunit of topoisomerase IV.[10,11] Mutations in *acrR*, *marR* and *soxR*, which are the repressor genes of operons *acrRAB*, *marRAB* and *soxSR*, respectively, cause the overexpression of the multidrug efflux pump AcrAB and its direct activators MarA and SoxS. In addition, MarA and SoxS downregulate the outer membrane porin OmpF, resulting in decreased cell permeability and resistance to oxidants as well as antibiotics.[7,8,12,13]

From this perspective, the present study attempted to investigate topoisomerase mutations (in *gyrA*, *parC* and *parE*) and AcrAB efflux pump regulator mutations (in *acrR*, *marRA* and *soxSR*) in *Escherichia coli* and associate these mutations with our proposed different levels of levofloxacin resistance. Cavaco *et al.*[14] made similar efforts to correlate resistance mechanisms to minimum inhibitory concentrations (MICs) in quinolone-resistant *E. coli* isolates.

The Clinical and Laboratory Standards Institute (CLSI) publishes annual guidelines for antimicrobial susceptibility testing and the interpretation breakpoints of a large number of organism–drug combinations.[15] For quinolones, the CLSI provides a three-level interpretation

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(resistant (R), intermediate (I) and susceptible (S)) that is primarily for clinical use. In this study, the distribution of the isolates covered 16 MIC dilutions and some isolates scored MIC values ×1000 greater than wild-type MIC values. It was necessary to cluster this broad distribution into a number of manageable classes that could be correlated to the genotypes under investigation. Therefore, attempts were made to establish a five-level interpretation system using 'epidemiological' breakpoints that were generated by a statistical method similar to the method of Kronvall.[16] The resultant categories were labeled as follows: highly resistant (HR), R, I, reduced susceptibility (RS) and S. These classifications were satisfactory for the purposes of this study.

MATERIALS AND METHODS

Chemicals, biochemical reagents and media

Mueller-Hinton agar and broth were from LabM (Lancashire, UK); Luria-Bertani (LB) medium was from CONDA Pronadisa (Madrid, Spain). Levofloxacin (5 mg/mL) was purchased from Sanofi-Aventis (Cairo, Egypt); levofloxacin disks (5 μg) were from Bioanalyse (Ankara, Turkey).

Bacterial isolates

A total of 103 clinical *E. coli* isolates were used in this study. They were under investigation for their virulence factors in two separate, previous studies. Eighty-eight isolates were associated with enteric infections,[17] and 15 were associated with urogenital infections (El-Far M *et al*., unpublished data, 2014). Therefore, these isolates represented convenient samples that were received as identified stock cultures preserved at −80 ℃ in 15% glycerol. *E. coli* K-12 was kindly provided by the Biotechnology Centre, Faculty of Pharmacy at Cairo University.[18] Unless otherwise specified, all isolates were propagated aerobically at 35 ℃ in LB broth or on LB agar. Stock cultures were stored at −80 ℃ in 15% glycerol.

Antimicrobial susceptibility testing

Susceptibility profiles were initially screened using the disk diffusion method and verified by determining the MIC using the broth microdilution method. Both methods were performed according to the CLSI 2012 documents M02-A11 (for the disk diffusion method) and M07-A9 (for the broth microdilution method).[14] *E. coli* K-12 was included as a control and the same lots of broth and agar media were used for susceptibility testing of all isolates.

Instead of considering the collected isolates as a single population with a normal distribution, they were treated as a mixture of different subpopulations, each with its own normal distribution. To define the subpopulations in MIC and inhibition zone histograms, the modal MICs and zone diameters on the histograms were located and the cutoff values around the mean of each subpopulation were calculated to be at ±2.0 standard deviations (SDs). The adopted method is similar to the normalized resistance interpretation method,[16] which is used to establish epidemiological breakpoints separating wild-type isolate subpopulations from those harboring resistance mechanisms.

Polymerase chain reaction (PCR) amplification

Initially, the PrimerQuest program (IDT, Coralville, IA, USA) was used to design primers in the conserved regions flanking the target region of each gene, which were located by blasting the sequences from *E. coli* K-12 MG1655 using NCBI's BLAST for the taxid *Escherichia*. Primer compatibility and specificity in the multiplex reaction were checked using OligoAnalyzer 3.1 (IDT) and Primer-BLAST software from NCBI,[19] respectively. The six newly designed pairs of oligonucleotide primers are listed in Table 1.

Multiplex PCR protocols were designed for the simultaneous amplification of the QRDRs of topoisomerase genes (*gyrA*, *parC* and *parE*) and/or the genes involved in regulating the expression of the AcrAB efflux pump in their entirety (*acrR*, *marRA* operon and *soxSR* operon) (with a maximum of six PCR products). The PCRs were performed using the EmeraldAmp GT PCR Master Mix (Takara, Otsu, Shiga, Japan) in a TECHNE thermocycler (Bibby Scientific Ltd, Staffordshire, UK). Colony lysates of each bacterial isolate were used as the PCR amplification template. Multiplex PCR was optimized by varying the cycling conditions and primer ratios until the final settings listed in Table 2 were reached.

Gel electrophoresis and DNA extraction

DNA fragments from the multiplex PCRs were separated using the ADVANCE electrophoresis system (Mupid-exu, Chuo-ku, Tokyo, Japan) at 5 V/cm and a combination of 1% agarose and 0.2% agarose (Lonza SeaKem LE Agarose, Walkersville, MD, USA). A similar method was described in a previous study, whereby sequencing of

Table 1 A list of the designed primers in this study that were used for amplification and sequencing

Gene	Primer[a,b]	T_m (℃)[c]	Amplified region	Amplicon size (bp)
gyrA	5′-GCT CCT ATC TGG ATT ATG CGA TGT (F)	64	Nucleotides 56–1007	952
	5′-GCC ACC ATG TTG ATA CCG AAA GA (R)	65		
parC	5′-AAC GCC TAC TTA AAC TAC TCC ATG T (F)	64	Nucleotides 46–403	358
	5′-CGC TCA ATA GCA GCT CGG AAT AT (R)	64		
parE	5′-TAT CCT GTG GCT GAA CCA GAA CG (F)	66	Nucleotides 1062–1644	583
	5′-AAG TAC GCC CTC TTT CTC TTC TTC (R)	64		
acrR	5′-CGT CGT GCT ATG GTA CAT ACA TTC (F)	63	Nucleotides −65 to +112[d]	825
	5′-CTG AAC CTG AAG AAC GAC CTG AA (R)	64		
marRA	5′-CCG ATT TAG CAA AAC GTG GCA TC (F)	65	Nucleotides −101 *marR* to 341 *marA*[d]	896
	5′-CCC TGC ATA TTG GTC ATC CGG TA (R)	66		
soxSR	5′-GCA GGT GTT TAT GCA ATG GAT GG (F)	64	Nucleotides −63 *soxS* to +57 *soxR*[d]	994
	5′-GCG GGA TAG AGA GAA AGA CAA AGA (R)	64		

[a] (F), forward; (R), reverse.
[b] Underlined primer, sequencing primer.
[c] T_m, melting temperature.
[d] −ve sign denotes upstream, +ve sign denotes downstream.

Table 2 Conditions for the uniplex and multiplex amplification reactions

Target amplicon	Primers ratio[a]	Cycling conditions
Individual genes	—	Initial denaturation at 94 ℃ (5 min), 35 cycles: denaturation at 98 ℃ (10 s), annealing at 60 ℃-touch-down-55 ℃ (30 s), extension at 72 ℃ (1 min) and final extension at 72 ℃ (5 min)
gyrA, *parC* and *parE* or *soxSR*, *acrR* and *marRA*	3:1:1	**Annealing at 57 °C-touch-down-54 °C (45 s)** [b]
gyrA, *soxSR*, *acrR*, *marRA*, *parC* and *parE*	6:6:4:4:1:1	**Annealing at 60 °C-touch-down-54 °C (45 s), extension at 72 °C (3.5 min)**[b]

[a] Listed in their respective order.
[b] The cycling conditions are the same as in the uniplex reactions except for the bolded steps.

the separated fragments was performed directly with little additional purification.[20] A 1% agarose gel was prepared using a gel mold, subsequently, a rectangle was cut out from the interior portion and a 0.2% agarose gel was poured into this space (Figure 1A). The fragments were initially separated on the 1% agarose portion of the gel and then allowed to enter the 0.2% portion of the gel, where they were cut, pooled, and purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Vilnius, Lithuania). Because of the subsequent purification and dilution steps, this method guaranteed that agarose concentrations were <0.2% in the sequencing reaction, because agarose concentrations above 0.2% could inhibit the reaction.[21]

DNA sequencing

Mutations in the QRDRs of the *gyrA*, *parC* and *parE* genes and mutations in the entire genes of the *marRA* system, *soxSR* system and *acrR* were identified by sequencing the purified PCR amplicons using the dideoxynucleotide chain termination method[22] with fluorescent cycle sequencing using dye-labeled terminators (BigDye Terminator version 3.1 cycle sequencing kit; Applied Biosystems, Grand Island, NY, USA) on an ABI prism 3730 automated DNA sequencer.

Genetic analysis

The online software Clustal Omega (EMBL-EBI, Hinxton, UK)[23] was used to perform the multiple sequence alignment. A mutation was considered evident if it resulted in a unique amino acid change when compared to the publicly available NCBI sequences of the *E. coli* K-12 susceptible substrains MG1655 and DH10B.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, USA). The general association of mutations with MIC values was analyzed using the Kruskal–Wallis (KW) test. The difference between isolates with and without mutations at specific MIC values was assessed using the Fisher's exact (FE) test. A P value of <0.05 for a whole family of tests was considered statistically significant. The Bonferroni correction method was used to

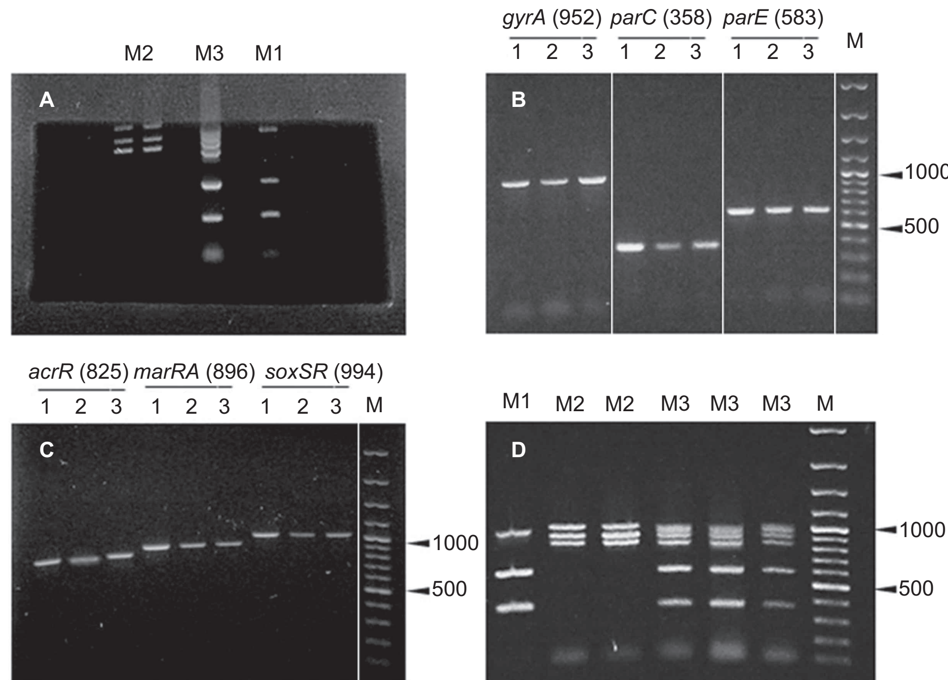

Figure 1 (**A**) Multiplex PCR reactions were electrophoresed in a 1.0% agarose gel frame with an embedded 0.2% agarose gel reservoir; (**B** and **C**) uniplex PCR reactions were electrophoresed in a 1.0% agarose gel; (**D**) multiplex PCR reactions were electrophoresed in a 1.0% agarose gel. Lane 1, isolate ZE14; lane 2, isolate ZU29; lane 3, *E. coli* K-12 as a positive control; lane M, 100 bp molecular size marker; lanes M1, M2 and M3, represent the (*gyrA*, *parC* and *parE*), (*acrR*, *marRA* and *soxSR*) and (*gyrA*, *parC*, *parE*, *acrR*, *marRA* and *soxSR*) multiplex amplification patterns, respectively.

re-correct for multiple comparisons in the KW and FE entire family of tests.

RESULTS

Antibiotic susceptibility profiles

Both the inhibition zones and MIC distributions for *E. coli* against levofloxacin (Figures 2A and 2B, respectively) showed three peaks (three modes) on their histograms. The constructed normal distribution curves showed an intersection between RS and S subpopulations over the range of the inhibition zones from 28 to 32 mm and a MIC of 0.062 μg/mL (Figures 2A and 2B, respectively). Cutoff values around the mean of each subpopulation were calculated to be at ±2.0 SDs, which corresponded to 95% of the subpopulation except for the HR suppopulation in which MIC cutoff values were set based on the genotype (Figure 3). The resultant cutoff values are listed in Table 3.

The susceptibility of the 103 *E. coli* isolates against levofloxacin was distributed as follows: 14 (13.6%) were resistant; two (1.9%) were intermediate; 16 (15.5%) showed reduced susceptibility; and 71 (86.9%) were susceptible.

PCR amplification products and fragment separation

Uniplex PCR reactions resulted in fragments of expected sizes using the primer pairs listed in Table 1 under the same cycling conditions (Figures 1B and 1C). Equimolar concentrations of the primers were then used to perform the multiplex reactions, which resulted in multiple bands; in larger fragments with weak intensities, this was optimized by increasing the primer pair ratio for weak fragments and prolonging the PCR extension time. The products of multiplex reactions were amplified in nearly equal amounts when the conditions listed in Table 2 were applied (Figure 1D). Interestingly, when fragments close in size (896 (*marRA*), 952 (*gyrA*) and 994 (*soxSR*) bps) moved into the 0.2% agarose region dedicated for extraction, they were more discernible (Figure 1A).

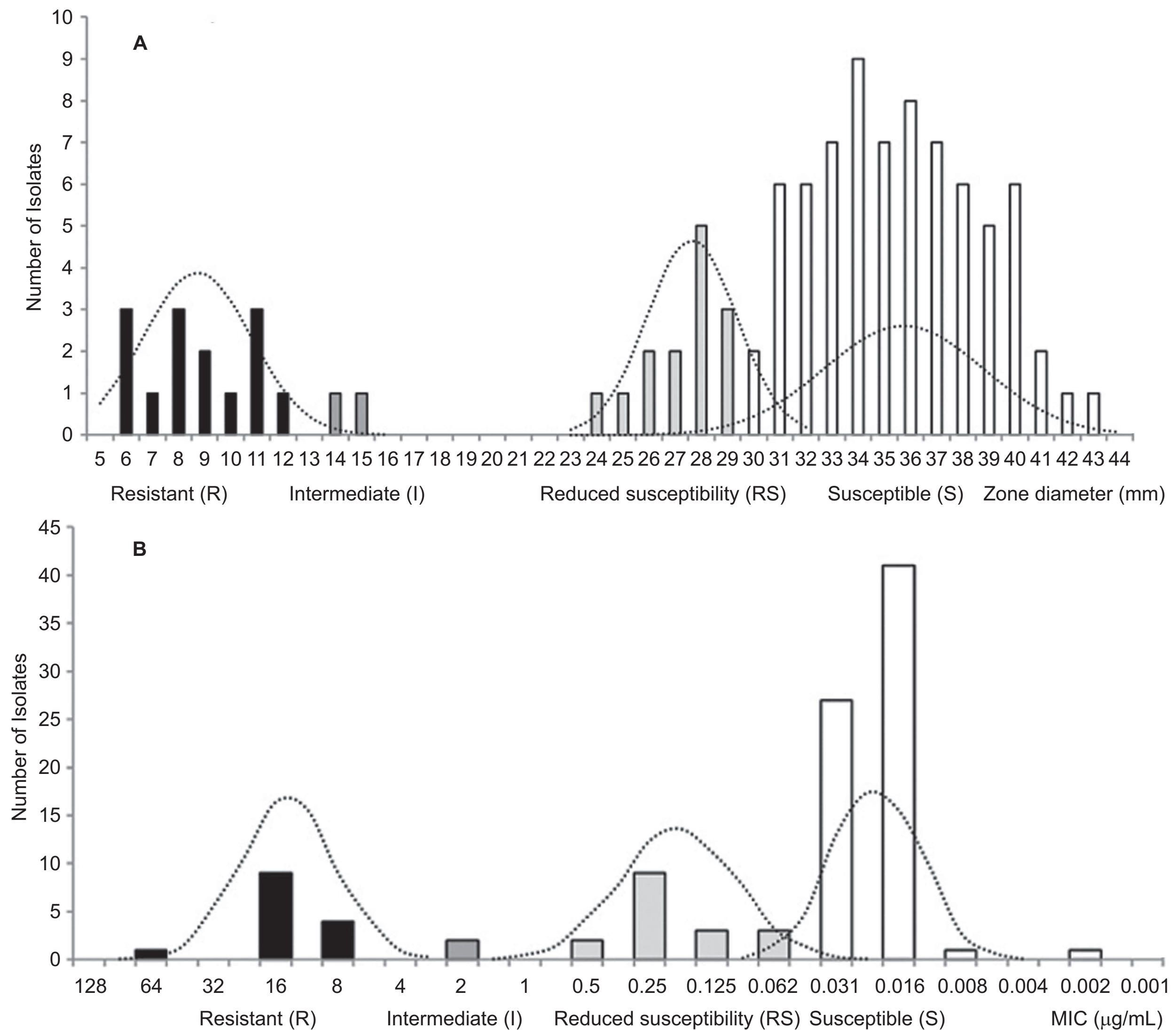

Figure 2 The recorded inhibition zone diameter of *E. coli* (**A**) and MIC (**B**) against levofloxacin. The diagram bars show the number of *E. coli* isolates. The generated normal distributions of the subpopulations are indicated by dotted line graphs. The normal distribution curves could not be generated over the intermediate (I) range due to too few data points. For MIC values, the generated normal distributions are based on the distribution of binary logarithms of MIC dilutions. The graphs represent the average of two determinations.

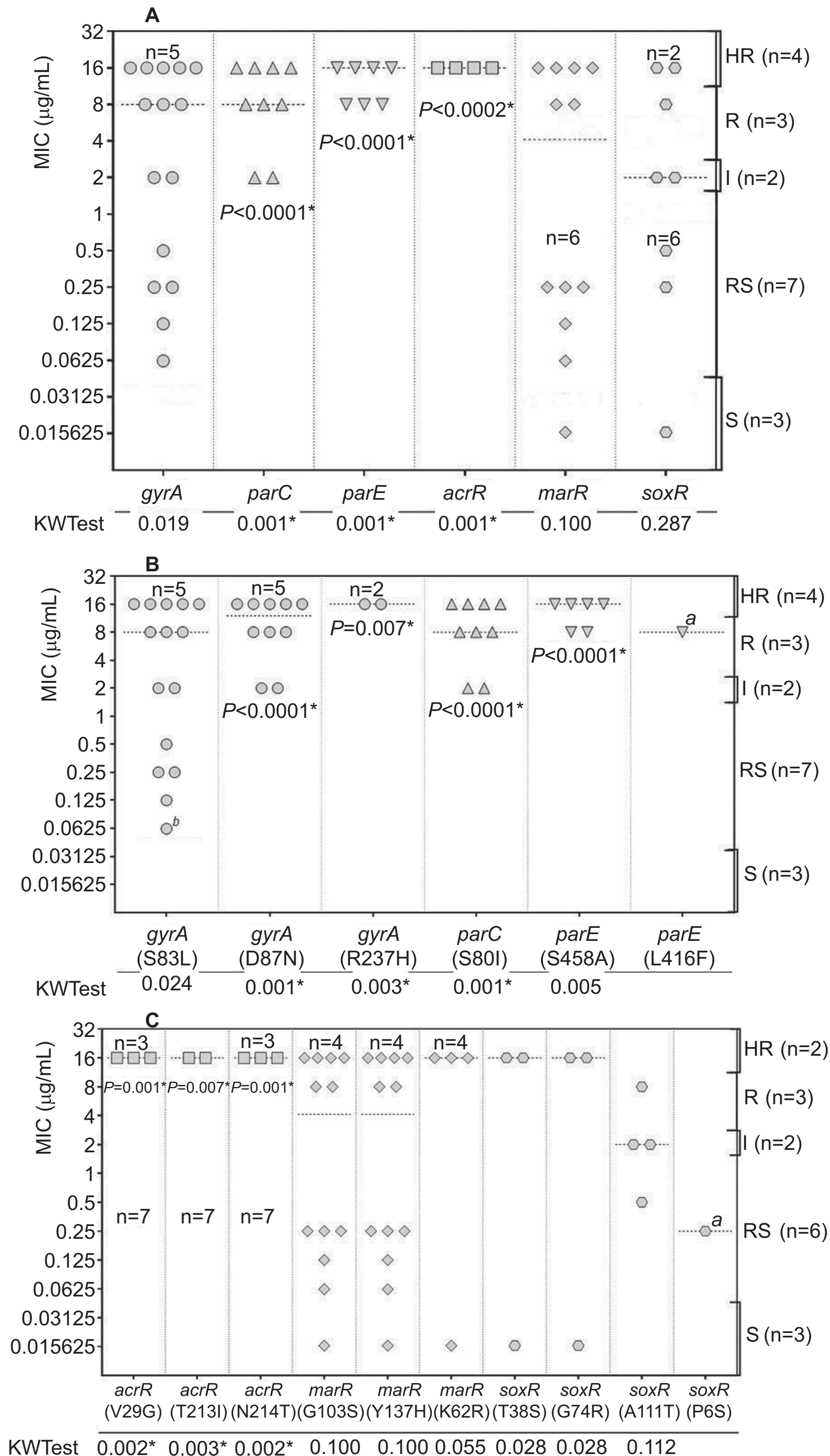

Figure 3 Resistance level and MIC for levofloxacin of *E. coli* isolates carrying mutated genes (**A**), point mutations in the topoisomerase genes (**B**) and point mutations in the efflux pump regulator genes (**C**). The dashed transverse lines represent the median MIC for each mutation. The difference between isolates with and without mutations at specific MIC levels was assed using the FE test with the *P* values presented on the graphs. The KW test was used to test the association of mutations with MIC values with the *P* values presented under the graphs. A *P* value with an asterisk is significant below the corrected levels of 0.0035 and 0.0071 for individual KW and FE tests, respectively. *n*=the number of sequences tested for each level of resistance unless otherwise stated on the data point. [a]Single observations, which could not be included in the statistical tests. [b]S83A rather than S83L.

Table 3 Cutoff values of *E. coli* subpopulations for inhibition zone and MIC distributions

	Inhibition zone cutoff values (mm)				MIC cutoff values (μg/mL)			
Subpopulation[a]	−2 SD	+2 SD	App.[b]	CLSI break points	−2 SD	+2 SD	App.[b]	CLSI break points
HR	4.6	**(12.8)**[c]	⩽13	⩽13	**(5)**[c]	40	⩾8	⩾8
R								
I	13.1	**(15.9)**[c]	14–16	14–16	**(2)**[c]	2	2–4	4
RS	24.2	31[d]	17–29	⩾17	0.05[d]	0.7	0.062–1	⩽2
S	**(29.6)**[c,d]	42	⩾30		0.008	**(0.055)**[c,d]	⩽0.031	

[a] (HR) subpopulation was distinguished from (R) subpopulation by the difference in their genotypes and the HR cutoff value was set at (⩾16) μg/mL for MIC.
[b] The approximated cutoff values that have been used in this study.
[c] (bold) These values were used for establishing the cutoff values defining the subpopulations after approximation to the next zone diameter or MIC dilution.
[d] The larger sample size of the susceptible isolates favored using the values of S over those of RS as cutoff values defining the two subpopulations.

Identification of mutations

DNA sequencing was performed on a random sample from each subpopulation using the sequencing primers listed in Table 1, with a total number of 18 samples sequenced (S (n=1), RS (n=7), I (n=2), R (n=3) and HR (n=5)). The NCBI sequences of the susceptible *E. coli* K-12 substrains MG1655 and DH10B were included in the analysis. The samples were sequenced only if they were consistently reported to belong to one of the subpopulations by both the disk diffusion and broth microdilution methods. The best qualities and longest chromatogram reads were obtained when uniplex PCR fragments were pooled and copurified (data not shown). The mutation profiles of the sequenced isolates are listed in Table 4. All HR isolates showed mutations in all the sequenced genes, whereas all R isolates had mutations in only four genes (three topoisomerases and one efflux regulator), and all I isolates had mutations in only three genes (two topoisomerases and one efflux regulator) (Figure 3A).

Interestingly, each of the two HR isolates (ZU14 and ZE29) harbored a total of 13 mutations in six different genes (Table 4). It was also observed that R isolates lacked mutations in *acrR*, and I isolates lacked mutations in *acrR*, *parE* and *marR*. Similarly, RS isolates lacked mutations in *acrR*, *parE*, *parC*, and the double mutation in *gyrA*, whereas S isolates lacked mutations in *acrR*, *parE*, *parC* and *gyrA*. None of the sequenced isolates carried mutations in *marA* (n=16) or *soxS* (n=14) at any level of resistance (Figure 3A).

On the level of single positions, the following results were observed: (i) none of the R isolates carried mutations in *gyrA*-R237H, *marR*-K62R, *soxR*-T38S, *soxR*-G74R or any position of *acrR*; (ii) none of the I isolates carried mutations in *gyrA*-R237H, *soxR*-T38S, *soxR*-G74R or at any position of *acrR*, *parE* or *marR*; (iii) none of the RS isolates harbored mutations in *gyrA*-R237H, *gyrA*-D87N, *marR*-K62R, *soxR*-T38S, *soxR*-G74R or any position of *acrR*, *parE* or *parC*; and (iv) none of the S isolates harbored mutations in any position of *acrR*, *parE*, *parC* or *gyrA* (Figures 3B and 3C).

Associations between different mutations and levels of resistance

A total of 20 sets of sequences (18 from the sequencing step in this study and two from NCBI) were subjected to statistical analysis tests. The KW test was first used to pinpoint whether there was an association between any of the levels of resistance with the different mutations. Because the KW test does not identify where the associations occur or how many actually occur, the FE test was then used to locate the associations between specific levels of resistance and specific mutations. Significant associations were found between the following mutations and levels of resistance: (i) the mutations in *acrR* and HR level, *parE* and R/HR levels, *parC* and I/R/HR levels (Figure 3A); (ii) the mutation *gyrA*-R237H and HR level, *gyrA*-D87N and I/R/HR levels (Figure 3B); and (iii) mutations in *acrR* (all positions) and HR level, *parE*-S458A and R/HR levels, *parC* S80I and I/R/HR levels (Figures 3B and 3C). Corrected P values for individual KW and FE tests are shown in Figure 3.

DISCUSSION

In this study, we attempted to obtain a panoramic view of the changes in the genotype of *E. coli* isolates and to pinpoint possible associations between these changes and their corresponding level of resistance to levofloxacin. In previous studies, the mutation pattern of *gyrA*-S83L/D87N was shown to be related to levofloxacin resistance, whereas the single mutation *gyrA*-S83L was not.[26,27] Faye *et al.*[10] explained that double-mutant enzyme-DNA complexes had a lower affinity for quinolones than wild-type complexes. This explanation is confirmed in this study through the significant association between the double mutation and I/R/HR levels. Moreover, we located a third mutation outside the conventional QRDR, which associated the triple mutant *gyrA*-S83L/D87N/R237H with HR levels.

Although the role of *acrR* in establishing a clinically significant HR level could be attributed to the overexpression of the AcrAB efflux pump via *acrR* mutations, other mechanisms should be considered.[7,13,28,29] Similarly, the effect of single and double mutations in *parC* on resistance were previously reported whereby complementation with the wild-type allele significantly reduced the resistance level.[11,13] The contribution of the double mutation *soxR*-T38S/G74R to resistance may be through the overexpression of SoxS, which could also be overexpressed by other mechanisms.[9] No significant association was found between the double mutation *marR*-G103S/Y137H and any level of resistance. This supports the notion that the contribution of *marR* mutations to resistance levels is through the accumulation of mutations in other sites in the *mar* mutants, a phenomenon that was not found in wild-type *E. coli* isolates.[8] The *marR*-K62R mutation found in some of the HR isolates in this study was previously reported as irrelevant to the loss of MarR function.[6]

Interestingly, two isolates (found amongst the HR clinical isolates) harbored 13 mutations on six different genes. To the best of our knowledge, this number of mutations has not been previously reported and thus these two isolates are labeled as 'supermutators'. It should be noted that these isolates were obtained from two different specimens in two different hospitals in Egypt. In fact, the variation in sequence (including silent mutations) and phenotype indicated that all sequenced isolates were from different clones.

There may be additional resistance mechanisms in such supermutator isolates, including the following mechanisms: mutations in *mutM*, *ligB* and *recG* encoding other putative DNA binding enzymes;[30] mutations in *tolC* encoding the outer part of the AcrAB-TolC efflux

Table 4 Topoisomerases and efflux pumps regulators mutations in sequenced *E. coli* isolates/strains

Isolate/strain[a]	MIC (μg/mL)	Topoisomerases mutations[b]			Efflux pumps regulators mutations[b,c]			Number of mutations[d]
		gyrA	*parC*	*parE*	*acrR*	*marR*	*soxR*	
ZE72	0.016	-	-	-	-	G103S, Y137H, K62R	T38S, G74R	5 (2)
ZE37	0.062	S83A	-	-	-	G103S, Y137H	-	3 (2)
ZE54	0.125	S83L	-	-	-	G103S, Y137H	-	3 (2)
ZE39	0.25	S83L	-	-	-	G103S, Y137H	-	3 (2)
ZE19	0.25	-	-	-	-	G103S, Y137H	-	2 (1)
ZE11	0.25	S83L	-	-	-	G103S, Y137H	P6S	4 (3)
ZE12	0.5	S83L	-	-	-	-	A111T	2 (2)
ZE62	0.5	-	-	-	-	ND	ND	0 (0)
ZE7	2	S83L, D87N	S80I	-	-	-	A111T	4 (3)
ZE16	2	S83L, D87N	S80I	-	-	-	A111T	4 (3)
ZE41	8	S83L, D87N	S80I	S458A	-	-	A111T	5 (4)
ZE83	8	S83L, D87N	S80I	L416F	-	G103S, Y137H	-	6 (4)
ZE21	16	S83L, D87N, ND	ND	ND	ND	ND	ND	2 (1)
ZE29	16	S83L, D87N, R237H	S80I	S458A	V29G, T213I, N214T	G103S, Y137H, K62R	T38S, G74R	13 (6)
ZU11	8	S83L, D87N	S80I	S458A	-	G103S, Y137H	-	6 (4)
ZU7	16	S83L, D87N, ND	S80I	S458A	N214T, ND	G103S, Y137H	ND	7 (5)
ZU12	16	S83L, D87N, ND	S80I	S458A	V29G, ND	G103S, Y137H, K62R	ND	8 (5)
ZU14	16	S83L, D87N, R237H	S80I	S458A	V29G, T213I, N214T	G103S, Y137H, K62R	T38S, G74R	13 (6)
DH10B[e]	0.008	-	-	-	-	-	-	0 (0)
MG1655[e]	0.016	-	-	-	-	-	-	0 (0)

[a] Grouped by source and listed by levofloxacin MIC. Isolates with (ZE) designation were from stool samples, while those starting with (ZU) were from urine samples.
[b] ND, not determined; -, wild type.
[c] No mutations were found in *marA* or *soxS* in all the isolates.
[d] Numbers in brackets were the numbers of the genes involved.
[e] MIC values were obtained from references 24 and 25, respectively.

pump;[31] the acquisition of plasmid-borne genes *qnr* and *aac(6′)-Ib-cr* that encode drug-blocking and drug-modifying proteins, respectively; the overexpression of quorum-sensing regulator SdiA; mutations in *robA* encoding the global regulator RobA; and mutations in *gyrB* encoding the B subunit of the gyrase enzyme.[4] Such resistance mechanisms present themselves as interesting locations for investigation in future studies. A previous study showed that QRDR mutations of *gyrA*, *parC* and *parE*, generally had a greater effect on FQ MICs than the acquisition of plasmid-borne genes or overexpression of the efflux pump.[32]

The novel mutations *gyrA*-R237H and *acrR*-V29G associated with the HR isolates require further study to reveal their possible roles in resistance. These mutations may contribute to increased fitness rather than increased levels of resistance.[33] The same role could be played by *soxR*-P6S and *parE*-L416F, which could not be included in the statistical tests as each was recorded once in the sequenced isolates. Hence, more investigation is needed to sequence a larger number of *E. coli* isolates with a greater focus on isolates collected from blood samples. This could increase the power of the statistical tests and possibly reveal more significant associations especially for *soxR*-T38S/G74R and the single mutation *gyrA*-S83L. The *parE*-L416F mutation was reported in small ratios of the isolates screened in a previous study.[34]

The appearance of three modes on the zone diameter and MIC histograms indicated that the collected isolates included non-wild-type isolates that harbored resistance mechanisms. This was previously observed for the MIC distribution of *E. coli* obtained from The European Committee on Antimicrobial Susceptibility Testing (n=3219) on moxifloxacin showing a pattern with two major peaks and a tendency toward a third peak in between these two peaks. For levofloxacin, the same study set the epidemiological breakpoint for the wild-type population (n=9144) at $\leqslant$0.082, whereas European Committee on Antimicrobial Susceptibility Testing reported $\leqslant$0.25 and the CLSI reported $\leqslant$2 μg/mL.[16] Additionally, in this study, the established five-level interpretation criteria included the RS range. Such classifications have been reported before by the CLSI for vancomycin against *Staphylococcus aureus*.[15] In fact, mutations in the *gyrA* and AcrAB efflux pump regulator genes were found in the RS isolates, which were not distinguished from the S range.

Direct sequencing of products from a multiplex reaction is usually unsuccessful. This could be attributed to the high percentage of the mixture of primers that remains after the purification step. Hence, the separation of the products on an agarose gel would remove the interference of the primer mixture. However, the cut and pooled target fragments would have a high percentage of agarose, which could inhibit subsequent sequencing reactions.[21] In this regard, the present study introduced a modification to the method of Ma *et al.*[20] to recover the products from a two-gel system with a minimum agarose percentage and no primer mixture interference. Additionally, we designed multiplex PCR protocols for high-throughput sequencing of the QRDRs of topoisomerase genes *gyrA*, *parC* and *parE*, and/or the multidrug efflux pump AcrAB expression regulation systems *acrRAB*, *marRA*, and *soxSR* (with maximum of six PCR products). Interestingly, a recent study developed a multiplex PCR protocol to detect up to eight plasmid-mediated quinolone-resistance determinants, which enabled the detection of resistance mechanisms in 37% of ciprofloxacin-resistant *E. coli* isolates.[35] Combining this method (to detect plasmid-mediated mechanisms) with the method developed in our study (to detect mechanisms due to chromosomal mutations) could further expand the understanding of quinolone-resistance in future studies.

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