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CRISPR/Cas9: A Novel Weapon Against Antibiotic Resistance Through Plasmid Gene Editing

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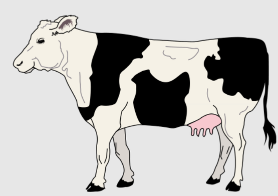
Background

- Antimicrobial resistance (AMR) is a significant global threat, with a projected annual fatality rate of approximately 10 million by 2050 [1].
- Factors driving AMR include excessive antibiotic prescription, agricultural overuse, and natural microorganism adaptation [2].
- AMR spreads beyond clinical settings through plasmid-mediated gene transfer in natural aquatic ecosystems [2].
- The pharmaceutical industry has not introduced new antibiotic classes in the past three decades, raising doubts about the efficacy of existing antibiotics [3].
- Carbapenem antibiotics are the last resort for bacterial infections [4].
- The gene blaNDM confers resistance to carbapenems [4]
- CRISPR/Cas9 offers a promising solution to target and disable AMR genes.
- This project designed 13 sgRNA sequences to target blaNDM-5 for *in vitro* digestion in an effort to demonstrate the potential of CRISPR/Cas9 in combating AMR.


Unnecessary agricultural use

Overprescription

Unfinished prescriptions

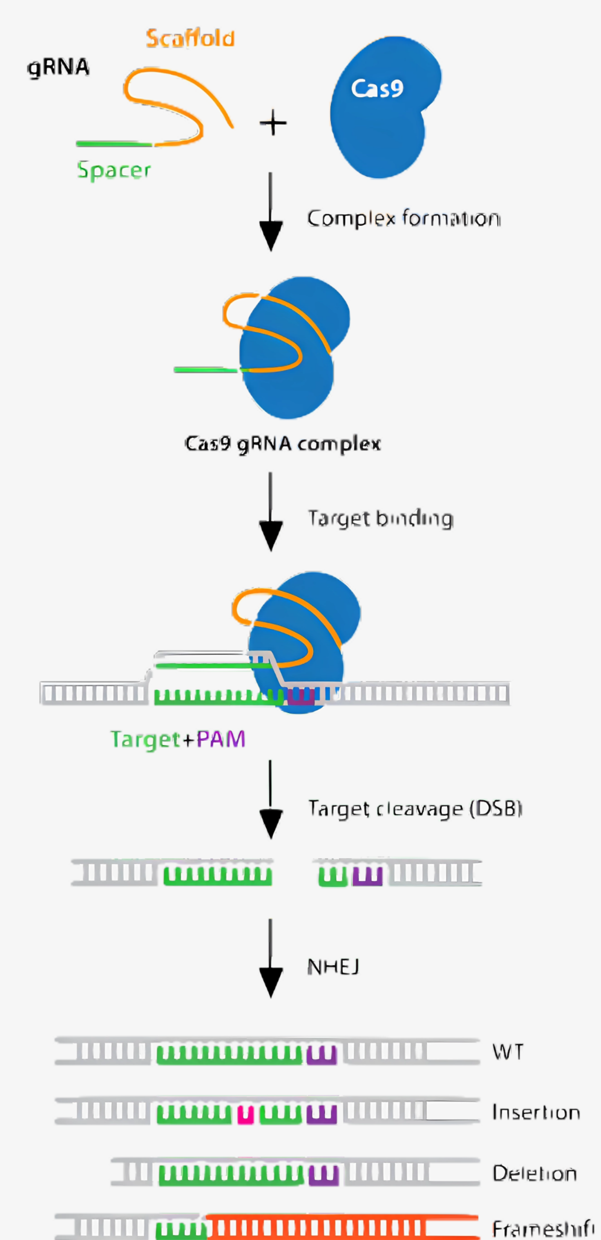
Poor infection control in medical settings

Poor hygiene and lack of sanitation

Lack of new antibiotics being formulated

Method

- Obtained the first 15 alleles sequences of blaNDM.
- Used Python to identify base pair differences.
- USED bioinformatics sources (CHOPCHOP, CRISPOR) to select sgRNA targets that were present in all 15 alleles, ranked highly in Moreno-Mateos et al.'s method, had a CFD specificity score of 100, and ≤1 off-target compared to K. pneumoniae genome.
- *K. pneumoniae* clinical isolates were used as source of blaNDM-5, which was used the NDM-5 as target for CRISPR/Cas9 cleavage
- Determined DNA and RNA concentrations using a NanoDrop spectrophotometer for in vitro digestion optimisation with Cas9 protein.
- Performed digestion with a Cas9 to sgRNA to target dsDNA ratio of 5:5:1



Master protocol steps:

1. Resuspend DNA oligos for RNA synthesis.
2. Lysing clinical isolates to obtain digestion target DNA.
3. PCR amplification of target NDM-5 DNA (Q5® High-Fidelity 2X Master Mix).
4. Gel electrophoresis to verify correct gene amplification.
5. DNA purification (QIAquick® PCR Purification Kit).
6. sgRNA synthesis from DNA oligos (EnGen® sgRNA Synthesis Kit, S. pyogenes, NEB #E3322V/S).
7. RNA cleanup (Monarch® RNA Cleanup Kit, 50 µg, #T2040L).
8. Nanodrop analysis of sgRNA and DNA concentrations.
9. In vitro DNA digestion with Cas9 nuclease, S. pyogenes (M0386).
10. Gel electrophoresis (1.5% Agarose DNA Grade, 110 min, 80 V, ethidium bromide, Hyperladder 1kb weight marker).

Results

- Gel electrophoresis results matched the predicted pattern, shown in Figure A
- All 13 target sites were successfully digested by sgRNA/Cas9 complexes as shown in Figure B
- The sgRNA targeting nucleotide position 720 (sense strand) exhibited partial digestion.
- Partial digestion was also observed with the pBR322 controls.

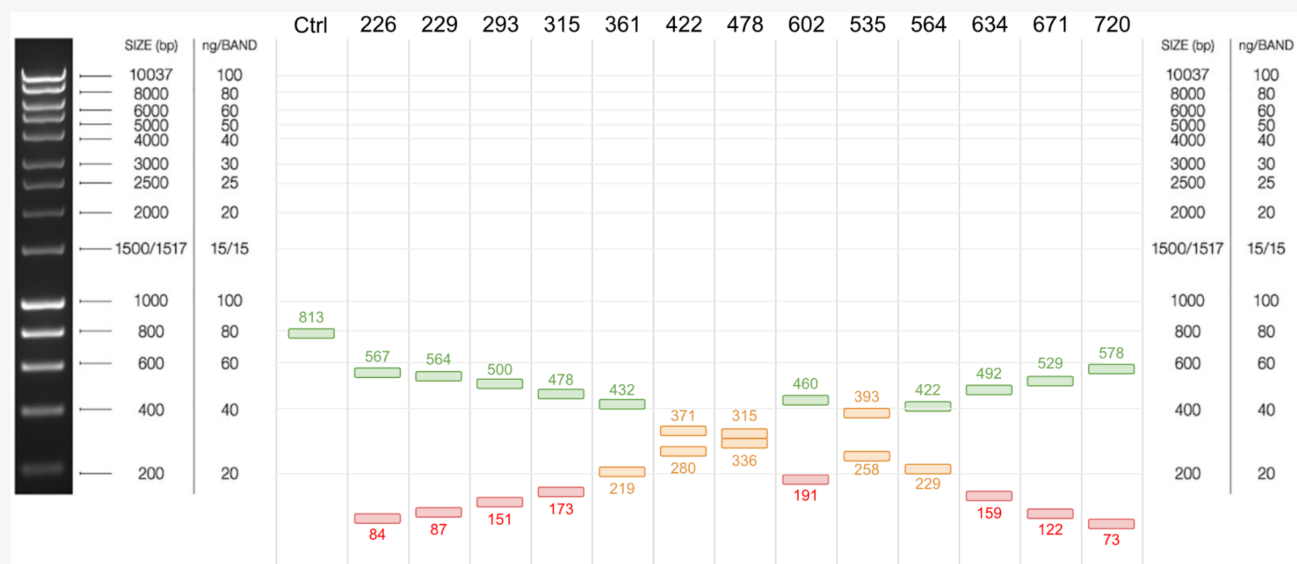


FIGURE A: Predicted gel profile. High likelihood of visibility indicated in green, medium likelihood in yellow, and poor likelihood in red

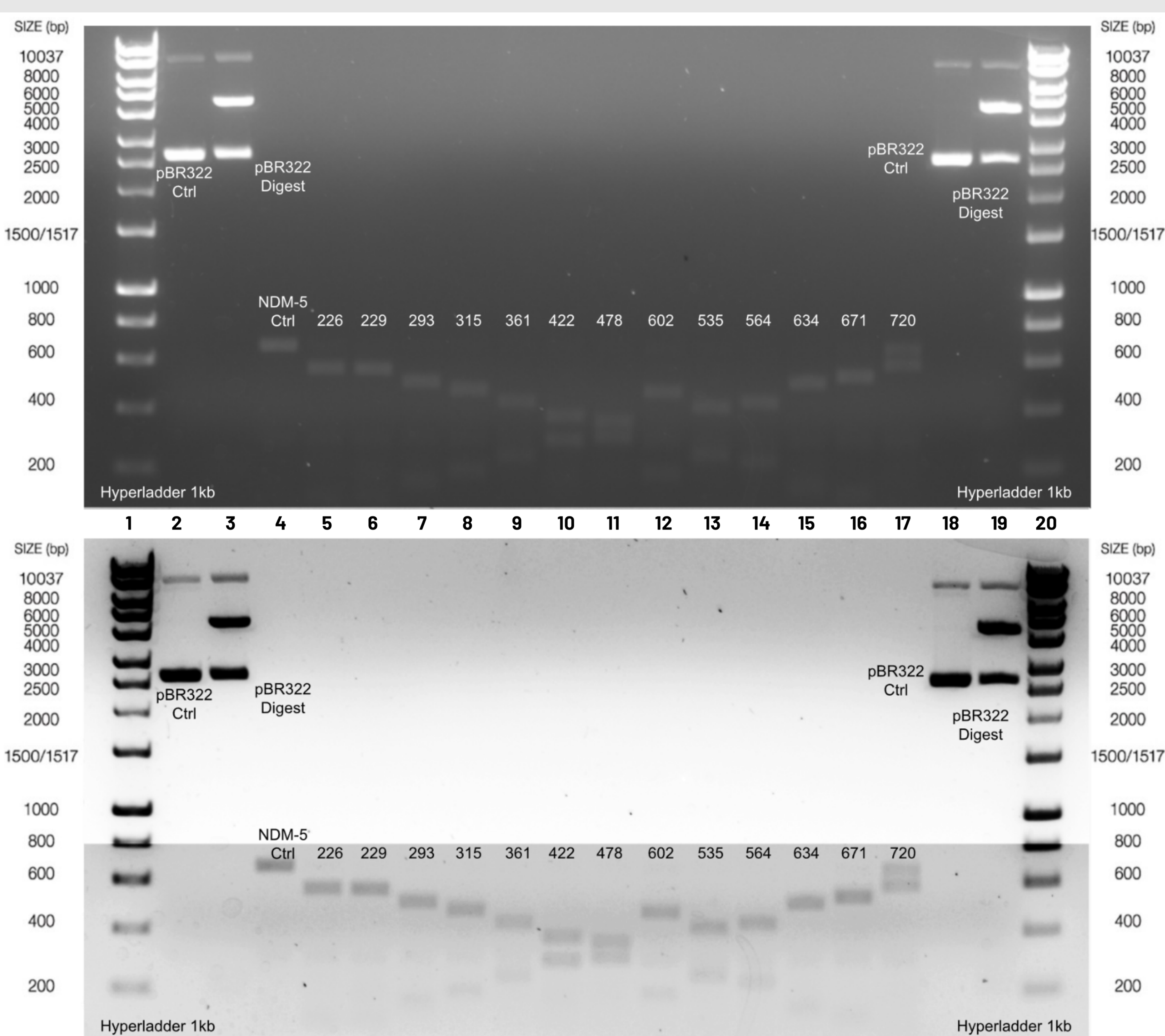


FIGURE B: Gel electrophoresis results. Unedited raw image above, and edited image with inverted colours and increased contrast below.

Discussion

- Results matched predictions, except for partial digestion of sgRNA 720 and pBR322 controls.
- Partial digestion of pBR322 controls possibly due to inaccessibility of Cas9/sgRNA complex to coiled plasmid sites.
- DNA fragments of 8000-10000 bp in wells 2, 3, 18, and 19 likely caused by nicked plasmid DNA due to temperature degradation during PCR or shear forces during mixing [5].
- Initially, the New England Biolabs protocol for in vitro digestion with Cas9 yielded inconclusive results.
- Ultimately, optimized the current protocol to increase the visibility of digested DNA.
- Increased target DNA concentration from 30 nM to 60 nM.
- Implemented shaking at 250 rpm to maintain reaction efficiency.
- Increased agarose concentration from 1% to 1.5%, ethidium bromide concentration to 3 µl / 100 ml, voltage decreased from 100 V to 80 V, and run time extended from 60 minutes to 110 minutes.

Conclusion

- Gel profile confirmed successful digestion of blaNDM-5 at 13 separate positions.
- Suggests CRISPR/Cas9 techniques as a potentially viable option for AMR inactivation.
- Future work could focus on developing compatible vectors, such as plasmids, for *in vivo* work

References

[1] M. E. A. de Kraker, A. J. Stewardson, and S. Harbarth, “Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050?,” PLOS Medicine, vol. 13, no. 11, p. e1002184, Nov. 2016, doi: https://doi.org/10.1371/journal.pmed.1002184.

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[3] “2021 antibacterial agents in clinical and preclinical development: an overview and analysis,” www.who.int. https://www.who.int/publications/i/item/9789240047655

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[5] C.-H. Lin, Y.-C. Chen, and T.-M. Pan, “Quantification Bias Caused by Plasmid DNA Conformation in Quantitative Real-Time PCR Assay,” PLoS ONE, vol. 6, no. 12, p. e29101, Dec. 2011, doi: https://doi.org/10.1371/journal.pone.0029101.