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Original article

Investigating the association between the CRISPR-Cas system and antibiotic resistance genes in *Neisseria* spp.

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Abstract

The study aims to examine the CRISPR Cas-systems in the *Neisseria* species, with a specific focus on its potential role in antibiotic resistance (AR). A total of 360 *Neisseria* strains belonging to different species were retrieved from the NCBI database. The CRISPR Cas arrays were found among 89 *Neisseria* genomes with 140 distinct direct repeats and 1661 spacer regions. While, 69% were determined to have the type II-C system and 28% had the I-C system. The CRISPR type II-C was found to have efflux pump AR (71%) majorly. It was found that species with several CRISPR arrays often had either no or just one AR genes in their genomes. The study highlights multiple CRISPR array in *Neisseria* spp. might have played a prominent role in the prevention of horizontal gene transfer of AR genes.

Keywords

Antibiotics, CRISPR Cas, Direct repeats, *Neisseria*, Spacer

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Introduction

The *Neisseria* genus encompasses a group of closely related Gram-negative bacterial species, with some appearing as coccoid shapes (such as *N. gonorrhoeae*, *N. lactamica*, *N. meningitidis*, and *N. subflava*) and others as rod-shaped (*N. bacilliformis* and *N. elongata*). While most of these species are typically harmless and coexist on mucosal surfaces, but two of them, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, have the potential to cause diseases in humans [1]. In 2015, there were approximately 395,200 reported cases of multidrug resistant gonorrhea, which represented a notable 27% increase compared to 2012. This increase is likely even more pronounced in the aftermath of the COVID-19 pandemic, as limitations in both sensitive diagnostic capabilities and accessible testing centers in resource-constrained regions may have led to an underreporting of cases [2]. The rise of antibiotic resistance (AR) in *Neisseria* is a significant global public health concern. Horizontal gene transfer (HGT) is a fundamental process driving the development of AR in bacteria. In nature, this phenomenon occurs through mechanisms like transformation, transduction, and conjugation, enabling the transfer of mobile genetic elements (MGEs), including transposons, integrons, and gene cassettes, between different bacterial species [3]. Nevertheless, efforts to detect and diagnosis the presence of AR genes in *Neisseria spp*. face challenges due to the lack of rapid diagnosis and high costs associated with traditional methods.

The genome editing technique, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas is a highly specific and effective gene knockout approach. It has been investigated as a potential strategy for targeting bacteria and AR genes in a sequence-specific manner. A typical CRISPR–Cas system has a CRISPR array which are made up of unique spacer sequences interspaced by repeat sequences, and CRISPR-associated (Cas) proteins [4]. This technique has recently been designed to facilitate genome editing and expression analysis in a wide variety of organisms, notably human cells. The genome editing studies have also been reported in bacterial species, like *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *and Escherichia coli* etc [5]. Recent studies indicate that the CRISPR-Cas systems may have a role in influencing AR in bacteria. For example, in *Streptococcus pneumoniae*, it was observed that the native state of the CRISPR-Cas system prevented plasmid transformation. However, some statistical models have not shown evidence that the CRISPR-Cas system can effectively prevent horizontal gene transfer (HGT) over extended periods of bacterial evolution. Previous studies have demonstrated that the impact of CRISPR-Cas systems on AR differ among various bacteria. In *Klebsiella pneumoniae*, certain types of CRISPR-Cas systems may limit the acquisition of AR, while in *Francisella* bacteria, it facilitate AR [6]. Further investigations into the functions of the CRISPR-Cas systems, its interactions with HGT mechanisms, and its relationship with AR could provide valuable insights into bacterial defense mechanisms against antibiotics and aid in the development of effective approaches to tackle AR infections.

In this study, the prevalence of CRISPR Cas systems in *Neisseria spp.* was analyzed by retrieving the genome sequence from the NCBI dataset. By examining the genetic structure and functionality of CRISPR array and their relationship with AR, we sought to shed light on the defense mechanisms of *Neisseria* against genetic invaders and explore the possible link between CRISPR-Cas systems and AR genes.

Methodology

Identification of CRISPR-Cas system

Genomic data of *Neisseria* genome sequences from NCBI RefSeq (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/ *Neisseria*) were consider for the study. Complete genomes were only considered for the analysis. The CRISPR miner (http://www.microbiome-bigdata.com/CRISPRminer2/index/) and CRISPR Cas finder (https://CRISPRCas.i2bc.paris-saclay.fr/) were used to detect the existence of the CRISPR locus region. CRISPR Cas Finder uses Shannon's entropy and entropy-based conservation to provide evidence levels to putative CRISPR array. CRISPR Cas Finder version 2.0 was used to find the Cas types in genomes with anticipated CRISPR array [7]. CRISPR miner is a web-based programme that offers a collection of CRISPR Cas array, similar to CRISPR Cas Finder, but additionally includes information on self-targeting, anti-CRISPR regions, and host phage interaction [8].

Detection of AR genes

The presence of AR genes was found using Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca) and Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) tool [9, 10]. The *Neisseria* genome which shows presence of CRISPR Cas system was only considered for the further analysis. In both the tools, fasta format of the genome sequence was given as an input. The major criteria for finding resistance genes were high-quality sequence coverage and the exclusion of incomplete gene predictions.

Structural stability of direct repeats

The structure of direct repeats was analyzed using the RNA fold web server (http://rna.tbi.univie.ac.at/cgi-bin/

RNAWebSuite/RNAfold.cgi). It's part of the Vienna RNA package, and it was created to estimate the minimal free energy (MFE) for each RNA secondary structure using the dynamic programming technique outlined by Zuker and Stiegler [11, 12].

Evaluation of spacer region

In this analysis, spacer sequences were initially extracted from predicted CRISPR arrays. To link these spacers with potential phage and plasmid associations, it was compared to a database containing plasmid and phage sequences using the BLASTN algorithm. Spacer sequences were considered associated if it exhibited characteristics such as greater than 90% sequence identity, query coverage greater than 85%, and an e-value - 0.001 in their BLAST hits. Spacer sequences that met these criteria were retained for further analysis. Subsequently, spacer targeting phage regions were grouped into distinct categories, including lytic, temperate, and non-lytic, to provide insights into the types of phages being targeted by the CRISPR system. This approach helps to characterize the host's defense mechanisms against plasmids and phages based on the specific interactions observed in the spacer sequences.

Statistical analysis

The statistical analysis was performed to examine the correlation of two variables: the presence of a CRISPR-Cas system and the existence of AR genes in *Neisseria spp*. For each species under investigation, the total number of *Neisseria* genomes (denoted as N) and the subset of genomes that exhibited both a CRISPR-Cas system and AR genes (denoted as O) was calculated. To determine whether this co-occurrence was statistically significant or merely a chance outcome, the estimation was done by calculating the expected number of genomes (E) where the presence of both the CRISPR array and the AR genes would occur purely by random chance. This estimation was derived using the formula $E = N \times Pb(CRISPR) \times Pb(AR\ genes)$, where Pb signifies the probability associated with each event occurring.

Results

Identification and analysis of CRISPR Cas array

A total of 360 complete genome sequences of *Neisseria spp,* were evaluated for the presence of the CRISPR Cas system was using the CRISPR miner and CRISPR Cas finder tools, with 89 genomes (24%) containing the CRISPR locus. Most of the genomes (82) were reported as known species: *animalis*(2), *animaloris* (1), *arctica* (1), *brasiliensis* (1), *canis* (1), *chenwenguii* (1), *cinerea* (2), *dentiae* (1), *dumasiana* (1), *elongata* (4), *flavescens* (1), *lactamica* (2), *macacae* (1), *meningitidis* (44), *mucosa* (2), *musculi*(1), *shayeganii* (1), *sicca* (2), *subflava* (8), *wadsworthii* (1), *weaveri* (2), *weixii* (1), and *zoodegmatis* (1). The remaining seven genomes were from unnamed species and will be referred as *Neisseria* from here on. The CRISPR-Cas positive isolates were from the United States of America (USA; n = 31), United Kingdom (UK; n=16), Sweden (n = 11), Canada (n = 8), China (n = 8), Singapore (n = 6), Australia (n = 2), Germany (n = 2), Korea (n = 2), France (n = 2), and Japan (n = 1). The source of isolation of these genomes were from *Homo sapiens* (79), Marmot (2), Plateau pika (2), *Anser albifrons* (1), Bovine (1), *Felis catus*(1), Guinea pig (1), *Mus musculus*(1) and Rhesus monkey (1) (Table 1). The number of CRISPR array discovered in *Neisseria spp*. varies from each other. The data revealed that 71 *Neisseria spp.* had just one identified CRISPR locus, 16 *Neisseria spp.* had two verified CRISPR arrays, and only two *spp.* had three CRISPR arrays.

CRISPR types and Cas genes in *Neisseria spp.*

In this study, the presence of the CRISPR type and Cas gene cluster was investigated in 89 different *Neisseria spp.*

Table 1. Information of *Neisseria spp*. utilized in this study

S. No	Acc. No	Species	NCBI Submission Date	Size (bp)	Country	Source	Strain
1	CP000381.1	*Neisseria meningitidis*	31-Jan-14	2153416 bp	China	*Homo sapiens*	053442
2	CP016672.1	*Neisseria meningitidis*	02-Aug-16	2172926 bp	USA	*Homo sapiens*	M22828
3	FR774048.1	*Neisseria meningitidis*	27-Feb-15	2227255 bp	Germany	*Homo sapiens*	WUE2594
4	CP002422.1	*Neisseria meningitidis*	31-Jan-14	2287777 bp	USA	*Homo sapiens*	M01-240355
5	FM999788.1	*Neisseria meningitidis*	27-Feb-15	2277550 bp	UK	*Homo sapiens*	8013
6	CP016671.1	*Neisseria meningitidis*	02-Aug-16	2180570 bp	USA	*Homo sapiens*	M22783
7	CP016654.1	*Neisseria meningitidis*	02-Aug-16	2185698 bp	USA	*Homo sapiens*	M22811
8	CP016647.1	*Neisseria meningitidis*	02-Aug-16	2182171 bp	USA	*Homo sapiens*	M22809
9	AL157959.1	*Neisseria meningitidis*	06-Feb-15	2184406 bp	UK	*Homo sapiens*	Z2491
10	CP016646.1	*Neisseria meningitidis*	02-Aug-16	2173686 bp	USA	*Homo sapiens*	M22819
11	CP016660.1	*Neisseria meningitidis*	02-Aug-16	2174791 bp	USA	*Homo sapiens*	M22804
12	CP007524.1	*Neisseria meningitidis*	21-May-14	2188020 bp	China	*Homo sapiens*	510612
13	CP007726.1	*Neisseria elongata*	22-Jul-15	2256647 bp	Canada	*Homo sapiens*	ATCC 29315
14	FN995097.1	*Neisseria lactamica*	03-Nov-16	2220606 bp	UK	*Homo sapiens*	020-06
15	CP012392.1	*Neisseria meningitidis*	02-Aug-16	2170619 bp	Germany	*Homo sapiens*	DE10444
16	CP031332.1	*Neisseria meningitidis*	05-Aug-18	2190201 bp	USA	*Homo sapiens*	M22814

S. No	Acc. No	Species	NCBI Submission Date	Size (bp)	Country	Source	Strain
17	CP020401.2	*Neisseria meningitidis*	02-Oct-19	2397461 bp	USA	*Homo sapiens*	FDAARGOS_214
18	CP021723.1	*Neisseria meningitidis*	09-Sep-19	2170095 bp	Sweden	*Homo sapiens*	13-600
19	CP020420.2	*Neisseria meningitidis*	30-Sep-19	2181232 bp	USA	*Homo sapiens*	FDAARGOS_209
20	CP021518.1	*Neisseria meningitidis*	09-Sep-19	2168615 bp	Sweden	*Homo sapiens*	12-176
21	CP021523.1	*Neisseria meningitidis*	09-Sep-19	2167995 bp	Sweden	*Homo sapiens*	98-182
22	CP039887.1	*Neisseria subflava*	07-May-19	2195659 bp	UK	*Homo sapiens*	ATCC 49275
23	CP021516.1	*Neisseria meningitidis*	09-Sep-19	2166707 bp	Sweden	*Homo sapiens*	14-563
24	CP021725.1	*Neisseria meningitidis*	09-Sep-19	2165984 bp	Sweden	*Homo sapiens*	95-134
25	CP023429.1	*Neisseria weixii*	21-Sep-17	2511904 bp	China	*Plateau pika*	10022
26	CP021521.1	*Neisseria meningitidis*	09-Sep-19	2198497 bp	Sweden	*Homo sapiens*	09-292
27	CP031255.1	*Neisseria elongata*	01-Aug-18	2534634 bp	USA	*Homo sapiens*	M15910
28	CP021522.1	*Neisseria meningitidis*	09-Sep-19	2167920 bp	Sweden	*Homo sapiens*	06-178
29	CP045960.1	*Neisseria meningitidis*	17-Nov-19	2166248 bp	Australia	*Homo sapiens*	AUSMDU00005726
30	CP020402.2	*Neisseria meningitidis*	02-Oct-19	2305818 bp	USA	*Homo sapiens*	FDAARGOS_215
31	CP021520.1	*Neisseria meningitidis*	09-Sep-19	2157444 bp	Sweden	*Homo sapiens*	strain 11-7
32	CP039886.1	*Neisseria flavescens*	07-May-19	2231882 bp	USA	*Homo sapiens*	ATCC 13120
33	CP040504.1	*Neisseria*	29-May-19	2502158 bp	Australia	*Homo sapiens*	F0314
34	CP031699.1	*Neisseria animalis*	03-Oct-19	2236930 bp	USA	*Guinea pig*	ATCC 49930
35	CP021724.1	*Neisseria meningitidis*	09-Sep-19	2169717 bp	Sweden	*Homo sapiens*	12-330
36	CP031334.1	*Neisseria meningitidis*	05-Aug-18	2314390 bp	USA	*Homo sapiens*	M22293
37	CP012694.1	*Neisseria meningitidis*	03-Oct-16	2191116 bp	China	*Homo sapiens*	331401
38	CP031253.1	*Neisseria lactamica*	01-Aug-18	2200224 bp	USA	*Homo sapiens*	M17106
39	CP016883.1	*Neisseria meningitidis*	11-Aug-16	2168169 bp	USA	*Homo sapiens*	M22790
40	CP031324.1	*Neisseria meningitidis*	05-Aug-18	2291778 bp	USA	*Homo sapiens*	M23347
41	CP046027.1	*Neisseria brasiliensis*	19-Nov-19	2617510 bp	USA	*Homo sapiens*	N.177.16
42	CP022527.1	*Neisseria*	31-Jul-17	2371912 bp	Korea	*Homo sapiens*	KEM232
43	CP021517.1	*Neisseria meningitidis*	09-Sep-19	2167947 bp	Sweden	*Homo sapiens*	12-221
44	CP022278.1	*Neisseria chenwenguii*	10-Jul-17	2496444 bp	China	*Plateau pika*	10023
45	CP031251.1	*Neisseria subflava*	01-Aug-18	2321871 bp	USA	*Homo sapiens*	M18660
46	CP016682.1	*Neisseria meningitidis*	02-Aug-16	2175832 bp	USA	*Homo sapiens*	M24705
47	CP016680.1	*Neisseria meningitidis*	02-Aug-16	2173901 bp	USA	*Homo sapiens*	M22822
48	CP021519.1	*Neisseria meningitidis*	09-Sep-19	2156539 bp	Sweden	*Homo sapiens*	11_14
49	CP031252.1	*Neisseria elongata*	01-Aug-18	2397276 bp	USA	*Homo sapiens*	M15911
50	CP020422.2	*Neisseria meningitidis*	30-Sep-19	2305805 bp	USA	*Homo sapiens*	FDAARGOS_211
51	CP031328.1	*Neisseria meningitidis*	05-Aug-18	2223855 bp	USA	*Homo sapiens*	M18755
52	CP020452.2	*Neisseria mucosa*	27-Sep-19	2783943 bp	USA	*Homo sapiens*	FDAARGOS_260
53	CP065653.1	*Neisseria meningitidis*	14-Dec-20	2181321 bp	USA	*Homo sapiens*	FDAARGOS_914
54	CP073116.1	*Neisseria subflava*	11-Jul-22	2409157 bp	Singapore	*Homo sapiens*	TT0073
55	CP053939.1	*Neisseria mucosa*	04-Jun-20	2224757 bp	USA	*Homo sapiens*	FDAARGOS_758
56	CP065726.1	*Neisseria cinerea*	14-Dec-20	1832901 bp	USA	*Homo sapiens*	FDAARGOS_871
57	CP059570.1	*Neisseria dentiae*	04-Aug-20	2755930 bp	Canada	*Cattle*	DSM 19151
58	CP073119.1	*Neisseria subflava*	11-Jul-22	2277784 bp	Singapore	*Homo sapiens*	HP0069
59	CP091522.1	*Neisseria*	11-Apr-22	2749212 bp	Canada	*Felis catus*	Dent CA1/247
60	CP073115.1	*Neisseria subflava*	11-Jul-22	2479061 bp	Singapore	*Homo sapiens*	TT0077
61	CP091509.1	*Neisseria dumasiana*	11-Apr-22	2679563 bp	Canada	*Homo sapiens*	LMG 30012
62	CP059566.1	*Neisseria sicca*	04-Aug-20	2864419 bp	Canada	*Homo sapiens*	DSM 17713
63	CP060414.1	*Neisseria musculi*	28-May-21	2928421 bp	USA	*Musmus culus*	NW831
64	CP059567.1	*Neisseria shayeganii*	04-Aug-20	2419744 bp	Canada	*Homo sapiens*	DSM 22244
65	CP091510.1	*Neisseria arctica*	11-Apr-22	2378219 bp	Canada	*Anser albifrons*	KH1503
66	CP073114.1	*Neisseria subflava*	11-Jul-22	2243952 bp	Singapore	*Homo sapiens*	HP0048
67	CP059565.1	*Neisseria wadsworthii*	04-Aug-20	2501534 bp	Canada	*Homo sapiens*	DSM 22245
68	CP094241.1	*Neisseria macacae*	29-Mar-22	2801968 bp	Korea	*Rhesus monkey*	ATCC 33926
69	CP064367.1	*Neisseria meningitidis*	11-Apr-22	2181327 bp	USA	*Homo sapiens*	PartJ-N meningitidis-RM8376
70	CP073118.1	*Neisseria subflava*	11-Jul-22	2332965 bp	Singapore	*Homo sapiens*	CG0073
71	CP072524.1	*Neisseria sicca*	05-Apr-21	2566407 bp	China	*Homo sapiens*	NS20201025
72	CP062976.1	*Neisseria*	20-Oct-20	2645607 bp	China	*Marmot*	ZJ785
73	AP024489.1	*Neisseria meningitidis*	27-Feb-21	2158475 bp	Japan	*Homo sapiens*	NIID777
74	CP073117.1	*Neisseria subflava*	11-Jul-22	2213981 bp	Singapore	*Homo sapiens*	HP0015
75	CP116766.1	*Neisseria*	05-Feb-23	2065000 bp	China	*Marmot*	ZJ106
76	LT906434.1	*Neisseria zoodegmatis*	15-Aug-17	2552522 bp	UK	*Homo sapiens*	NCTC12230
77	LR134287.1	*Neisseria animalis*	19-Dec-18	2240945 bp	UK	*Homo sapiens*	NCTC10212
78	OW969598.1	*Neisseria*	22-May-22	2024518 bp	France	*Homo sapiens*	Marseille-Q6792
79	LR134533.1	*Neisseria weaveri*	19-Dec-18	2238481 bp	UK	*Homo sapiens*	NCTC12742
80	LS483369.1	*Neisseria cinerea*	17-Jun-18	1832904 bp	UK	*Homo sapiens*	NCTC10294
81	OX336253.1	*Neisseria*	21-Sep-22	2354813 bp	France	*Homo sapiens*	Marseille-Q5346
82	LR134516.1	*Neisseria animaloris*	19-Dec-18	2283939 bp	UK	*Homo sapiens*	NCTC12227
83	LT571436.1	*Neisseria weaveri*	17-May-16	2188497 bp	UK	*Homo sapiens*	NCTC13585
84	LS483435.1	*Neisseria elongata*	17-Jun-18	2249415 bp	UK	*Homo sapiens*	NCTC11050
85	LR134313.1	*Neisseria canis*	19-Dec-18	2569389 bp	UK	*Homo sapiens*	NCTC10296
86	LR134525.1	*Neisseria meningitidis*	19-Dec-18	2186098 bp	UK	*Homo sapiens*	NCTC10025
87	LR134522.1	*Neisseria meningitidis*	19-Dec-18	2182188 bp	UK	*Homo sapiens*	NCTC3372
88	LR134526.1	*Neisseria meningitidis*	19-Dec-18	2305833 bp	UK	*Homo sapiens*	NCTC10026
89	LR134528.1	*Neisseria meningitidis*	19-Dec-18	2228346 bp	UK	*Homo sapiens*	NCTC12163

The CRISPR-Cas system was classified into six types: I-A, I-C, I-F, II-C, III-A, and III-B. Among the tested *spp.*, 69% (16 out of 89) were found to have type II-C CRISPR system, while 28% (31 out of 89) had type I-C system. Sixteen *spp.* of *Neisseria* were identified to possess two CRISPR Cas array in their genome. Among this *spp.* a majority (69%) had both type II-C and I-C systems. Especially, two *spp. Neisseria subflava* and *Neisseria dumasiana*, were discovered to have three CRISPR Cas array in their genomes. The study examined 44 CRISPR positive *spp.* of *Neisseria meningitidis* and found that it possessed the II-C CRISPR system alone exclusively (Table 2). However, in contrast, no CRISPR arrays were detected in the *Neisseria gonorrhoeae* strains indicating the absence of the typical CRISPR-Cas system in this species. But observed the presence of an orphan CRISPR, which means that a CRISPR locus was identified without the associated Cas genes that are typically part of the CRISPR-Cas system. This suggests that although *Neisseria gonorrhoeae* lacks the complete CRISPR-Cas system, it still retains some remnants of the CRISPR machinery, possibly reflecting evolutionary changes or previous interactions with foreign genetic elements.

In all the CRISPR-positive *spp.* investigated in this study, the essential components of the active CRISPR system, namely the cas1 and cas2 genes, were identified. These two genes are essential for the CRISPR system's ability to acquire and incorporate additional viral or foreign DNA sequences into the bacterial CRISPR array. The Type II-C CRISPR system depends on a single effector protein that can target and cleave both single-stranded and double-stranded DNA utilising a dual RNA-guided mechanism, in contrast to the Type I-C CRISPR system, which uses a multi-subunit complex (Csy) to target and cleave single-stranded DNA [13]. The signature protein for type I-C is Cas8c whereas for II-C is Cas9. CRISPR Cas I-F system utilizes a multi-subunit effector complex known as the Csy-F (Cascade-F complex). The Cascade- complex includes various Cas proteins such as Cas8f, Cas7f, Cas6f, Csy2 and Cas3 in the *Neisseria spp*. The CRISPR Cas Type III-A and III-B were detected in two and five *spp.* of *Neisseria,* respectively. In which type III-B and type I-C co-occurred in four out of five *spp.* of *Neisseria*. The presence of unique genes for small subunits of respective effector complexes, specifically csm2 for III-A and cmr5 for III-B, distinguishes these subtypes. In subtype III-A, cas1, cas2, and cas6 genes are often present. Additionally, III-A systems have been shown to target DNA, providing them with DNA-targeting capabilities.

Analysis of spacer sequences in CRISPR arrays

There were 3093 CRISPR spacer sequences in 89 species of the *Neisseria* altogether. After eliminating the duplicate sequences, 1661 unique spacer sequences were screened manually. The direct repeats found were of 26 – 37 bp in length and spacer sequences of 30 – 48 bp in length. The maximum number of the spacer sequence in a genome analyzed was 151, while the least was merely two. A bacteriophage interaction is seen as a critical event in CRISPR-Cas spacer acquisition because it gives selective pressure to stay intact, particularly in clinically relevant pathogens. The amount of phage-targeting spacers was shown to be positively associated with the overall number of spacers in each genome. In this study, totally 366 sites were found to be spacer targeted phage regions and the total number of self-targeting regions were about 156 in the sequence analyzed (Figure 1). Since phage interaction is believed to be a potent evolutionary process for sustaining CRISPR-Cas systems, a sizable portion of spacers (22%) were estimated to target phage DNA. Only 8% of spacers were anticipated to target plasmids. Because

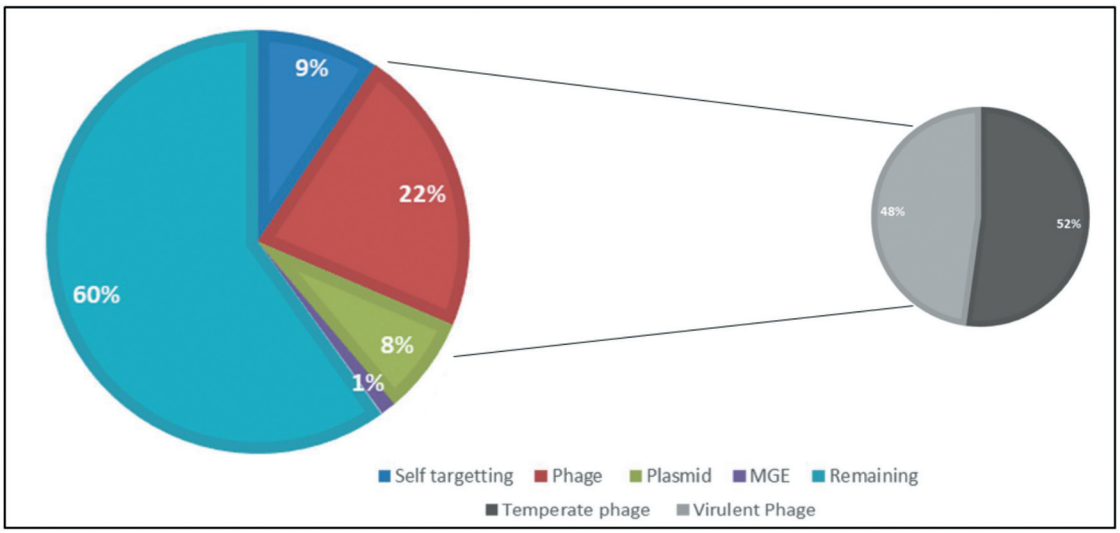

Figure 1. Spacer targeting sequences in CRISPR arrays

Table 2. CRISPR Cas regions present in the *Neisseria spp.*

S. No	Acc. No	CRISPR Types	Region	Repeats	No of Repeats	Repeat Length	No of Spacers	Spacer length
1	CP000381.1	II-C	401433 - 405981	GATTCCCGCCTGCGCGGGAATGACGG	3	26	2	42
				GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	10	36	9	48
2	CP016672.1	II-C	2099389 - 2103937	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	26	36	25	30
3	FR774048.1	II-C	375710 - 380258	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	23	36	22	30
4	CP002422.1	II-C	1919555 - 1924101	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	18	36	17	30
5	FM999788.1	II-C	1917073 - 1921621	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	26	36	25	30
6	CP016671.1	II-C	401 - 4949	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	5	36	4	30
7	CP016654.1	II-C	549773 - 554321	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	26	36	25	30
8	CP016647.1	II-C	2117129 - 2121677	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	26	36	25	30
9	AL157959.1	II-C	609568 - 614116	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	17	36	16	30
10	CP016646.1	II-C	950918 - 955466	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	27	36	26	30
11	CP016660.1	II-C	1326170 - 1330718	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	16	36	15	30
12	CP007524.1	II-C	612661 - 617209	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	24	36	23	30
13	CP007726.1	I-C	1479046 - 1483607	GTTTCAATACACAGCCGCCCGAAGGCGGCTG	11	31	10	34
14	FN995097.1	II-C	1890078 - 1894625	GTTTCAACACACAGCCGCCTAGAGGCGGCTGA	11	32	10	34
				ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	10	36	9	30
15	CP012392.1	II-C	1805076 - 1809623	ATTGTAGCACTGCGAAATGAGAATGGGAGCTACAAC	19	36	18	30
				ATTGTAGCACTGCGAAATGAGAATGGGAGCTACAAC	4	36	3	30
16	CP031332.1	II-C	318131 - 322675	ATTGTAGCACTGCGAAATGAGAATGGGAGCTACAAC	25	36	24	30
17	CP020401.2	II-C	1048461 - 1053009	CCGTCATTCCCGCGCAGGCGGGAATC	3	26	2	44
				GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	30	36	29	30
18	CP021723.1	II-C	84429- 88976	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
19	CP020420.2	II-C	915140 - 919688	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	19	36	18	30
20	CP021518.1	II-C	84427 - 88974	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
21	CP021523.1	II-C	83506 - 88053	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	18	36	17	30
22	CP039887.1	II-C	1775428 - 1779972	CCAGCCGCCTTCAGGCGGCTGTGTGTTGAAAC	152	32	151	34
		I-C	570933 - 581613	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	21	36	20	30
23	CP021516.1	II-C	84423 - 88970	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
24	CP021725.1	II-C	83483 - 88030	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	18	36	17	30
25	CP023429.1	I-C	2060384 - 2070426	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	14	36	13	30
		II-C	815633 - 820185	GTTTCAACACACAGCCGCCCGTGGGCGGCTGA	24	32	23	34
26	CP021521.1	II-C	81087 - 85634	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	23	36	22	30
27	CP031255.1	II-C	426210 - 430763	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAGC	25	36	25	30
		I-C	1708507 - 1720752	GTTTCAACACACAGCCGCCCGAAGGCGGCTGG	26	32	16	34
28	CP021522.1	II-C	84428 - 88975	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
29	CP045960.1	II-C	84193 - 88740	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	17	36	16	30
30	CP020402.2	II-C	848677 - 853225	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	37	36	36	30
31	CP021520.1	II-C	83475 - 88022	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	18	36	17	30
32	CP039886.1	I-C	515098 - 526750	CCAGCCGCCTTCAGGCGGCTGGTGTGTTGAAAC	7	33	6	34
33	CP040504.1	II-C	1856374 - 1860948	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	28	36	27	30
		I-F	2005639 - 2016144	TTTCTAAGCTGCCTATTCGGCAGGTAAC	44	28	43	32
				TTTCTAAGCTGCCTGTGCGGCAGGTAAC	18	28	17	32
34	CP031699.1	II-C	1387090 - 1391609	ATTGTAACACTACGAGATGAGAGAGGGAGCTACAAC	12	36	11	30

S. No	Acc. No	CRISPR Types	Region	Repeats	No of Repeats	Repeat Length	No of Spacers	Spacer length
35	CP021724.1	II-C	84362 - 88909	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	14	36	13	30
36	CP031334.1	II-C	309325 - 313869	ATTGTAGCACTGCGAAATGAGAATGGGAGCTACAAC	43	36	42	30
37	CP012694.1	II-C	2049829 - 2054377	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	19	36	18	30
38	CP031253.1	II-C	168076 - 172624	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	12	36	11	30
39	CP016883.1	II-C	371884 - 376432	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	32	36	31	30
40	CP031324.1	II-C	996535 - 1001079	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	30	36	29	30
41	CP046027.1	I-C	1631803 - 1640082	TCAGCCACCTTCGGGTGGCTGTGTGTTGAAAC	20	32	19	34
42	CP022527.1	I-C	177305 - 185856	GTTTCAACACTCAGCCGCCCGAAGGCGGCTGC	26	32	25	34
43	CP021517.1	II-C	84425 - 88972	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
44	CP022278.1	I-C	1936631 - 1946895	GTTTCAATACACAGCCACCCGCGAGGGTGGCTG	38	33	37	34
45	CP031251.1	I-C	1193128 - 1202381	GTTTCAACACACAGCCGCCCGAAGGCGGCTGG	172	32	171	35
		III-B	1331696 -1350397	GTCGGAAGACTTGCCCCACTAATCGGGGATTAAGAC	14	36	13	34
				GTCTTAATCCCCGATTAGTGGGGCAAGTCTTCCGAC	19	36	18	34
		I-A	2100402 - 2106823	GTCTTAATCCCCATGTGGTGGGGAGGTTTTTCAGAG	21	36	20	34
				GTCTTAATCCCCATGTGGTGGGGAGGTTTTTCAGAG	20	36	19	35
46	CP016682.1	II-C	25073 - 29621	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	20	36	19	30
47	CP016680.1	II-C	1226815 - 1231363	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	26	36	25	30
48	CP021519.1	II-C	85186 - 89733	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	15	36	14	30
49	CP031252.1	I-C	223404 - 234018	CAGCCGCCTTTAGGCGGCTGTGTGTTGAAAC	25	31	24	35
50	CP020422.2	II-C	1284066 - 1288614	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	37	36	36	30
51	CP031328.1	II-C	2209402 - 2213946	ATTGTAGCACTGCGAAATGAGAATGGGAGCTACAAC	23	36	22	30
52	CP020452.2	III-B	1698165 - 1717071	GTCGGAAGACTTGCCCCACTAATCGGGGATTAAGAC	20	36	19	34
				GTCTTAATCCCCGATTAGTGGGGCAAGTCTTCCGAC	18	36	17	34
		I-C	1780305 - 1790271	CCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	76	32	75	34
53	CP065653.1	II-C	1516025 - 1520573	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	19	36	18	30
54	CP073116.1	II-C	1877716 - 1882260	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	10	36	9	30
55	CP053939.1	I-C	483233 - 497654	CCAGCCGCCTTCAGGCGGCTGTGTGTTGAAAC	16	32	15	34
56	CP065726.1	II-C	1601771 - 1606315	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	19	36	18	30
57	CP059570.1	III-A	1125145 - 1125533	TCTCAATCCCCGTGTTGATGGGGCTTTTTTGTGTCC	6	36	5	34
		II-C	2469750 - 2474265	GTTGTAGCTTCCTCTCTCATCTCGTAGTGCTACAAT	23	36	22	30
58	CP073119.1	II-C	95952 - 100497	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	23	36	22	30
		I-C	1584845 - 1595472	GTTTCAACACACAGCCGCCCGAAGGCGGCTGG	27	32	26	35
59	CP091522.1	I-C	178250 - 189166	TCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	74	32	73	35
60	CP073115.1	II-C	2248300 - 2252845	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	7	36	6	30
61	CP091509.1	II-C	1138380 - 1143001	GTCTTAATCCCCGAATGGTGGGGCTTTGTTTTCAAT	50	36	49	33
		III-A	464699 - 481428	GTTGTAGCTTCCTCTCTCATCTCGTAGTGCTACAAT	15	36	14	30
		I-C	2469067 - 2482172	GTTTCAACACACAGCCACGCGAAGGTGGCTGA	8	32	7	34
				GTTTCAACACACAGCCGCCTGAAGGCGGCTGG	8	32	7	35
62	CP059566.1	III-B	863873 - 879969	GTCGGAAGACTTGCCCCACGTATCGGGGATTAAGAC	7	36	6	33
				GTCGGAAGACTTGCCCCACTAATCGGGGATTAAGAC	6	32	82	34
				GTCTTAATCCCCGATTCGTGGGGCAAGTCTTCCGAC	3	36	2	33
				GTCTTAATCCCCGATGCGTGGGGCAAGTCTTCCGAC	4	36.2	3	34
		I-C	1179516 - 1192803	CCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	83	35.2	5	35

S. No	Acc. No	CRISPR Types	Region	Repeats	No of Repeats	Repeat Length	No of Spacers	Spacer length
63	CP060414.1	II-C	2238654 -2243142	ATTGTAGCACTGCGAGATGAAAGAGGAAGCTACAAC	22	36	21	30
				GTTGTAGCTTCCTCTTTCATCTCGCAGTGCTACAAT	22	36	21	30
64	CP059567.1	I-C	1474197 - 1484682	GTTTCAACACACAGCCGCCCGAAGGCGGCTGA	3	32	2	33
65	CP091510.1	II-C	2367224 - 2371744	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	17	32	3	35
		I-C	232682 - 2339182	GTTTCAACACACAGCCACCCGAAGGTGGCTGA	4	36	16	30
66	CP073114.1	II-C	1369084 - 1373627	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	26	36	25	30
67	CP059565.1	I-C	6517 - 15886	GTTTCAACACACAGCCGCCCGAAGGCGGCTGA	6	32	5	35
				GTTGTAGCTTCCTCTCTCATCTCGTAGTGCTACAAT	4	36	3	30
		II-C	863809 - 868414	GTTGTAGCTTCCTCTCTCATCTCGTAGTGCTACAAT	26	36	25	30
68	CP094241.1	III-B	1517659 - 1534284	AGTCGGAAGACTTACCCCACTAGTCGGGGATAAAACT	4	37	3	33
				GTCTTAATCCCCGATTAGTGGGGCAAGTCTTCCGAC	11	36	10	34
				GTCTTAATCCCCGATTCGTGGGGCAAGT	9	28	8	42
		I-C	1835421 - 1848345	CAGCCGCCTTTAGGCGGCTGTGTGTTGAAAC	7	31	6	35
69	CP064367.1	II-C	869274 - 873822	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	19	36	18	30
70	CP073118.1	I-C	920 - 10662	GTTTCAACACACAGCCGCCTGAAGGCGGCTGG	11	32	10	34
				ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	5	36	4	30
		II-C	757607 - 762151	GTTTCAACACACAGCCGCCTGAAGGCGGCTGG	23	32	22	34
71	CP072524.1	III-B	779344 - 797781	GTCGGAAGACTTGCCCCACTAATCGGGGATTAAGAC	16	36	15	33
				GTCTTAATCCCCGATTAGTGGGGCAAGTCTTCCGAC	26	36	25	33
		I-C	1096743 - 1106398	GTTTCAACACACAGCCGCCCGAAGGCGGCTGG	62	32	61	34
72	CP062976.1	I-C	484640 - 495508	TCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	12	32	11	34
				TCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	37	36	32	30
		II-C	870835 - 875393	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	33	32	36	34
73	AP024489.1	II-C	80955 - 85499	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	10	36	9	30
74	CP073117.1	II-C	742194 - 746741	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	28	36	27	30
		I-C	1724281 - 1734277	CCAGCCGCCTTCAGGCGGCTGTGTGTTGAAAC	98	32	97	35
				CCAGCCGCCTTCAGGCGGCTGTGTGTTGAAAC	10	32	9	35
75	CP116766.1	I-C	920001 - 931498	GTTTCAACACACAGCCGCCTGAAGGCGGCTGA	31	32	30	34
76	LT906434.1	I-C	188995 - 198248	GTTTCAACACACAGCCGCCCGAAGGCGGCTGC	62	32	61	34
77	LR134287.1	II-C	473845 - 478364	ATTGTAACACTACGAGATGAGAGAGGGAGCTACAAC	12	36	11	30
78	OW969598.1	II-C	1953431 - 1957964	GTTGTAGCTCCCATTCTCATTTCGCAGTGCTACAAT	27	36	26	30
79	LR134533.1	I-C	153056 - 159598	GTTTCAACACACAGCCGCCCGAAGGCGGCTGT	63	32	62	35
80	LS483369.1	II-C	106683 - 111227	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	8	36	35	30
81	OX336253.1	II-C	1223399 - 1227947	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	7	36	35	30
82	LR134516.1	II-C	1226810 - 1231355	ATTGTAGCACTACGAGATGAGAGAGGAAGCTACAAC	7	36	35	30
		I-C	1889746 - 1900991	GTTTCAACACACAGCCGCCCGAAGGCGGCTGA	4	32	31	35
83	LT571436.1	I-C	918256 - 929848	GTTTCAACACACAGCCGCCCGAAGGCGGCTGTTT	79	34	33	34
84	LS483435.1	I-C	1905654 - 1910215	GTTTCAATACACAGCCGCCCGAAGGCGGCTG	11	31	30	34
85	LR134313.1	I-C	2423410 - 2433620	GTTTCAACACACAGCCGCCCGAAGGCGGCTG	36	31	30	35
86	LR134525.1	II-C	1519022 - 1523570	ATTGTAGCACTGCGAAATGAGAAAGGGAGCTACAAC	19	36	35	30
87	LR134522.1	II-C	81278 - 85826	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	22	36	35	30
88	LR134526.1	II-C	91517 - 96065	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	39	36	35	30
89	LR134528.1	II-C	82689 - 87237	GTTGTAGCTCCCTTTCTCATTTCGCAGTGCTACAAT	15	36	35	30

Table 3. Stability of CRIPSR direct repeats in *Neisseria spp*.

S. No	CRISPR Type	Repeat Sequence	Frequency (%)	Folding score (kcal/mol)
DR1	I-A	GTCTTAATCCCCATGTGGTGGGGAGGTTTTTCAGAG	47.57 %	-10.66
DR2	I-C	CCAGCCGCCTTCAGGCGGCTGGTGTGTTGAAAC	90.22 %	-19.36
DR3	I-C	GTTTCAATACACAGCCACCCGCGAGGGTGGCTG	69.64 %	-19.22
DR4	I-C	TCAGCCGCCTTCGGGCGGCTGTGTGTTGAAAC	90.70 %	-17.06
DR5	I-C	CAGCCGCCTTTAGGCGGCTGTGTGTTGAAAC	90.00 %	-16.06
DR6	I-F	TTTCTAAGCTGCCTGTGCGGCAGGTAAC	38.57 %	-8.69
DR7	II-C	GTTTCAACACACAGCCGCCTAGAGGCGGCTGA	80.56 %	-16.63
DR8	II-C	ATTGTAGCACTGCGAGATGAAAGAGGAAGCTACAAC	33.55 %	-7.37
DR9	II-C	CCGTCATTCCCGCGCAGGCGGGAATC	79.71 %	-13.84
DR10	II-C	GATTCCCGCCTGCGCGGGAATGACGG	38.57 %	-8.69
DR11	II-C	GTTGTAGCTTCCTCTTTCATCTCGCAGTGCTACAAT	64.43 %	-8.07
DR12	III-A	TCTCAATCCCCGTGTTGATGGGGCTTTTTTGTGTCC	56.17 %	-9.46
DR13	III-B	AGTCGGAAGACTTACCCCACTAGTCGGGGATAAAACT	47.57 %	-9.96
DR14	III-B	GTCGGAAGACTTGCCCCACTAATCGGGGATTAAGAC	84.00 %.	-9.31
DR15	III-B	GTCTTAATCCCCGATTCGTGGGGCAAGTCTTCCGAC	28.48 %	-7.77
DR16	I-C , II-C	GTTTCAACACACAGCCGCCCGAAGGCGGCTG	79.10 %	-16.04
DR17	II-C, III-A, I-C	GTTGTAGCTTCCTCTCTCATCTCGTAGTGCTACAAT	64.83 %	-8.07

of the presence of Anti CRISPR (Acr) genes or the lack of homologous Cas genes, self-targeting spacers were prevalent in genomes anticipated to have inactivated CRISPR-Cas systems. The identified phages were further categorized into temperate and virulent groups. Among these groups, approximately 52% of the phages were classified as temperate, while the remaining 48% were classified as virulent. Notably, phage sequences from *Haemophilus* phage, *Ralstonia* phage, *Enterobacteria* phage, *Burkholderia* phage, and *Pseudomonas* phage were observed at a higher frequency in the dataset. Interestingly, some spacers were found to be identical as *Neisseria* plasmid sequences, despite not being derived from the current host bacteria. Additionally, there were 16 spacer sequences that exhibited matches with plasmid and phage sequences, suggesting potential interactions and exchange of genetic material between these mobile genetic elements. Among the spacers analyzed, approximately 60% did not show any recognizable target in our database searches.

Stability of CRIPSR direct repeats

The structural stability and intramolecular structure of distant direct repeats were performed using RNAfold web server for the dataset (Table 3). One hundred and forty direct repeat sequences of *Neisseria spp.* were grouped into 17 categories based on sequence homology. The tool will design the RNA structure based on the bit score that represents the stability of repeats. In this study, the repeat regions DR2, DR3, DR4, DR5,DR7 and DR16 found to have folding scores between – 16 to -19 kcal/mol which indicates stable secondary structure whereas other direct repeat regions found to have fold scores. The difference in the structural stability of CRISPR repeats has a significant consequence in pre-crRNA processing since it helps in forming tracrRNA. The formation of tracrRNA with closed hairpin structure will elevate the genome editing efficiency by 10 folds and also it will minimize the prescreening of gRNAs towards targeting the gene of interest [14, 15].

Relation between the CRISPR Cas system and bacterial drug resistance

The AR gene analysis in CRISPR positive *Neisseria spp.* was conducted by performing BLASTN search against the Resfinder and CARD databases. The analysis findings reveal that 30 out of the 89 genomes showed no detected AR genes, constituting approximately 33.7% of the sampled data. In the analyzed *Neisseria* genomes with CRISPR type II-C, a notable finding was the presence of efflux pump resistance genes in the majority of the sequences (71%). These efflux pump genes include *farB*, *mtrF*, *mtrC*, *mtrA*, and *norM*. Among the *Neisseria spp.* possessing both III-B and I-C CRISPR types, the majority of the *spp.* (75%) were found to harbor only the *norM* efflux gene (Table 4).The *norM* gene encode an efflux pump that facilitates the removal of hydrophobic agents, which can include antibiotics, nonionic detergents, certain antibacterial peptides, bile salts, and steroidal hormones. This gene's activity leads to a decrease in susceptibility to fluoroquinolones [16]. However, there was one exception, where a *Neisseria mucosa* genome was identified to harbor additional resistance genes. This particular strain was found to carry genes such as *aph(6)-Id*, *aph(3")-Ib*, *sul2*, *blaTEM-1*, and *tet(B)*, in addition to the *norM* gene. *blaTEM* genes confer resistance to amoxicillin-clavulanate in clinical settings. However, they maintain susceptibility to inhibition by tazobactam, which subsequently renders them susceptible to the combination of piperacillin and tazobactam [17]. Determinants of tetracycline resistance were more susceptible to tigecycline whereas aminoglycoside resistances are susceptible to amikacin [18, 19].

Statistical analysis was computed to measure the association of CRISPR and the AR genes in the *Neisseria*

Table 4. Correlation of CRISPR Cas system and AR genes

S. No	Acc. No	CRISPR Types			Antibiotic Resistance Genes	Species	Country
1	CP039887.1	II-C	I-C		No AR genes	*Neisseria subflava*	USA
2	CP023429.1	I-C	II-C		No AR genes	*Neisseria weixii*	China
3	CP031255.1	II-C	I-C		No AR genes	*Neisseria elongata*	USA
4	CP040504.1	II-C	I-F		*norM*	*Neisseria*	Australia
5	CP031251.1	III-B	I-A	I-C	No AR genes	*Neisseria subflava*	USA
6	CP020452.2	III-B	I-C		*aph(6)-Id*, *aph(3")-Ib*, *sul2*, *blaTEM-1*,*tet(B)*,*norM*	*Neisseria mucosa*	USA
7	CP059570.1	III-A	II-C		No AR genes	*Neisseria dentiae*	UK
8	CP073119.1	I-C	II-C		No AR genes	*Neisseria subflava*	Singapore
9	CP091509.1	II-C	III-A	I-C	No AR genes	*Neisseria dumasiana*	USA
10	CP059566.1	III-B	I-C		*norM*	*Neisseria sicca*	UK
11	CP091510.1	II-C	I-C		No AR genes	*Neisseria arctica*	USA
12	CP059565.1	I-C	II-C		No AR genes	*Neisseria wadsworthii*	USA
13	CP094241.1	III-B	I-C		*norM*	*Neisseria macacae*	South Korea
14	CP073118.1	II-C	I-C		No AR genes	*Neisseria subflava*	Singapore
15	CP072524.1	I-C	III-B		*norM*	*Neisseria sicca*	China
16	CP062976.1	II-C	I-C		No AR genes	*Neisseria*	China
17	CP073117.1	I-C	II-C		No AR genes	*Neisseria subflava*	Singapore
18	LR134516.1	II-C	I-C		No AR genes	*Neisseria animaloris*	UK

Table 5. Presence of CRISPR Cas loci in *Neisseria spp.* and its associations with AR genes

Species	Total No of Species	No of CRISPR positive Species	Observed no of Species has both CRISPR and AR genes (O)	Expected no of Species possessing both CRISPR and AR genes (E)	Log frequency-ratios (Log(O/E))
Neisseria animalis	2	2	2	0.36	0.75
Neisseria animaloris	2	1	1	0.32	0.5
Neisseria arctica	1	1	0	0	0
Neisseria bacilliformis	1	0	0	0.14	0
Neisseria brasiliensis	1	1	1	0.16	0.81
Neisseria canis	1	1	1	0.16	0.81
Neisseria chenwenguii	1	1	1	0.16	0.81
Neisseria cinerea	2	2	2	0.36	0.75
Neisseria dentiae	1	1	0	0	0
Neisseria dumasiana	1	1	0	0	0
Neisseria elongata	4	4	3	0.68	0.65
Neisseria flavescens	1	1	1	0.16	0.81
Neisseria gonorrhoeae	169	0	0	50.94	0
Neisseria lactamica	4	2	2	0.36	0.75
Neisseria macacae	1	1	1	0.16	0.81
Neisseria meningitidis	136	44	44	119.94	-0.44
Neisseria mucosa	3	2	2	0.36	0.75
Neisseria musculi	1	1	1	0.16	0.81
Neisseria perflava	1	0	0	0.14	0
Neisseria polysaccharea	1	0	0	0.14	0
Neisseria shayeganii	1	1	1	0.16	0.81
Neisseria sicca	3	2	1	0.32	0.5
Neisseria	8	7	3	0.91	0.52
Neisseria subflava	8	8	7	2.22	0.5
Neisseria wadsworthii	1	1	0	0	0
Neisseria weaveri	2	2	1	0.17	0.76
Neisseria weixii	1	1	0	0	0
Neisseria zalophi	1	0	0	0.14	0
Neisseria zoodegmatis	1	1	1	0.16	0.81

spp (Table 5). A positive log frequency-ratio signifies a positive association, suggesting that AR genes tend to coexist with CRISPR Cas. Conversely, a negative association is observed when the presence of CRISPR Cas tends to exclude AR genes. It was found that the *Neisseria spp.* with several CRISPR arrays often had either no AR genes or only one AR gene in their genomes. The presence of efflux pump genes has been identified in the majority of the *Neisseria spp.* Efflux pumps are specialized transporters in bacterial cells that play a crucial role in AR. It actively eliminate antibiotics from the bacterial cell, lowering their intracellular concentration and decreasing their ability to fight infections. However, when more than one array region was present in the *Neisseria* genome along with CRISPR type II-C, no similar pattern of harboring efflux pump genes was observed.

Discussions

Prokaryotes, in response to daunting survival challenges, have evolved CRISPR-Cas systems as their defense mechanisms. Within the gastrointestinal tract, a rich array of natural phages exists, setting the stage for an unending struggle between bacteria and bacteriophages. Bacterial *spp.* equipped with these CRISPR-Cas systems are prime candidates for industrial applications because of their robust resistance to bacteriophages. The interplay between AR and CRISPR-Cas

systems in *Neisseria* pathogens is a critical concern due to the rise of AR strains. *Neisseria* species, like *N. gonorrhoeae* and *N. meningitidis*, have developed resistance to multiple antibiotics, diminishing our ability to treat infections effectively. *N. meningitidis* can lead to various clinical conditions, including meningococcemia, pneumonia, septic arthritis, pericarditis, and urethritis. *N. gonorrhoeae* primarily causes sexually transmitted infections, with symptoms such as genital discharge and discomfort during urination [20]. However, CRISPR-Cas systems, which function as a bacterial immune system, offer a unique avenue for addressing this issue. These systems capture and store genetic material from invading elements like plasmids carrying AR genes, and later use this information to target and destroy matching sequences. Consequently, researchers are exploring the use of CRISPR technology to selectively eliminate AR genes within *Neisseria* pathogens, potentially restoring their susceptibility to antibiotics and providing a novel strategy to combat AR strains. This approach not only has the potential to extend the efficacy of existing antibiotics but also represents a significant development in the ongoing battle against AR, a public health crisis of global significance.

A comprehensive investigation into the prevalence and diversity of CRISPR-Cas systems was conducted in a collection of 360 *Neisseria spp.* These bacterial strains were sourced from a variety of hosts, including *Homo sapiens* (humans), *Felis catus* (cats), *Mus musculus* (mice), *Anser albifrons* (white-fronted goose), *Plateau pika* (a small mammal), Rhesus monkeys, marmots, cattle, poultry and guinea pigs. Among these *spp.* 89 were identified to harbor CRISPR arrays. Notably, 69% of the tested *spp.* (16 out of 89) were found to harbor the type II-C CRISPR system, while 28% (31 out of 89) exhibited the type I-C system. Type I-C systems employ a complex of multiple Cas proteins, known as the cascading complex, to target and cleave foreign DNA during interference. In contrast, Type II-C systems, exemplified by Cas9, rely on a single effector protein for both target recognition and DNA cleavage, making them simpler and widely used in genome editing applications. Earlier research in Gram-negative bacteria, particularly *Pseudomonas*, revealed a high prevalence of the CRISPR type I-F system [21]. On the other hand, studies involving *Klebsiella* species identified the presence of the typical Type I-E and I-F CRISPR-Cas systems within their genomic makeup [22]. These findings illustrate the diversity and distribution of CRISPR-Cas systems across different bacterial species, highlighting their adaptability in various microbial environments. The results of this particular study appear to diverge from earlier literature, notably the research by (Burstein *et al*, 2016) [23]. Burstein and colleagues reported that Class I CRISPR systems were predominant among prokaryotes. In contrast, the study suggests that within *Neisseria spp.* Class II Type C CRISPR systems are the most commonly encountered.

In this study, 366 regions within phage genomes that were targeted by CRISPR spacers were found, indicating the potential role of the CRISPR-Cas system in defending against these specific viral regions. Additionally, the analysis revealed 156 regions within the examined sequences where the CRISPR-Cas system could target its own genetic material (self-targeting spacers). This discovery underscores the intricate nature of CRISPR-Cas systems, encompassing both their defensive capabilities and the intriguing phenomenon of self-targeting, which could have ramifications for understanding the immune response and genetic regulation in these organisms. In a comparative analysis conducted by (Parra *et al*, 2023) the examination of *Pseudomonas* genomes revealed the presence of 2050 spacers within their CRISPR arrays [24]. Approximately, 52% of these spacers exhibited similarity to bacteriophage sequences, while 26% matched chromosomal DNA and 22% corresponded to plasmid DNA. Notably, no instances of potential self-targeting spacers were identified within the CRISPR arrays, suggesting the existence of a protective mechanism preventing autoimmunity in *Pseudomonas*. Conversely, a study by (Devi *et al*, 2019), focusing on *Klebsiella*, uncovered a different scenario. Here, 3% of the spacers were found to be self-targeting and less than 9% of the spacer sequences in *Klebsiella* displayed matches to known plasmids (6%) or phages (2.8%) in existing databases, underscoring the limited understanding of the various adversaries that bacteria encounter in their environment [25].The frequency of self-targeting spacers in the CRISPR array is likely to have correlation with phage targeting regions. The inclusion of a greater number of phage and plasmid sequences to the database was thought to be responsible for the considerable fall in the proportion of self-targeting spacers [26].These findings emphasize the dynamic interplay between CRISPR systems and the microbial challenges it faces, shedding light on the ongoing evolutionary arms race between bacteria and their viral and genetic adversaries.

A total of 140 direct repeat sequences from *Neisseria spp.* were categorized into 17 groups, primarily based on their sequence homology. The number of repeats and its structural stability in a CRISPR–Cas system serves as an important indicator of its functionality and integrity. A higher number of repeats usually denote that the CRISPR–Cas system is complete and functioning effectively. In such cases, the system is fully capable of defending the organism against foreign genetic elements like viruses and plasmids. Conversely, when the number of repeats is intermediate, it indicates that

the CRISPR–Cas system has experienced recent erosion or degradation. This erosion may have been caused by the loss of functional Cas genes or other factors that compromise the system's ability to protect against invaders effectively. In instances where the number of repeats is low, only relics of the CRISPR–Cas system are noticed [27]. This suggests that the system might have been severely reduced in its functionality, potentially leaving the organism more susceptible to viral and plasmid infections. The presence of specific secondary structure motifs within CRISPR repeats is essential for the generation and loading of crRNAs in many CRISPR–Cas systems. These repeats exhibit structural diversity, and (Kunin *et al*, 2007) research findings suggested that the system likely relies on an RNA intermediate, as evidenced by compensatory base changes, including G:U base pairs, within the stem regions of structured repeats [18].

Numerous studies have highlighted the genetic exchange in the development of AR in the pathogenic *Neisseria spp* [28, 29]. By examining the genomic and phylogenetic distributions of CRISPR-Cas systems in various bacteria, have sought evidence of how these systems might function in preventing the acquisition of foreign DNA elements. A study by (Wheatley *et al*, 2020) supporting this hypothesis in the case of *Pseudomonas aeruginosa*, a bacterial species known for having both large core genome and accessory genome [30]. In such organisms, the presence of CRISPR-Cas systems may indeed contribute to genome reduction by inhibiting the acquisition of foreign DNA elements. Similarly, previous research on 16 *E. faecalis* genomes indicated that the presence of CRISPR-Cas systems was negatively correlated with AR. To validate and extend this finding, a more comprehensive analysis was conducted, involving 514 *E. faecalis* genomes [31]. The results revealed that approximately two-thirds of these genomes (338 out of 514) lacked CRISPR-Cas systems. Interestingly, these 338 genomes without CRISPR-Cas systems also exhibited multiple AR genes, conferring them resistance to various drug classes. This suggests that the absence of CRISPR-Cas systems may contribute to the prevalence of AR in *E. faecalis spp.* Additionally, a prior study using 672 clinical isolates of *P. aeruginosa* similarly found that bacteria with CRISPR-Cas systems had lower sulfonamide resistance [32].This convergence of results shows that the presence of CRISPR-Cas systems in pathogens may be associated with a decreased likelihood of carrying AR genes, thus acting as a defense mechanism against AR. In-depth investigations by (Pursey *et al*, 2021) focused on modeling the association between CRISPR-Cas systems and indicators of HGT [33]. The study by (García et al, 2018) made an intriguing observation regarding *E. coli* genomes. They found that approximately 30% of these genomes, specifically 1706 out of 5661 analyzed, contained resistance genes related to antibiotics such as beta-lactam, quinolone, macrolide, and trimethoprim, but surprisingly lacked CRISPR-Cas systems [34]. It was align with another prior research that has shown how CRISPR-Cas systems can impede natural transformation, a key mechanism for HGT, in specific bacterial species, as illustrated in the case of *N. meningitides* [35]. The genome-wide correlation analysis conducted by (Shehreen et al, 2019) revealed that the majority of bacterial species showed no strong correlation between the presence of CRISPR-Cas systems and AR genes, their study identified specific clinically important bacterial species where this relationship exhibited either a positive or negative correlation [36]. This indicates that the connection between CRISPR-Cas systems and AR genes is not uniform across all species and emphasizes the need for a tailored, species-specific approach to understand these interactions fully in the context of AR mechanisms. One plausible explanation could be the selective pressure exerted by antibiotic exposure, which might favor the acquisition of AR genes through HGT over the maintenance of CRISPR-Cas systems. It is conceivable that in the evolutionary history of these strains, ancestors lost their CRISPR-Cas systems due to their reduced relevance in the face of antibiotic-driven selection.

Conclusion

In-silico examination of the CRISPR-Cas system in *Neisseria spp.* which was identified across genomes of varied geographical location was considered for the analysis. The CRISPR Cas arrays were discovered in 89 *Neisseria* genomes, 69% of which contained the type II-C CRISPR system and 28% had the type I-C system. In this investigation, 366 regions were identified to be spacer targeted phage regions, with about 156 self-targeting regions out of 1661 distinct spacers. The structural stability of the direct repeat regions was also studied. The direct repeat regions found to have fold score between – 16 to -19 kcal/mol, it indicates stable secondary structure. AR genes were absent in 30 of the 89 *Neisseria spp.* A striking observation was the existence of efflux pump resistance genes in the vast majority of the sequences examined harboring CRISPR type II-C. It was found that *spp.* with several CRISPR arrays frequently have no AR genes or only one AR gene in their genomes. The presence of the CRISPR-Cas system was linked to a decrease in the number of AR genes. The finding raises interesting questions about the potential mechanisms underlying the absence or presence of CRISPR Cas system in relation with AR genes. Therefore, gaining a deeper understanding of the complex relationship between CRISPR-Cas systems

and AR in *Neisseria spp* requires further investigation to identify additional factors that contribute to the emergence and dissemination of AR genes.

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The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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