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In silico designing of CRISPR gRNA and vector to Knockout Tetracycline Resistant gene (TetA gene) of *Escherichia coli.*

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ABSTRACT

The rise in antibiotic resistance is major issue, primarily driven by the horizontal gene transfer. Antibiotic resistance can't be eliminated as long as antibiotics continue to be used for treating infectious diseases. TetA gene, encoding the efflux carrier in plasma membrane of bacteria, is major contributor of resistance against the tetracycline. *E. coli*, one of the opportunistic pathogens, presents serious threat to healthcare due to resistance against various antibiotics including tetracycline. CRISPR cas system, a form of bacterial acquired immunity, has gained much attention recently for its potential use in overcoming the spread of antibiotic resistance genes. In the present study, we performed *in-silico* gRNA and vector designing specific to *TetA* gene of *E. coli* to determine the effect of CRISPR cas system on spread of antibiotic resistance. Three computational tools, ChopChop Crispor and Benchling, were used to design the gRNAs. These *in-silico* tools were employed to predict a relevant list of gRNAs targeting the TetA gene. Furthermore, the RNA fold web service was used to analyse the secondary structures of the lead single guide RNAs (sgRNAs) and gRNA1 was identified as the best candidate. The selection of the lead guide RNA involved designing target-specific oligonucleotides and sgRNAs using the NEBioCalculator tool. Subsequently, an *in-silico* construction of the guide RNA expression vector was carried out using SnapGene software. However, *in vitro* studies are required to determine whether the computationally predicted guide RNAs can effectively inhibit the TetA gene.

Keywords: *TetA, CRISPR, Guide RNA (sgRNA), Tetracycline Drug resistance, In silico, Escherichia coli.*

1. INTRODUCTION

Antibiotic resistance is an increasingly pervasive global concern and it is projected to cause more than 39 million deaths in the period of 2025-2050 (53) .As per the World Bank report, it causes US$ 1 trillion additional healthcare cost by 2050, thus leads to huge economic burden (**54**). Antibiotic resistance can't be eliminated as long as antibiotics continue to be used for treating infectious diseases. The discovery of new antibiotics has markedly slowed down over the past decade [1].This a broad-spectrum antibiotic commonly used to treat a variety of bacterial infections, including those caused by Gram-negative pathogens. However, many Gram-negative bacteria, such as *E. coli*, express the tetracycline efflux protein TetA. The tetA gene encodes a class B tetracycline resistance protein, which functions as a metal-tetracycline/H^+ antiporter. Through an active efflux mechanism, TetA reduces intracellular tetracycline concentrations, thereby conferring resistance. This energy-dependent process prevents the antibiotic from accumulating to therapeutic levels within the bacterial cell, rendering the treatment less effective [2]. Horizontal gene transfer, particularly through conjugation, is a major mechanism responsible for the spread of antibiotic resistance genes including tetA [3].Developing novel strategies to combat the spread of antimicrobial resistance (AMR) genes will demand substantial efforts in the years ahead [4].

In 2014, Doudna and Charpentier demonstrated how CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR), widely used technique for genome editing, can selectively avoid the spread and remove AMR genes from bacterial

populations.

CRISPR-Cas is an adaptive immune system in bacteria and *archaea* that protects against foreign nucleic acids, such as those from phages and plasmids. It works by incorporating short spacer sequences from invaders into CRISPR loci, which are later used to guide Cas proteins to recognize and cleave matching sequences. CRISPR systems are classified into two classes: Class 1 (Types I, III, IV) involves multi-protein complexes, while Class 2 (Types II, V, VI) uses a single Cas enzyme. The Class 2, Type II CRISPR-Cas9 system has become a powerful tool for genome editing due to its simplicity and precision. While extensively applied in genetic engineering, its potential to eliminate antimicrobial resistance (AMR) genes remains underexplored [5,6,7].

Bikard et al 2012, in last decade postulated and confirmed that a synthetic CRISPR-Cas system could be utilised as an antimicrobial to kill specific bacterial genotypes [8]. Various articles claims the potential use of the CRISPR Cas system to precisely remove bacterial strains that carry genes, including those determining drug resistance, from populations and to re-sensitise bacteria to antibiotics by selectively removing AMR-encoding plasmids [9].

Moreover, CRISPR-Cas antimicrobials are very specific to target bacteria, individual bacterial strains having antibiotic resistance gene can be selectively removed from a mixed population of *E. coli* genotypes by transforming the population with a plasmid encoding CRISPR-Cas programmed to target a sequence unique to each genotype [10].

Many studies demonstrated that CRISPR-Cas9 antimicrobials can be delivered to target bacteria using different methods like phagemids (plasmids packaged in phage capsids) to selectively kill the clinically relevant bacterial pathogens *E. coli* [8] and *Staphylococcus aureus* [11]. Bikard and his group (2012) investigated the phagemid mediated delivery of CRISPR-Cas9 constructs programmed to target AMR genes harboured on plasmids, which effectively removed these plasmids from bacteria [8]. In one more study CRISPR Cas9 was delivered by conjugative plasmids to kill bacteria carrying AMR genes in the chromosome [9].

The current study performed an *in-silico* gRNA and vector designing specific to TetA gene of *E. coli*. The study investigates three computational tools, ChopChop Crispor and Benchling, to pick the gRNA [12,13,14]

To identify suitable guide RNAs (gRNAs), three *in-silico* tools—**CHOPCHOP, CRISPOR, and Benchling**—were used to predict and shortlist candidate gRNAs targeting the tetA gene. The predicted gRNAs were then analyzed using the RNAfold web server to assess their secondary structures. Based on structural stability and target accessibility, gRNA1 was identified as the most promising candidate [12,13].

The lead gRNA selection also included the design of target-specific oligonucleotides and single guide RNAs (sgRNAs) using the NEBioCalculator. Subsequently, an *in-silico* expression vector was constructed using SnapGene software for downstream cloning and expression analysis [14]. However, *in vitro* studies are required to confirmation of computationally designed gRNAs which could effectively inhibit tetA expression [15].

Systematic studies have evaluated the DNA sequence features influencing sgRNA efficiency, leading to improved design algorithms. For instance, Doench et al. (2016) developed predictive models that consider base preferences—such as a preference for cytosine near cleavage sites—to enhance sgRNA performance. Designing effective sgRNAs also requires adapting strategies for different CRISPR applications (e.g., knockout vs. CRISPRi/a), which further highlights the importance of purpose-specific design tools [15,16].

Despite these advances, off-target effects remain a major concern. Tools such as sgRNAcas9 have been developed to predict off-target sites and improve sgRNA specificity. These computational platforms allow for safer and more effective genome editing by reducing unintended cleavage events. In therapeutic settings, *in-silico* designing tools play a critical role in predicting and mitigating off-target effects [14, 15].

A significant trend in CRISPR development is the growing demand for tools that integrate efficiency prediction with off-target risk analysis, such as CHOPCHOP v2. However, many current platforms still lack real-time feedback from experimental data. Future directions may include the development of adaptive design algorithms that refine gRNA selection based on iterative experimental results. This will improve the robustness of genome editing strategies, particularly in complex systems [16].

The design of sgRNA is necessary to increase the success rate of knockouts in the *E. coli.* The efficacy of sgRNA should be confirmed in silico before its synthesis and use **[18, 19].** Online bioinformatics tools such as sgRNA scorer 2. 0 CRISPRlnc CHOPCHOP and CRISPR RGEN tools are available to design these sgRNAs [20, 21]. All of these tools along with *in silico* designs enable effective sgRNA design.

The present study was focused on *in-silico* designing of sgRNAs for the CRISPR/Cas9-targeted TetA gene in *E. coli.* Furthermore, the study aims to determine the effect of CRISPR-Cas on bacterial conjugation in *E. coli*, through engineering a recipient by CRISPR-Cas that targets the tetA gene and comparing it to a recipient without CRISPR..

2. METHODOLOGY

Phylogenetic Tree Analysis

The TetA-gene sequence of E. coli from genome sequence was selected and phylogenetic tree was created according to the Neighbor-Joining method using the open-source software, MEGA 11.0 version [22].

Computational Designing of TetA sgRNAs

The targeting sgRNAs for TetA gene were predicted using web based bioinformatics tools by using E-coli genome. The advancement in bioinformatics, web-based tools have been developed to design the efficient sgRNAs for successful gene knock-out experiments. The challenge in CRISPR-Cas system is on-target and off-target efficiencies. The available sgRNA designing tools have been classified into scoring and alignment-based functions. By using alignment-based method, the sgRNA sequences were aligned by using reference genome by locating the PAM (Protospacer Adjacent Motif). On the other hand, the scoring based approach aligned the sgRNAs and scored by features like GC content, exon position, etc. Studies report that the Scoring based methods perform much better than the alignment based [23]. Appropriate sequences were chosen and assessed based on the criteria of GC content ranging from 35% to 65% [24], efficiency of at least 40, and self-complementarity below 1. Appropriate sgRNA sequences were identified by Mismatches (MM) ranging from 0 to 3, aiming to avoid or minimize off-target sequences. In the current study, three highly recommended scoring-based online tools: CHOPCHOP, CRISPOR, and Cas-Designer [22] were selected, which predict off-targets and are not limited to a few species. In CRISPR-Cas system, the PAM sequence can be designed using various bioinformatics tools.

CHOPCHOP

A web-based CHOPCHOP tool was utilized for CRISPR genome editing by applying parameters like, off-target scores, efficiency score, self-complementarity regions not longer than 3 nt, ideal GC content, and on-target location of sgRNAs in a targeted gene. The presence of guanine in 20th position of the sgRNA target region [12]. CHOPCHOP supports 378 reference genomes and its database was updated regularly.

Crispor

Crispor was a user friendly sgRNA designing web-based tool that uses genomes lesser than 2300 bp length and should contains multiple PAM sequences. The output from this tool aligned the sgRNA sequence by calculating the MIT specificity, efficiency, off-target, on-target, and CFD (Cutting Frequency Determination) specificity scores. This tool currently available with 811 genomes [13].

Benchling

Benchling is a cloud-based platform to design CRISPR sgRNA for various genome editing experiments. This tool designs the effective sgRNAs by computing the gene target region, cut position, on-target scores, off-target score and strand type. This tool allows the users to share their experiments over cloud-based [25]. This tool has 164 reference genomes for the users [26].

Validation of the gRNA

Besides labelling the target, structural characteristics of the guide RNA also play an important role in how it interacts with the cas9 protein. Consequently, the secondary structure of the gRNA must be examined. While there are many tools available for designing gRNAs, none can provide information on their secondary structure. So RNAfold, a web based secondary structure prediction server [17] was used to predict the secondary structure of the guide RNA. It relies on the minimum free energy (MFE) algorithm by Zuker and Stiegler [27] and the RNA folding model [28].

Generation of target specific oligos

EnGen sgRNA Template Oligo Designer (https://switch.org/#!/sqrna) was used to design target-specific oligomers for the 20-nucleotide guide sequence. An oligo sequence is generated by arranging T7 promoter region at 5’ end and 14 nucleotides sequence near 3’ end. If the guide sequence doesn't begin with 'G' at the 5′ tip, the tool will include a second occurrence of -G in the generated oligo.

In silico designing of a Vector to TetA Gene

An expression vector can be inserted into to produce the guide sequence for in vitro transcription. Keith Joung [52] provided the ID 42250, a guideRNA expression vector known as ID 2250 to be obtained from Addgene plasmids. The SnapGene software was employed to generate the expression vector that incorporated the chosen gRNA sequence.

4.3. RESULTS

Phylogenetic Tree Analysis

The evolutionary distances were calculated using the Poisson correction method, focusing on the total number of amino acid

substitutions per site. In the tree, each internal node displays a plot of sites where there's at least one clear base present in at least one sequence for every descendant clade (Figure No.1).

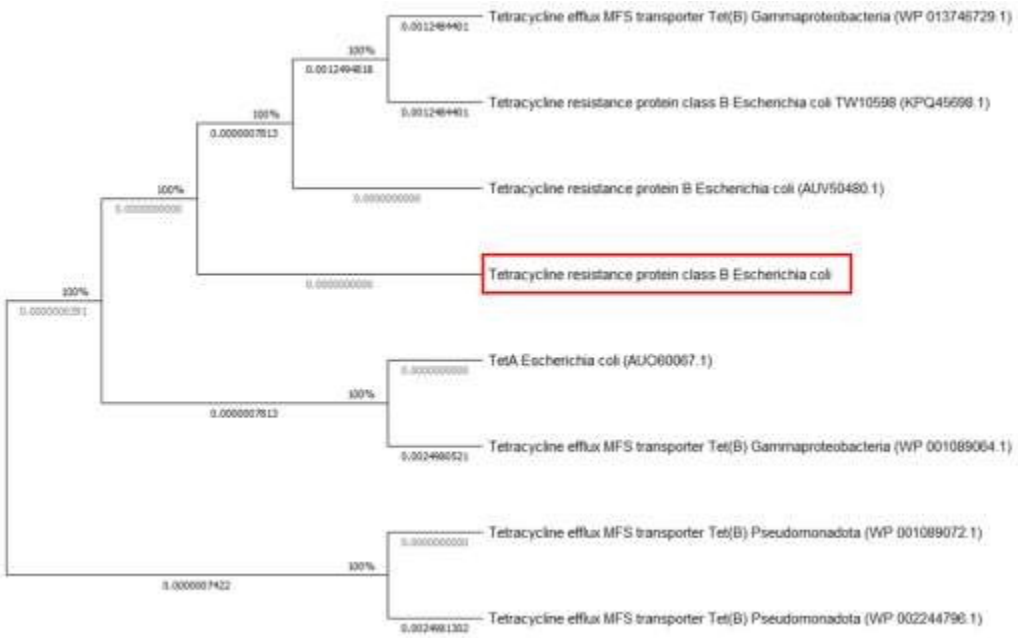


Figure No. 1: The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree is shown (next to the branches).

Our research involved analysing 8 different amino acid sequences and eliminating any uncertain positions with the help of the pairwise deletion option. There was a total of 401 positions in the final dataset. Evolutionary tree was constructed using MEGA11 software

CHOP-CHOP

In Chopchop, a collection of 160 guide RNAs were created, and the top-performing gRNA hits were determined by examining several factors, including GC content between 40% and 70% [12], efficiency of at least 40, and a self-complementarity score of 0. Appropriate sgRNA sequences were filtered by Mismatches (MM) set to 0, in order to avoid or minimize off-target sequences.

The results showed that 89 out of 160 gRNAs were most effective in the 40–70 percent target range and had a GC content percentage in this range. Furthermore, gRNAs that contain too much or too little GC are useless [29, ,30]. The existence of self-complementarity regions exceeding 3nt signifies the quantity of self-complementarity regions anticipated in the guide sequence. As stated earlier, self-complementarity in or among the guide RNA and RNA backbone may hinder gRNA efficiency and cas9 function [31]. Consequently, it is highly advised to steer clear of gRNAs that exhibit self-complementarity. Chopchop offers a mismatch search over all bases preceding the PAM to forecast off-targets (gRNA binding beyond the target gene) (Table 1).

Table No. 1: Predicted guide RNA hits along with PAM from Chopchop

S. No.	Guide sequence + PAM	Strand	GC content (%)	Self-complementarity	Mismatches
1.	AATAAAGCCCAGCAGCACCGCGG	-	55	0	0
2.	GATAACACCGATACCGAAGTGGG	+	45	0	0
3.	CAAATGGGGCGAAAAAACCGCGG	+	50	0	0

4.	CAGACCAAAAGCCATCAGCAGGG	+	50	0	0
5.	GTGCTGATTCTGCTGGCGGGCGG	+	65	0	0
6.	GAACATTGTGACCTTTCTGGTGG	+	45	0	0
7.	CGATAACACCGATACCGAAGTGG	+	50	0	0
8.	TTCGGTAAACAGCACCCACACGG	-	50	0	0
9.	CTTCGGTATCGGTGTTATCGCGG	-	50	0	0
10.	AATAATCAGCAGAATCGGCATGG	-	40	0	0
11.	GTGAGCCTGACCAACGCGACCGG	+	65	0	0
12.	CGCAATAAATTCGCGCAGCAGGG	-	50	0	0
13.	CATAGCCTGCCGATTTGGGATGG	+	55	0	0
14.	CAGCAGCGGGCCAATCACGCCGG	-	70	0	0
15.	GGCTCAGCAGCAGCACCGGGCGG	-	75	0	0
16.	ATAAATTCGCGCAGCAGGGTCGG	-	50	0	0
17.	GGCTGGAACAGCATGATGGTGGG	+	55	0	0
18.	TCCAGCCATCCCAAATCGGCAGG	-	60	0	0
19.	TTTGCTGCCCTGCGCCTGCGGGG	-	70	0	0
20.	TGGCTGGAACAGCATGATGGTGG	+	55	0	0
21.	TCAGGCTCAGCAGCAGCACCGGG	-	65	0	0
22.	CTGGGTTTCCACGCCCACTTCGG	-	60	0	0
23.	TCAGACCAAAAGCCATCAGCAGG	+	50	0	0
24.	TTTCTGGCGTTTATTAGCGAAGG	+	40	0	0
25.	AATATACACGCTGTTGCTCTGGG	-	40	0	0

26.	TTCGGTATCGGTGTTATCGCGGG	-	50	0	0
27.	ATGCTGACCCCGCAGGCGCAGGG	+	70	0	0
28.	CACCGATACCGAAGTGGGCGTGG	+	65	0	0
29.	AACAGCACCCACACGGTCGCCGG	-	65	0	0
30.	TCGCTCATTTTGCCCAGCCACGG	-	55	0	0
31.	ATTGCGGGCCCGATTATTGGCGG	+	55	0	0
32.	GTTTCCGGTGCTGATTCTGCTGG	+	55	0	0
33.	GCAGCGCCGCAATAAAAAACGGG	-	50	0	0
34.	GACCTTTATGCTGACCCCGCAGG	+	60	0	0
35.	TGTTTGCTGCCCTGCGCCTGCGG	-	65	0	0
36.	TATTTTAGCGCGCAGCTGATTGG	+	45	0	0
37.	CAAATCCAGCCATCCCAAATCGG	-	45	0	0
38.	GCGCTGTGGATGCTGTATCTGGG	+	55	0	0
39.	GCGCGAGCCAGCGCGTGAAATGG	+	70	0	0
40.	AGCCAAACCATTTCACGCGCTGG	-	55	0	0
41.	CGATCGCTTTGGCCGCCGCCCGG	+	75	0	0
42.	TTCCACGCCCACTTCGGTATCGG	-	55	0	0
43.	TCGCAATAAATTCGCGCAGCAGG	-	50	0	0
44.	CTGATTCTGCTGGCGGGCGGCGG	+	70	0	0
45.	CAATAATCGGGCCCGCAATCAGG	-	55	0	0
46.	CGCCTGCGGGGTCAGCATAAAGG	-	65	0	0
47.	AGCCAGCGCGTGAAATGGTTTGG	+	55	0	0

48.	AGCAGCGCCGCAATAAAAAACGG	-	45	0	0
49.	GAGCCTGGATTATCTGCTGCTGG	+	55	0	0
50.	CGCCGGTAATGCCGCTCAGCAGG	-	70	0	0
51.	GGCGCTGTATGCGCTGATGCAGG	+	65	0	0
52.	GCGCCAGCAGCACGCCAAAATGG	-	65	0	0
53.	TCACGCCGGTCGCGTTGGTCAGG	-	70	0	0
54.	CTCATCACGCCCTGCAGCGCCGG	-	70	0	0
55.	GTGCTGCTGCTGAGCCTGATTGG	+	60	0	0
56.	ATAACCATAGCCTGCCGATTTGG	+	45	0	0
57.	CGCCTGCTGAGCGGCATTACCGG	+	65	0	0
58.	CTGGGCGCGAGCTTTGGCCTGGG	+	70	0	0
59.	TCAGCAGCAGCACCGGGCGGCGG	-	75	0	0
60.	CTGTTTACCGAAAACCGCTTTGG	+	45	0	0
61.	CTGGATTTGGATTATTGGCCTGG	+	45	0	0
62.	CGCAGCAGGGTCGGCAGCACCGG	-	75	0	0
63.	GTTTGCTGCCCTGCGCCTGCGGG	-	70	0	0
64.	GAATCGGCATGGTTTTAAACAGG	-	40	0	0
65.	TAATATACACGCTGTTGCTCTGG	-	40	0	0
66.	GTTCCAGCCAAAGCGGTTTTCGG	-	50	0	0
67.	CGAAAAAACCGCGGTGCTGCTGG	+	60	0	0
68.	GGTGTTATCGCGGGTGTTTTTGG	-	50	0	0
69.	CGCGGTTTTTTCGCCCCATTTGG	-	55	0	0

70.	**CTGATTGCGGGCCCGATTATTGG**	**+**	**55**	**0**	**0**
71.	**GGCTTTAGCCTGGCGGGCCTGGG**	**+**	**70**	**0**	**0**
72.	**CCTTTCTGGTGGTGATGTTTTGG**	**+**	**45**	**0**	**0**
73.	**TAACCATAGCCTGCCGATTTGGG**	**+**	**45**	**0**	**0**
74.	**GATGGCTGGATTTGGATTATTGG**	**+**	**40**	**0**	**0**
75.	**GCTGAACATTGTGACCTTTCTGG**	**+**	**45**	**0**	**0**
76.	**CCTGCTGCATAGCGTGTTTCAGG**	**+**	**55**	**0**	**0**
77.	**GGGCTTTAGCCTGGCGGGCCTGG**	**+**	**75**	**0**	**0**
78.	**TAGCAGCGCGTTTGCGTTTCTGG**	**+**	**55**	**0**	**0**
79.	**GCTGGGCGCGAGCTTTGGCCTGG**	**+**	**75**	**0**	**0**
80.	**CGCGCTGTGGATGCTGTATCTGG**	**+**	**60**	**0**	**0**
81.	**ACGCCAGCAGCAGATAATCCAGG**	**-**	**55**	**0**	**0**
82.	**GGTGGCGGCGAGCGTGATTGCGG**	**+**	**70**	**0**	**0**
83.	**TCCGGTGCTGATTCTGCTGGCGG**	**+**	**60**	**0**	**0**
84.	**GAAAAAACCGCGGTGCTGCTGGG**	**+**	**55**	**0**	**0**
85.	**CCGGTGCTGATTCTGCTGGCGGG**	**+**	**65**	**0**	**0**
86.	**GCAGGCGCAGGGCAGCAAACAGG**	**+**	**70**	**0**	**0**
87.	**CCCGATTATTGGCGGCTTTGCGG**	**+**	**55**	**0**	**0**
88.	**GAACCATTTTGGCGTGCTGCTGG**	**+**	**55**	**0**	**0**
89.	**GAGCCTGATTGGCGCGAGCCTGG**	**+**	**70**	**0**	**0**
Note: PAM sequence highlighted in bold and underlined					

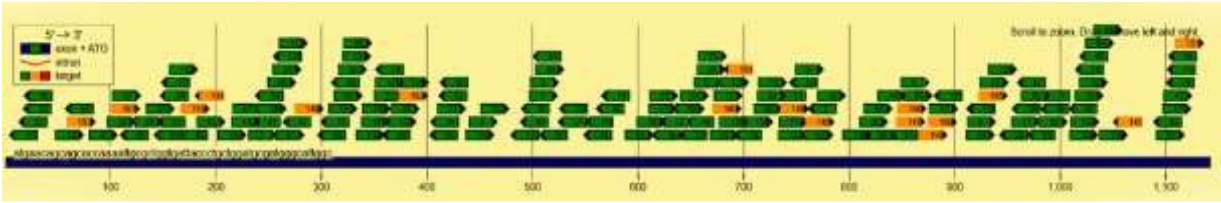


Figure 2: Figure is showing the results of CHOPCHOP analysis of sgRNAs targeting the tetA gene in E. coli using the Cas9 targeting mode and primer binding, restriction sites. Here higher efficiency sgRNAs are represented in Green, lower efficiency sGRNAs in red. Primer and restriction binding sites

Crispor

Crispor produced 40 potential gRNAs, with top selections made according to the MIT specificity score, cutting frequency determination (CFD) specificity score, off-target effects, Doench'16 score, and out-of-frame score. The MIT specificity score assesses how unique a guide RNA is within a genome; a gRNA sequence with lower uniqueness increases the chance that cas9 will cut additional sites in the genome. The specificity score varies between 0 and 100, with a higher score leading to reduced off-target effects. All the guide RNAs satisfy the recommended specificity score of greater than 50 [33]. The specificity score of CFD is another crucial factor that reliably forecasts guide RNAs exhibiting off-target activity. It is a weighted function considering position, number of mismatches, and nucleotide identities for all off-target sites with 2–3 mismatches to the sgRNA spacers [32]. All the guides meet the CFD score requirements. The efficiency score aims to forecast the cleavage efficiency of guide RNA regarding a target. It varies between 0 and 100. The efficiency score percentiles of <=30 stated as low predicted cleavage, >30 and <55 as medium predicted cleavage, and >55 as high predicted cleavage [34, 35]. Roughly 4 out of the 30 gRNAs displayed high predicted cleavage, while others had out-of frame score and off-target scores, with an outlier scoring 66, and 4 sgRNA have 1 off target score. Only 4 gRNAs were identified as the top hits in terms of all the parameters mentioned above, according to the overall analysis (Table No. 2) [36, 37].

Table No. 2: Guide sequence hits along with PAM predicted from CRISPOR tool

Guide sequence + PAM	MIT Specificity Score	CFD Specificity Score	Off targets	Doench'16-Score	Out of frame score
TTAATGACAACAACAGCACTGGG	100	100	1	60	70
GCGATAAAAAAGGGACTATGCGG	100	100	1	65	69
GAAAAAACGGCAGTACTGCTCGG	100	100	1	60	68
CAGCCAATAAAATTAAAACAGGG	100	100	1	61	64

Benchling

From a list of 135 possible gRNAs, 15 are benchmarked and scored on & off-target (BNP) per hit. As per the method for calculating off-target scores, scores above 50 are indicative of good gRNAs, which can be anywhere from 0 to 100 [33]. 135 gRNAs fulfil the off-target score requirement in this scenario. As per the score, only 13 gRNAs met the recommended on-target score of over 60 and their calculation is based on this information [15] (Table No 3).

Table No. 3: Predicted guide RNA hits along with the PAM sequence

Strand	Sequence	PAM	Off-Target Score	On-Target Score
Sense	TTACGTTACTCGATGCCATG	GGG	100	70.98
Anti-sense	ATAAATTCACGTAATAACGT	TGG	99.65	63.08
Anti-sense	GTGCAAGCAATACGCCAAAG	TGG	100	65.26
Sense	AAATGTCTGACCGATTTGGT	CGG	100	65.92
Anti-sense	CACGCGTTGAGAAGCTGAGG	TGG	100	64.63

Anti-sense	CTTCACGCGTTGAGAAGCTG	AGG	100	62.51
Anti-sense	GCGATAAAAAAGGGACTATG	CGG	99.73	64.61
Anti-sense	GGAACCAAAACATAACCACA	AGG	99.96	73.29
Sense	TTTTGGATGGAATAGCATGA	TGG	100	63.65
Anti-sense	CAGCCAATAAAATTAAAACA	GGG	99.53	61.05
Sense	CAAACAAAGAGTCATCAGCA	AGG	100	72.15
Anti-sense	CAGTAATGGGCCAATAACAC	CGG	99.76	64.84
Sense	TGTTAACCCCTCAAGCTCAG	GGG	100	71.59

Identification of top hits

The guide RNA hits obtained from all four online tools (Chopchop, Crispor, and Benchling) were compared, and about 2 gRNA hits (including 1 hit in three online tools and 1 hit in two tools) that met all the parameters mentioned in more than one tool were selected as top hits and thus analysed further (Table No.4).

Table No. 4: List of gRNA hits identified in more than one online tool

gRNA No.	gRNA hits + PAM	Online tools
1	CAGACCAAAAGCCATCAGCA**GGG**	Chop-Chop, Crispor and Benchling
2	GCGATAAAAAAGGGACTATG**CGG**	Crispor and Benchling

Next, the important step is to select from the lead gRNA that is chosen the best candidates. Along with the standards offered by computational gRNA design servers, multiple authors propose various parameters that could potentially influence amplification of target site activity. However, none of these criteria are exclusive to this group. Doench et al.(15) identified whether the locations of certain nucleotides in the gRNA are advantageous or detrimental. In line with this, guanine is highly preferred at position 20, but it's forbidden at location 16. Purine nucleotides located in the final four positions of the gRNA have a positive effect on the binding of gneRNA-cas9 [49]. Additionally, the adenine and guanine are favourable nucleotides to be presented at 20th position in the sequence, reinforcing the clamping for purines at this location. The Crispr-cas system's PAM is a trinucleotide sequence NGG, and studies suggest that the N family typically includes cytosine and thymine. In addition, the higher GC content in positions 4-8 implies greater gRNA activity [15, 34].' In Table No.5, the efficiency attributes are outlined.

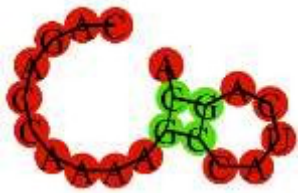
Table No. 5: Efficient and inefficient features of common gRNA hits

gRNA No	Efficient features				
	Guanine or Adenine at position 20	Cytosine at position16	GC content in positions 4–8	Purine nucleotides in the last four position	PAM (CGG)
1.	No(A)	Yes	Less (ACCAA) – 40%	3 (CA**GA**)	No
2.	Yes(G)	No	Less (ATAAA) – 0%	2 (**GCGA**)	Yes

Selection of lead gRNAs based on secondary structure analysis

In CRISPR-cas9 genome editing, the secondary structure secondary structure of the guide RNA plays an important role in recognizing the target sequence. The free energy value (ΔG) of the self-folding potential is higher in non-functional gRNA than functional gRNA [34,38]. The possibility of gRNA folding increases when the G value is negative, making it less likely to pair with the target sequence. In this study the minimum free energy (MFE) value prediction was calculated using the RNAfold web-based tool (Table No. 6).

Table No. 6: Secondary structural analysis of gRNAs

gRNA No.	gRNA	Minimum free energy (MFE)kcal/mol	gRNA Secondary Structure
1	CAGACCAAAAGCCATCAGCA	**-0.54** kcal/mol	
2	GCGATAAAAAAGGGACTATG	**-0.01** kcal/mol	

In the table referenced above (Table 6), the lowest free energy value of the top gRNA hits varies from −3 to 0 kcal/mol) [34, 38] state that the effectiveness of cas9 endonuclease in cleaving the target is enhanced when the gRNA self-folding free energy falls within the range of −2 to 0 kcal/mol. The leading hits gRNA 1 exhibit a ΔG value within the acceptable limits. Consequently, from the two highest gRNA hits evaluated on efficient attributes and Minimum free energy (MFE) kcal/mol, gRNA 1 (CAGACCAAAAGCCATCAGCA) was chosen as the primary gRNA. (Table No. 6). [34, 38]

Generation of target-specific oligo nucleotides

EnGen sgRNA Template Oligo Designer v1.121 from NEBiocalculator was used to create target-specific oligos by entering the selected lead gDNA1 and using an online tool. The predicted oligo sequence contained T7 promoter sequence in 5' end of sgRNA and a 14th nt overlapping sequence in 3' end. The primary sgRNA was not started with guanine to clamp the Guanine in 5' end, which is important for the precise transcription. Figure no. 3 displayed the fully transcribed sequence of lead sgRNA1.

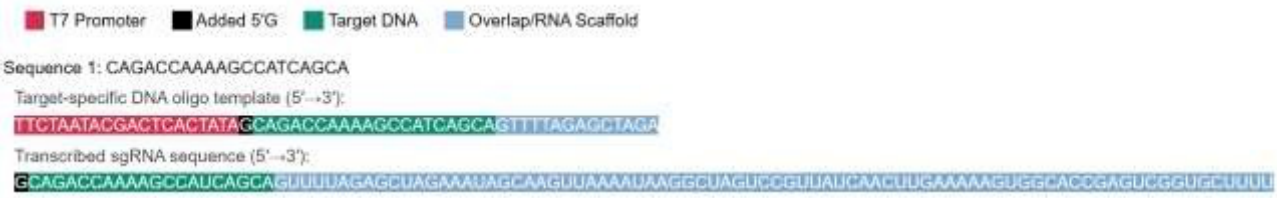


Figure No. 3: Design of Target specific oligos and transcribed sequence of lead sgRNA by NEBiocalculator

Secondary structure analysis of lead sgRNA

The complete sgRNA sequence of the lead was additionally analyzedanalysed for secondary structure using the RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Nevertheless, eExamination of the completely transcribed nucleotides of sgRNAs 1 reveals that they possessed an internal loop structure and hairpin loop structure, which are easily accessible, indicating their functionality (Figure No.4). The internal loop configuration within the core hairpin of a single-guide RNA (sgRNA) plays a crucial role in the effective cleavage of target DNA. The fundamental hairpin shape of sgRNA is regarded as vital for DNA cleavage by the SpCas9/sgRNA complex.

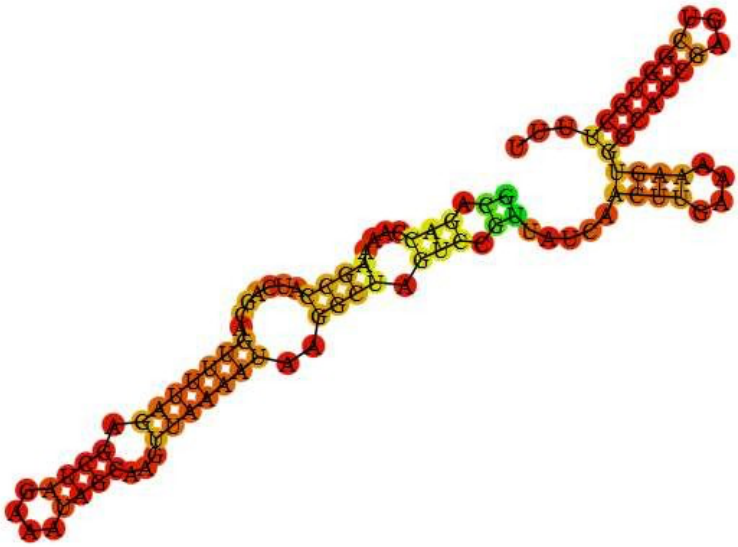


Figure No. 5: Secondary structure of lead sgRNA

Designing of Vector

The lead gRNA1 was input into SnapGene software to create a suitable guide RNA expression vector for cloning. In this study, the leading guide RNA was placed at the snaBI restriction site of the expression vector DR274, illustrated in Figure No. 6.

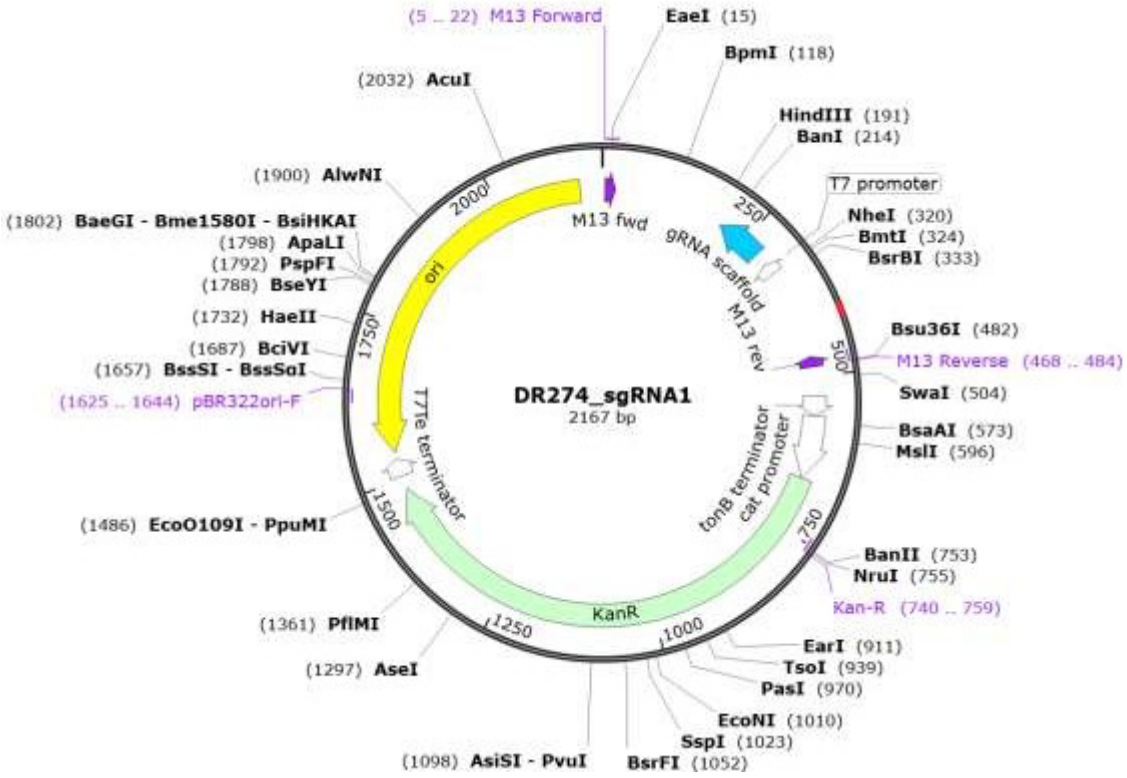


Figure No. 6: In-silicoIn-silico designed expression Vector (DR274) using SnapGene software

5.4. DISCUSSION

One of the major global problems in the world today is the quick emergence of drug resistant bacteria. E. coli serves as good indicator of antimicrobial resistance in bacterial communities as it is recognized as a major reservoir of the genes responsible for drug resistance [39]. Numerous studies reported that the presence of tetracycline resistance genes in both clinical and

non-clinical E. coli isolates across various regions of globe. There have been numerous several reports of tetracycline-resistant determinants from clinical and nonclinical isolates in different regions of global countries [40].

According to previous reports the most common tetracycline-resistant gene among E. coli is TtetA . E. Coli strains [41]. According to the earlier studiesy the majority of the tetracycline-resistant isolates (89–90%) had TtetA genes. The vast majority of TetA is specifically found in E.coli strains that have been isolated from soil poultry urine and stool [42,43,44]. It is hypothesized that TtetA genes are more easily found in the environment than other tetracycline determinants [45].

The remarkable specificity and programmability of CRISPR-Cas systems have captured the attention of researchers, leading to the development of new antimicrobials. Both in vitro and in vivo studies suggest that CRISPR-Cas gene editing can act as next-generation antimicrobial agents [46]. However, bBacterial cells are protected by the CRISPR-Cas system which recognizes and cleaves any invasive nucleic acids. It also affects the bacterial ability to produce a variety of virulence factors during infection including controlling gene expression creating biofilms repairing DNA adjusting to stress and acquiring resistance genes [47]. The most remarkable application of CRISPR cas gene editing is removal of drug resistance genes [48]. Firstly Zuberi et al.,(48) introduced the novel idea of CRISPR interference (CRISPRi) and its role in gene knockout. Authors reported This research suggests that CRISPRi which targets and knocks out the TetA gene is a promising method for treating nosocomial and environmental infections and preventing bacterial tetracycline drug resistance [48]. Hitherto, tThe most widely used method in recent years is CRISPR-cas9 due to its low-cost high efficiency and simple design [49]. Off-target effects are the main disadvantage of this approach and they are primarily caused by mismatches between sgRNA and PAM [49]. To reduce off-target effects a variety of computer programs that predict off-target locations have been developed. In this study an in-silicoin-silico gene editing technique was used to knock out TetA gene in E. Coli. Here we used three highly recommended computational tools—Chopchop Crispor and Benchling—that were used to design gRNAs for the TetA gene. Each of these tools contains crucial parameters for selecting gRNA hits (Table No. 1, Table 2 and Table 3). Approximately two gRNA hits that met all of the requirements specified by several online resources were selected for additional research. Present Studiesstudy shows that cytosines (C) are preferred in the N-position of the canonical 5′-NGG-3′ PAM whereas thymines (T) are not. When considering the gRNA sequence motif repetitive bases like five contiguous A, five contiguous C, four contiguous G or four contiguous Uuracils decrease cas9 activity [15, 34, 35]. With the help of tools like CRISPOR, CHOP-CHOP, Benchling, in present study we carried out some compositional changes in sgRNA to increase its efficiency like Nucleotide position 20 in the gRNA is critical, with guanine and adenine preferred over cytosine for efficient editing .

The secondary structure of gRNA is one of the critical factors involved in determining their activity. RNA folding free energy value for all gRNA self-folding for each of the top gRNA hits were assessed, as well as base accessibility data in the structure using the RNAfold server to determine most preferred leads. The self-folding free energy value indicated that gRNA sequence preferred to take the secondary structure. Non-functional gRNAs were more likely to fold onto themselves than functional gRNAs that had a high negative free energy value. Furthermore, since pairing must take place via the internal loop and hairpin loop regions, the accessibility of nucleotides at positions 18–20 (seed region) in a functional gRNA is of particular importance [15]. The lead hit was gRNAs 1 according to this structural analysis.

In the future the oligo sequence generated by in silico methods can be used to synthesize the ssDNA oligonucleotide primers required to make the in vitro transcription kit. The RNAfold server was used to analyzeanalyse the generated sgRNAs and found that both had internal and hairpin loops which are essential features for efficient gene editing [50]. Furthermore, the functional activity of these sgRNAs is further supported by the free accessibility of nucleotides at positions 51–53 [51]. Finally, gRNAs can be easily inserted into the gRNA expression vector DR274 using SnapGene for in silico vector construction.

Nevertheless, tThe gRNA design is crucial in Crispr-Cas9 editing. Due to technological advancements, many computational tools with unique functionalities dedicated to gRNA design have been developed. Future in vitro studies should validate the potency of gRNAs 1 as the lead from the current study that was able to knock out the TetA gene of multidrug-resistant E. coli. This recommendation was based on the evaluation of the results from computational resources and several other factors.

~~6.~~5. CONCLUSION

In recent years, the CRISPR-Cas9 gene editing method has yielded significant progress in subverting the genes of many bacteria to help prevent drug resistance. It can decrease bacterial death, reduce virulence, pathogenicity and biofilm development, and inhibit several important physiologic events responsible for infection. CRISPR-Cas system in E. coli has yet to be extensively explored, however. To knock down the TetA gene, the master regulator of drug resistance, the most recent technique was used to design guide RNAs with maximum efficiency and minimal off-target effect in the current study. The analysis of the results shows that gRNAs 1 was the lead guide RNAs for all were determined based on a number of criteria which includes GC content, self complementarityself-complementarity and number of mismatches as well as out of frame score, MIT specificity score, CFD specificity score, Doench's 16 score and off targets. The secondary structure analysis confirms their having unpaired seed region, internal loop, and hairpin loop with minimum free energy value of -2 to 0 kcal/mol that is permissible. The selected leads were then assembled into an in-silicoin-silico expression vector (DR274). There is still further work to establish the viability of the guide RNAs at knocking out the TetA gene in vitro. Additionally,

In-silicoIn-silico design of sgRNAs for CRISPR/Cas9 technology is a fast-emerging field with great promise to improve genome editing success. The use of computational tools in the design process allows researchers to optimize sgRNA sequences for specificity and efficiency of the CRISPR system., which are crucial problems like off-targeting. Moving forward, in efforts to capitalize on CRISPR/Cas9 technology to its fullest potential in research and therapeutic applications, continued exploration of knowledge gaps, along with the development of novel design and delivery strategies will be indispensable

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