International Journal of Antimicrobial Agents 56 (2020) 106097

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Adaptive laboratory evolution of *Vibrio cholerae* to doxycycline associated with spontaneous mutation

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ARTICLE INFO

Article history:
Received 15 April 2020
Accepted 13 July 2020

Keywords:
Adaptive laboratory evolution
Antimicrobial resistance
Co-resistance
Doxycycline
RpsJ
Vibrio cholerae

ABSTRACT

Cholera, caused by the Gram-negative bacterium *Vibrio cholerae*, remains a serious threat in underdeveloped countries. Although rehydration therapy has been the mainstay of disease management, antibiotics are also being used as an adjunct treatment, resulting in an increase in the circulation of antimicrobial-resistant *V. cholerae* strains. In the present study, adaptive laboratory evolution, whole-genome sequencing and molecular docking studies were performed to identify putative mutations related to doxycycline resistance in *V. cholerae* isolates. The V57L mutation in the RpsJ protein was identified to be important in conferring doxycycline resistance. As revealed by molecular docking studies, the mutation was identified to alter the ribosome structure near the doxycycline binding site. Doxycycline stress also induced co-resistance to colistin, a last-resort antibiotic to treat extensively drug-resistant bacteria. This study illustrates for the first time a possible mechanism of doxycycline-selected resistance in *V. cholerae* as well as doxycycline-selected co-resistance, warranting strict restrictions on the indiscriminate use of antibiotics.

1. Introduction

The Gram-negative bacterium *Vibrio cholerae* is responsible for 1.4–4.3 million cases of cholera and 21 000–143 000 deaths per year worldwide [1]. Although cholera is self-limiting and rehydration therapy serves as the primary treatment for the disease, selective use of antibiotics in patients who have a high purging rate is recommended [2]. However, over the years, an increasing frequency of antibiotic-resistant *V. cholerae* isolates has been observed in cholera-endemic countries, including India. The rationale for choosing an antibiotic to treat a disease is based on its efficacy, availability, safety, cost and, most importantly, the local resistance pattern of the pathogen. Presently, the drug of choice to treat cholera is doxycycline, a broad-spectrum synthetic antibiotic derived from tetracycline. Currently circulating strains of *V. cholerae* are susceptible to doxycycline and it also has lesser teratogenic effects and dental discoloration, thus making it safe for the treatment of pregnant women and young children [3].

Pathogens acquire resistance to antimicrobial compounds by different mechanisms. Common mechanisms of resistance acquisition are spontaneous mutation and horizontal gene transfer. Although the emergence of antimicrobial resistance by accumulation of spontaneous mutation is less common in *V. cholerae*, selective antibiotic pressure over a long period can cause spontaneous mutations that provide increased fitness of the bacteria [4]. Doxycycline is used as the first-line drug of choice to treat cholera patients in India. Apart from being used to treat bacterial and parasitic infections in humans, doxycycline is also widely used in veterinary medicine. Doxycycline has a longer period of bioavailability and is readily absorbed by cells owing to its lipophilic nature. Moreover, 90% of the drug is excreted in a non-degraded form in the urine and faeces. Several studies have shown the presence of doxycycline residues in the environment [5]. Recently, we reported the minimum inhibitory concentration (MIC) creep of doxycycline in *V. cholerae* over the years [6]. We hypothesise that antibiotic residues in the environment can alter the microbial community structure and function and cause selective pressure on them to evolve.

The main aim of this study was to gain insights into the mechanism of doxycycline resistance progression in *V. cholerae*. Adaptive laboratory evolution of *V. cholerae* starting in sub-MICs of doxycycline was performed to assess the rate of adaptive evolution of the pathogen to doxycycline and to determine the presence of proto-resistance genes.

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https://doi.org/10.1016/j.ijantimicag.2020.106097

2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

The antimicrobial susceptibility of a set of 39 *V. cholerae* clinical isolates collected from different geographical locations in South India over the period 2000–2018 was determined by the Kirby–Bauer disk diffusion method [7] on Mueller–Hinton agar (M173; HiMedia Laboratories, Mumbai, India). The following antibiotic disks (HiMedia Laboratories) were used: ampicillin (10 μg); chloramphenicol (30 μg); ciprofloxacin (5 μg); trimethoprim/sulfamethoxazole (SXT) (25 μg); gentamicin (10 μg); streptomycin (10 μg); trimethoprim (5 μg); tetracycline (30 μg); nalidixic acid (30 μg); norfloxacin (10 μg); erythromycin (10 μg); azithromycin (15 μg); polymyxin B (50 μg); imipenem (10 μg); cefoxitin (30 μg); ceftazidime (30 μg); ceftriaxone (30 μg); and cefepime (30 μg). *Escherichia coli* ATCC 25922 was used for internal quality control.

The MIC of doxycycline was determined by Etest (AB bioMérieux, Solna, Sweden) and cut-off levels for resistance were according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8].

2.2. Adaptive laboratory evolution (ALE) of Vibrio cholerae under doxycycline stress

A representative *V. cholerae* strain (W4-13) was used for ALE. The strain had previously undergone whole-genome sequencing (WGS) and the genome sequence was deposited at DDBJ/EMBL/GenBank under accession no. **NIWX00000000** [9]. An overnight broth culture of strain W4-13 was used as the initial inoculum for the ALE experiment. The optical density at 600 nm (OD_{600}) of the culture broth was recorded and then 100 μL of exponentially-growing cells were transferred into 2.0 mL of Mueller–Hinton broth (MHB) containing a sub-MIC (0.5 μg/mL) of doxycycline (D1822; Merck, St Louis, MO, USA) (ALEdoxy). Simultaneously, 100 μL of exponentially-growing cells were transferred to 2.0 mL of MHB without antibiotic supplementation (ALEcontrol) and the tubes were incubated under the same conditions. These batch cultures were transferred manually every 12 h at an initial OD_{600} of ~0.05. With each transfer, the concentration of doxycycline antibiotic was slowly scaled up (0.5 μg/mL per transfer) up to a concentration determined to be the resistance breakpoint (4.0 μg/mL) of doxycycline for *V. cholerae*. The doxycycline concentration was increased up to three times the concentration of the resistance cut-off (12.0 μg/mL).

2.3. Validation of stress adaptation

2.3.1. Growth curve assay

Growth curve assays of the adaptive laboratory-evolved *V. cholerae* strains in medium containing doxycycline (ALEdoxy) and medium without doxycycline (ALEcontrol) were performed both in medium containing doxycycline and without the antibiotic. The OD_{600} was measured for the collected samples at constant intervals and a graph was plotted of OD_{600} vs. time (in hours). The assay was performed in triplicate.

2.3.2. Minimum inhibitory concentration determination

The MIC of doxycycline was determined by Etest (AB bioMérieux) for the W4-13 ALEdoxy and ALEcontrol strains.

2.4. Determination of co-resistance

After ALE, the ALEdoxy and ALEcontrol strains were subjected to antibiogram profiling with the previously mentioned antibiotics and were compared with strain W4-13 to determine the presence of co-resistance.

2.5. Whole-genome sequencing and analysis

The ALEdoxy and ALEcontrol strains underwent WGS, and comparative genomics was performed to identify distinctive mutational patterns between the doxycycline-challenged (ALEdoxy) and unchallenged strains (ALEcontrol).

Whole bacterial genomic DNA was isolated using a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The purity and concentration of DNA was analysed using NanoDrop™ 2000 (Thermo Scientific, USA) and a Qubit 2.0 fluorometer (Thermo Scientific), and integrity was checked by running the DNA samples in 0.8% agarose gels. An Illumina HiSeq System (Illumina Inc.) was used for WGS. Pre-processed reads were aligned with the reference *V. cholerae* O1 biovar El Tor strain N16961 genome downloaded from the National Center for Biotechnology Information (NCBI). Single nucleotide polymorphism (SNP) calling was performed using SAMtools v.1.9 [10]

2.6. Molecular docking and simulation studies

Molecular modelling of the target proteins RpsJ-V57 and RpsJ-L57 was performed using a SWISS-MODEL protein folding server with 30S ribosomal protein template structure (PDB: 4v50). The high level of sequence homology between target and template proteins suggests that they may be functional homologues. Docking and molecular dynamics simulation methods were performed as described previously [11].

3. Results and discussion

3.1. Multidrug resistance and increasing doxycycline resistance in Vibrio cholerae

Increasing drug resistance in *V. cholerae* has been reported from all over the world, which is attributed to the indiscriminate use of broad-spectrum antibiotics to treat cholera. In the present study, the majority (97%) of isolates were multidrug-resistant (MDR) and a high percentage demonstrated resistance to trimethoprim, SXT, streptomycin and nalidixic acid. A few strains showed resistance to ampicillin (26%) and erythromycin (8%), and one strain (W1-11) showed resistance to ciprofloxacin in addition to the above antibiotics. However, all of the strains were susceptible to tetracycline, chloramphenicol, gentamicin, azithromycin and norfloxacin. All *V. cholerae* strains except one (A880) was identified to be resistant to successive generations of cephalosporin antibiotics (cefoxitin, ceftazidime and cefepime). However, it is noteworthy that all of the strains were susceptible to the third-generation cephalosporin ceftriaxone (Supplementary Table S1). A previous study documented *V. cholerae* strains isolated from India that were resistant to ceftriaxone [12]. Although doxycycline resistance in environmental non-O1/non-O139 *V. cholerae* has been documented previously [13], reports on *V. cholerae* O1 strains resistant to doxycycline are scarce [14]. We previously reported an increase in reduced susceptibility of *V. cholerae* to doxycycline [6], describing *V. cholerae* strains with susceptibility at the borderline of the susceptibility breakpoint (3.0 μg/mL) as well as Haitian variant strains with reduced susceptibility at 0.75 μg/mL [6]. Global dissemination of such MDR strains having reduced susceptibility to doxycycline across cholera-endemic countries may affect treatment efficacy.

3.2. Selection of doxycycline-resistant Vibrio cholerae strain

A well-known mechanism of doxycycline resistance in bacteria is ribosomal protection by cytoplasmic proteins with homology to

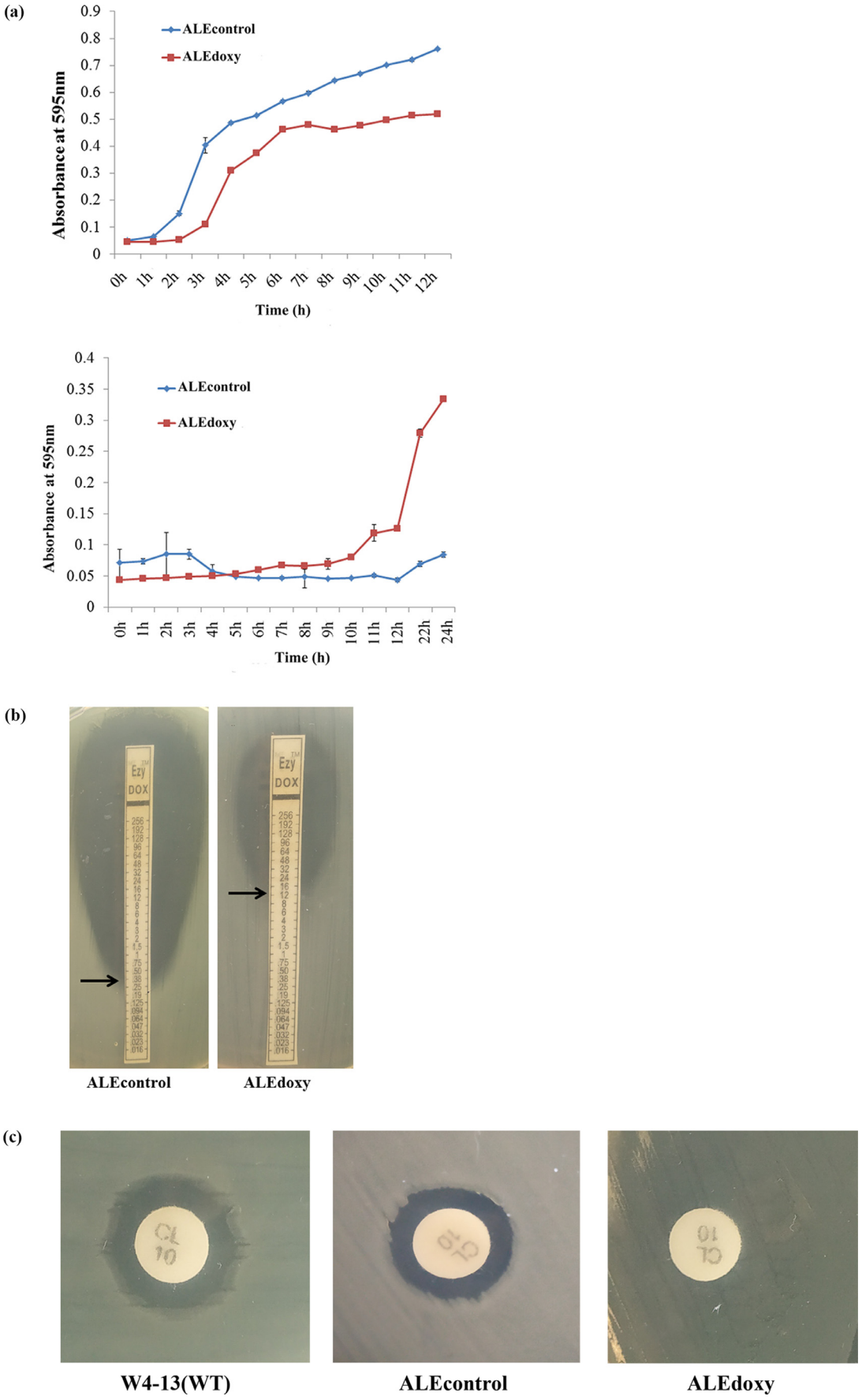

Fig. 1. (a) Growth curve assay of ALEcontrol and ALEdoxy strains of *Vibrio cholerae* in Luria–Bertani (LB) broth without doxycycline (top) and LB broth supplemented with 12.0 μg/mL doxycycline (bottom). (b) Minimum inhibitory concentration (MIC) analysis of ALEcontrol and ALEdoxy strains of *V. cholerae* against doxycycline by Etest. (c) Antimicrobial susceptibility assay of wild-type W4-13(WT), ALEcontrol and ALEdoxy strains against colistin, revealing complete resistance of ALEdoxy to the antibiotic.

elongation factors EF-Tu and EF-G [15]. In the current study, strains were observed to gain increased resistance to doxycycline at a slow pace over the years and hence the possibility of resistance by acquisition of genes was ruled out. Thus, it was hypothesised that it could be due to the development of resistance by spontaneous mutations owing to exposure to doxycycline from the environment or therapy. The objective of ALE of *V. cholerae* strains to reach 3-fold above the resistance breakpoint (12.0 μg/mL) of doxycycline was achieved after 270 days of constant application of selective doxycycline pressure in a progressive manner. Adaptation of *V. cholerae* to the initial stresses of doxycycline was slow and took ~210 days to exhibit resistance to 4.0 μg/mL doxycycline. However, the progression of *V. cholerae* to exhibit resistance to doxycycline above the resistance breakpoint and to reach resistance of 12.0 μg/mL was rapid (60 days). The ALEdoxy strain was observed to have a decreased growth advantage on a thiosulfate–citrate–bile salts–sucrose (TCBS) agar plate compared with the ALEcontrol strain. Visible growth of yellow *V. cholerae* colonies was observed on ALEdoxy TCBS plates only after 2–3 days of incubation at 37 °C. No growth of the ALEcontrol strain was observed on Luria–Bertani (LB) agar plates supplemented with doxycycline even after prolonged incubation, whereas the ALEdoxy strain grew well on doxycycline-supplemented LB plates. This is the first report to demonstrate that *V. cholerae* gains resistance to doxycycline by continuous exposure of the isolates to the antibiotic. Also, it is important to note that cells progressing towards resistance were slow growing and went unnoticed when the plates were incubated for only 18 h, which is the conventional incubation time. This slow growth of bacterial cells is considered as a strategy to be invulnerable against the effects of antibiotics.

3.3. Stress adaptation to doxycycline

Both ALEdoxy and ALEcontrol strains grew in LB + 0.5% NaCl broth. However, the ALEdoxy strain had a reduced growth rate compared with the ALEcontrol strain. The ALEdoxy strain had a prolonged lag phase of 3 h compared with the ALEcontrol strain that commenced log phase by the second hour. The ALEdoxy strain also achieved stationary phase earlier than the ALEcontrol strain (Fig. 1a, top). On the other hand, ALEdoxy had a growth advantage over the ALEcontrol strain in LB + 0.5% NaCl broth supplemented with 12.0 μg/mL doxycycline. There was a prolonged lag phase for both strains. However, after the tenth hour there was an increase in growth of the ALEdoxy strain, which increased up to 24 h. There was no increase in growth of the ALEcontrol strain, which remained static up to 24 h (Fig. 1a, bottom).

Etest revealed that the ALEdoxy strain showed increased resistance to doxycycline (12.0 μg/mL) compared with the ALEcontrol (0.25 μg/mL) (Fig. 1b). This demonstrated that there has been an adaptive evolution of the *V. cholerae* strain to doxycycline stress, indicating that a similar scenario is also possible in the environment. The susceptibility of the ALEcontrol strain to doxycycline proved that the development of doxycycline resistance in ALEdoxy was purely due to successive exposure of the strain to the antibiotic.

3.4. Co-resistance and collateral sensitivity of adaptive laboratory-evolved Vibrio cholerae

It was interesting to observe that there was a difference in the antibiotic susceptibility pattern between the ALEdoxy and wild-type W4-13 strain. The adaptive laboratory-evolved *V. cholerae* strain showed increased resistance to ampicillin, norfloxacin and chloramphenicol. Co-resistance was observed to different classes of antibiotics such as β-lactams, quinolones and chloramphenicol. However, the ALEdoxy strain developed susceptibility to antibiotics to which it was previously intermediate-resistant. Such

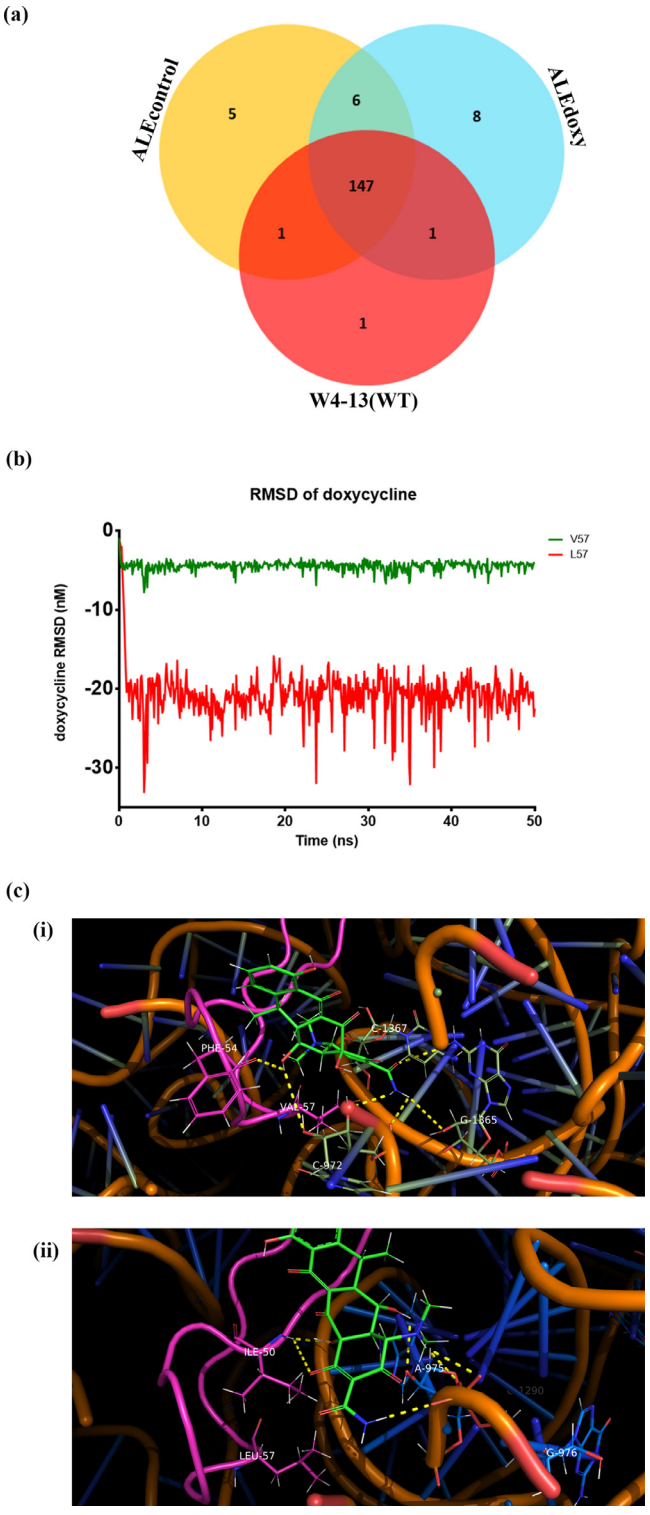

Fig. 2. (a) Number of common and unique variants identified via whole-genome sequencing of ALEcontrol, ALEdoxy and W4-13(WT) *Vibrio cholerae* strains. (b) Root-mean-square deviation (RMSD) analysis of doxycycline and RpsJ(V57) and RpsJ(L57). (c) Docking conformation of doxycycline with (i) RpsJ(V57) and (ii) RpsJ(L57). Residues interacting with the ligand are represented by lines.

increased susceptibility of bacteria is known as collateral sensitivity. The doxycycline-resistant strain showed collateral sensitivity to azithromycin and kanamycin. Strains becoming more susceptible to antibiotics on constant exposure to a particular antibiotic in long-term exposure experiments were recently reported by Lamrabet et al. [16]. The understanding of collateral sensitivity can be

Table 1
Unique variants identified in each *Vibrio cholerae* sample.

Sample	Gene	Variant position	Ref.	Alt.	Variant type	Protein change
ALEcontrol	*flgG*	NC_002505.1:2342560	A	C	SNP: missense	p.V206G
ALEdoxy	VC0040	NC_002505.1:38427	C	T	SNP: missense	p.H194Y
	VC0166	NC_002505.1:163926	G	A	SNP: nonsense	p.Q113X
	VC1311	NC_002505.1:1393477	C	T	SNP: silent	p.A43A
	rpsJ	NC_002505.1:2764613	C	G	SNP: missense	p.V57L
	cpxA	NC_002505.1:2861085	G	A	SNP: missense	p.A199T
	VCA1057	NC_002506.1:1008799	T	C	SNP: silent	p.A19A
W4-13(WT)	VCA1095	NC_002506.1:1050706 - 1050714	GTTGACCAT	G	indel: frameshift	p.L579fs

Ref., nucleotide in the reference genome; Alt., altered nucleotide in the current study; SNP, single nucleotide polymorphism; indel, insertion/deletion; V, valine; G, glycine; H, histidine; Y, tyrosine; Q, glutamine; A, alanine; L, leucine; T, threonine; X, protein truncation; fs, frameshift mutation.

used for collateral sensitivity cycling to counterselect a resistant subpopulation in laboratory conditions and is hypothesised to be a new treatment framework in which drugs with compatible collateral sensitivity profiles can be used sequentially to treat infection and select against the development of drug resistance.

3.4.1. Increased resistance to polymyxin antibiotics

Another major finding was that ALEdoxy became completely resistant to polymyxin antibiotics, namely polymyxin B and polymyxin E (colistin), a last-resort drug to treat MDR Gram-negative bacterial infections, compared with the wild-type W4-13 strain, which had a zone of inhibition of 11 mm (Fig. 1c). These antibiotics, which fall in the antimicrobial peptide (AMP) category, act on bacterial lipopolysaccharides and phospholipids by competitively displacing divalent cations from the membrane phospholipids and thereby disrupting the outer cell membrane. A major concern of increasing resistance to AMPs is that it induces cross-resistance to AMPs that are effectors in the human innate immune system and thus compromises natural defence against pathogens.

3.5. Common and unique variants identified

Comparison of WGS data and variant annotation between the ALEdoxy, ALEcontrol and wild-type W4-13 *V. cholerae* strains revealed a total of 237 variants, of which 225 were SNPs and 10 were indels (insertion/deletions). A total of 147 variants (145 SNPs and 2 indels) were present in all three samples (common variants). Five variants (all SNPs) were identified to be present only in ALEcontrol, eight variants (seven SNPs and one indel) were present only in ALEdoxy, and one variant (an indel) was present only in W4-13. The common and unique variants among the three samples are given in Fig. 2a.

Mutations unique to the ALEdoxy strain were in VC0040, VC0166, VC1311, *rpsJ*, *cpxA* and VCA1057 (Table 1). Interestingly, most of the genes that were identified to acquire SNPs in ALEdoxy strains were reported to be associated with resistance of *V. cholerae*, except VC0040 encoding a haemolysin/cytolysin protein. However, it was observed that a few genes acquired mutations that did not lead to an amino acid change (silent mutation) and thus there was no change in their expression or there was neither a loss of function nor gain of function.

3.6. Doxycycline resistance by rpsJ mutation

Genes that acquired missense mutations (a point mutation that leads to an amino acid change) were VC0040, VC0166, *rpsJ* and *cpxA*. Among the four genes, *rpsJ* mutations encoding changes in amino acids of the 30S ribosomal subunit protein S10 have been linked to tetracycline and tigecycline resistance in Gram-positive micro-organisms such as *Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis* and *Bacillus subtilis* [17] as well as in Gram- negative micro-organisms such as *Klebsiella pneumoniae* [18], *E. coli* and *Acinetobacter baumannii* [17]. In 2014, Villa et al. reported the identification of highly tigecycline-resistant *K. pneumoniae* with a similar V57L mutation in the RpsJ [18]. It was hypothesised that the specific mutation was acquired on constant exposure of the pathogen to tigecycline in hospital settings, and the V57L mutation in RpsJ alters the ribosome structure near the tigecycline binding site or disturbs the coordination of the Mg^{2+} ion, leading to weaker binding of tigecycline to 16S rRNA. Also, high levels of tetracycline resistance were described in *Neisseria gonorrhoeae* that possessed a point mutation in Val57 codon of the S10 protein [19]. This proves that tetracycline antibiotics can select for *rpsJ* mutation. It was interesting to note that associations among a few genes that acquired SNPs were as a result of doxycycline stress during ALE.

3.7. Validation of doxycycline resistance by RpsJ mutation through molecular docking studies

To validate the effect of RpsJ mutation (V57L), molecular docking was performed. Molecular docking methods investigated the binding affinity of doxycycline by docking it with the RpsJ binding pocket in each protein [RpsJ(V57) and RpsJ(L57)], which composes mutation in the S10 loop adjacent to 16S rRNA. Docking studies using AutoDock showed binding energies of −5.3 kcal/mol and −4.5 kcal/mol for doxycycline docked in the binding pocket of RpsJ(V57) and RpsJ(L57), respectively. The results showed that RpsJ(V57) has a stronger affinity with doxycycline and is likely to have more identified intermolecular interactions involving hydrogen and hydrophobic bonds compared with RpsJ(L57).

The docked complexes were further investigated using molecular dynamics simulations performed for 50 ns. Root-mean-square deviation (RMSD) values were calculated for the positional differences of doxycycline backbone atoms in the RpsJ(V57) and RpsJ(L57) complexes. A comparison of the trajectories illustrates that a long stable RMSD plateau for doxycycline of the RpsJ(V57) complex (lower RMSD) indicates good stability of the complex. However, the RpsJ(L57)–doxycycline complex experienced severe fluctuations throughout the simulation period, revealing an unstable complex, where the initial docked conformation of doxycycline deviated from the RpsJ binding pocket structure after ~5 ns (Fig. 2b). From visualisation, doxycycline is observed to interact with Val57 in the binding pocket of RpsJ(V57) and also stabilised by rRNA, whereas it does not make interactions with Leu57 and it appears far from the cavity of RpsJ(L57) and 16S rRNA (Fig. 2c).

3.8. Doxycycline stress induced mutations that cause co-resistance

Another major gene that was identified to acquire a SNP was the *cpxA* gene. The CpxA/CpxR two-component system is widely present in Gram-negative bacteria, especially Gammaproteobacte-

ria. It has been previously reported that CpxA mutants that constitutively activate *cpxR* are resistant to AMPs such as polymyxins. This could be the reason for the increased resistance to polymyxin antibiotics observed in the ALEdoxy strain. Also, the CpxA/CpxR two-component system is known to activate the multidrug efflux pump MarRAB that senses compounds such as tetracycline and acetaminophen [20].

4. Conclusion

In this study, 97% of the *V. cholerae* isolates were found to be MDR. MIC creep of doxycycline in *V. cholerae* strains over the years was observed, and ALE of *V. cholerae* in the presence of doxycycline as well as WGS revealed that it could acquire resistance by accumulating SNPs. Analysis of the functional effects of the accumulated SNPs revealed that that the RpsJ V57L mutation could be the most significant reason for the increased MIC of doxycycline in *V. cholerae*. However, only complementation studies in *rpsJ* V57L mutant with the wild-type *rpsJ* gene and checking the MIC of doxycycline to identify whether doxycycline susceptibility is restored would confirm the above statement. Hence, this study emphasises that antibiotics at sub-MICs present in many environments could drive the evolution of clinically relevant pathogens to acquire high-level resistance. *V. cholerae* autochthonous to marine and brackish water environments are highly susceptible to encounter such antibiotic challenges that can result in high-level resistance. The results of the present study reveal that resistance to antibiotics can be achieved by SNP accumulation, a disparate mechanism of resistance acquisition in *V. cholerae* unlike horizontal gene transfer. Hence, this study justifies strict regulations on the indiscriminate use of antibiotics and emphasis on the 'One Health' programme that works towards combatting antimicrobial resistance.

Acknowledgments

Lekshmi Narendrakumar is thankful to the Department of Science & Technology (DST), Govt. of India, for providing the INSPIRE fellowship [Fellow code: IF 140851]. The authors thank Dr Beena PS (OmicsGen LifeSciences Pvt. Ltd., Kochi, India) for whole-genome sequencing and analysis. The authors also are grateful to Prof. M. Radhakrishna Pillai (Director, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India) for the facilities provided.

Funding: This research was funded by an intramural grant of Rajiv Gandhi Centre for Biotechnology.

Competing interests: None declared.

Ethical approval: Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.106097.

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