Quantifying attenuation and vaccine potential of a codon-pair deoptimized RSV vaccine candidate

Respiratory syncytial virus (RSV) is a major cause of respiratory illness in infants and young children, responsible for over 3.6 million hospitalizations and approximately 100,000 deaths in children under 5 years of age each year.[1] With no specific treatment for RSV and no approved active immunization strategy directly for young infants, live-attenuated vaccines are being investigated for their ability to induce immunity at the site of viral entry while limiting viral replication.

Codon-pair deoptimization (CPD) is one strategy for developing live-attenuated vaccines, where synonymous mutations are introduced throughout viral genes to reduce viral replication fitness without changing the encoded proteins. Previous CPD RSV vaccine candidates containing different combinations of one or more CPD open reading frames (ORFs) demonstrated that synonymous recoding can produce attenuated viruses while retaining immunogenicity.[2] However, some candidates acquired compensatory mutations during serial passaging that partially restored viral replication.

Building on these findings, Levy *et al.* developed Min AL by combining CPD regions from two previously characterized RSV vaccine candidates, Min A and Min L.[2] Min AL contains CPD versions of seven of the 11 RSV ORFs and 2,073 silent mutations (Figure 1A). The researchers investigated the replication, temperature sensitivity, genetic stability, and acquisition of compensatory mutations in Min AL, as well as the effects of selected mutations on viral replication and immunogenicity. The study used the Celigo™ image cytometer to quantitatively assess plaque size as part of the *in vitro* evaluation of the vaccine candidates. Here, we explore the study's key findings.

Characterizing Min AL replication and temperature sensitivity

The researchers first compared Min AL with previously developed CPD RSV candidates under permissive (32 °C, representative of the upper respiratory tract) and restrictive (37 °C, representative of the lower respiratory tract and core body temperature) conditions. Min AL replicated efficiently at 32 °C, although replication was reduced compared with wild-type RSV (Figure 1B). At 37 °C, replication was substantially restricted, with Min AL showing a stronger temperature-sensitive phenotype than either of its parental candidates. Min AL had a shut-off temperature (T_{SH}) of 36 °C, compared with 40 °C and 38 °C for Min A and Min L, respectively.

The researchers also compared plaque formation as an additional measure of viral spread. Vero cell monolayers were inoculated with 250 plaque-forming units (pfu) per well of virus and incubated at 32 °C for seven days under a 0.8 % methylcellulose overlay. Following fixation and immunofluorescent staining with anti-RSV F monoclonal antibodies, plaques were visualized using the Celigo image cytometer and plaque area was quantified using Celigo software. An average of 3,451 (±1,200) plaques were analyzed per virus. As shown in Figure 1C, the CPD RSV candidates produced plaques approximately three- to five-fold smaller than wild-type RSV ($p<0.0001$), whereas the differences among the individual CPD candidates were not statistically significant.

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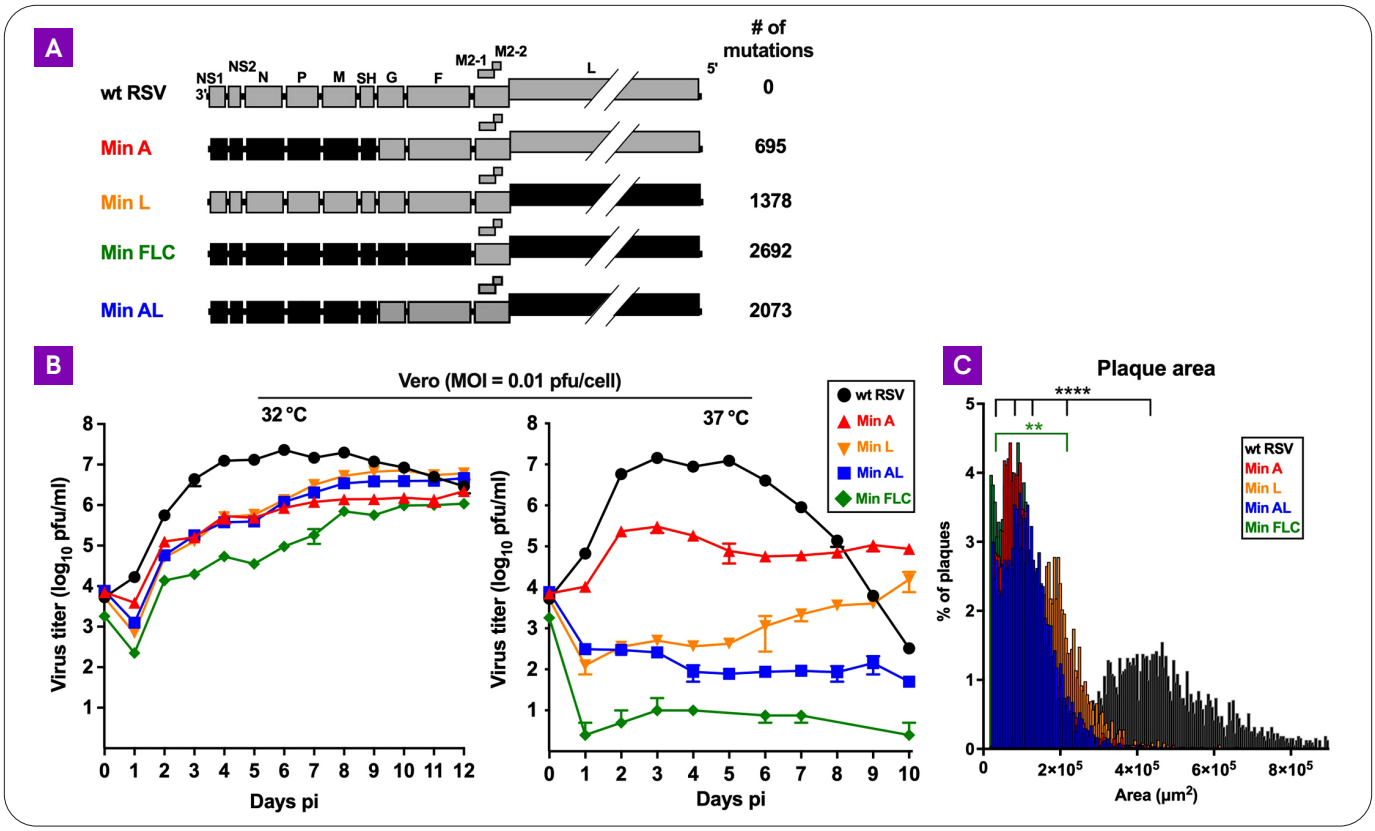

Figure 1: Construction of Min AL and characterization *in vitro*. (A) Genome map of the newly constructed Min AL virus compared to wild-type RSV and the previously described Min A, Min L, and Min FLC viruses. CPD and wild-type ORFs are shown in black and gray, respectively. The number of silent nucleotide substitutions introduced by CPD in each construct is indicated to the right. (B) Multi-cycle replication kinetics in Vero cells. Cell monolayers in six-well plates were infected at a multiplicity of infection of 0.01 pfu/cell with the indicated viruses and incubated at 32 °C (left panel) or 37 °C (right panel). At 24-hour intervals, cells from duplicate wells for each virus were scraped into the medium, vortexed to release cell-associated virus, and the suspensions were clarified by low-speed centrifugation. Media supernatants were harvested, aliquoted, and snap-frozen. Virus titers were determined by immunoplaque assay at 32 °C. Titers correspond to the mean of two replicate titrations of virus from each of two replicate wells at each time point. Day 0 titers correspond to the back-titration of the inocula. (C) Plaque size analysis. Vero cells in six-well plates were infected with 250 pfu/well of the indicated virus and incubated under a methylcellulose overlay at 32 °C. At day 7 post-infection, plates were fixed and stained with a mixture of three anti-RSV F monoclonal antibodies and a PE-labeled secondary antibody. Plaque area (μm^2) was evaluated using the Celigo image cytometer on an average of 3,451 (±1,200) plaques per virus (** = p≤0.01, **** = p≤0.0001, Wilcoxon rank-sum test with continuity correction). Image credit: Levy *et al.* 20242 licensed under CC BY 4.0.

Assessing genetic stability under temperature stress

Because previous CPD RSV candidates had acquired compensatory mutations during passaging, the researchers subjected Min AL to an *in vitro* stress test. Ten independent Min AL lineages were passaged in parallel, starting at 32 °C and increasing the temperature by 1 °C every two passages up to 40 °C. The lineages underwent 18 passages, corresponding to approximately four months of continuous culture, while two additional lineages were maintained at 32 °C as controls. Although most lineages replicated above the original 36 °C shut-off temperature, none replicated at 40 °C, suggesting that the lineages had undergone a partial, but not complete, loss of the temperature-sensitive phenotype.

Whole-genome sequencing identified multiple mutations in the temperature-stressed lineages. The M2-1 gene, which encodes a transcription processivity protein, accounted for 44 % of the 25 missense mutations identified. Mutations were also identified in both CPD and non-CPD ORFs, indicating that the acquired mutations were not limited to reversion of the CPD sequences. In contrast, Min AL lineages maintained at 32 °C as controls remained genetically stable. These findings indicate that Min AL can partially adapt to restrictive temperatures through the acquisition of compensatory mutations.

Testing compensatory mutations

The researchers then reintroduced selected mutations identified during the temperature-stress experiment into the original Min AL genome to determine whether they could restore viral replication. Several mutations increased viral replication at 37 °C, and combining mutations produced further increases. The corresponding T_{SH} increased from 36 °C to 39–40 °C, indicating that these mutations reduced the temperature-sensitive phenotype of Min AL.

The researchers used single-cycle experiments to investigate how the mutations affected viral replication. At 37 °C, the mutations increased the accumulation of positive-sense viral RNA, with some derivatives reaching levels comparable to wild-type RSV. Negative-sense genomic RNA also increased but remained below wild-type levels. Viral protein expression and virus production increased in parallel with the changes in viral RNA. However, virus yields remained at least 10-fold lower than wild-type RSV levels, indicating that the mutations improved replication without fully restoring the wild-type phenotype.

Evaluating Min AL in a hamster model

Finally, the researchers evaluated Min AL and selected derivatives in a hamster model. Min AL showed strong attenuation, with no detectable replication in the upper and lower airways, while still inducing serum and mucosal anti-RSV antibodies. Following challenge with wild-type RSV, the vaccinated animals showed protection against viral replication in the respiratory tract.

Conclusion

The study demonstrates how CPD can be used to generate attenuated RSV vaccine candidates and how subsequent genetic analysis can identify and characterize mutations that partially restore viral replication under restrictive conditions. By combining the CPD ORFs of Min A and Min L, the researchers generated Min AL with a strong temperature-sensitive phenotype of 36 °C and used temperature-stress passaging and genome sequencing to identify and characterize potential compensatory mutations.

The study also illustrates the use of quantitative imaging to characterize the phenotype of attenuated viruses. The Celigo image cytometer was used to visualize immunofluorescently stained RSV plaques and quantify individual plaque area, with an average of 3,451 (±1,200) plaques analyzed per virus. This provided a quantitative measure of plaque spread that complemented the other infectious virus measurements used in the study.

Overall, these findings advance understanding of the genetic determinants of RSV attenuation and provide a framework for assessing the genetic stability and immunogenicity of CPD-based vaccine candidates.

References

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2. Levy, M., Chen, J. W., Kaiser, J. A., Park, H., Liu, X., Yang, L., Santos, C., Buchholz, U. J., & Nouën, C. L. (2024). Intranasal respiratory syncytial virus vaccine attenuated by codon-pair deoptimization of seven open reading frames is genetically stable and elicits mucosal and systemic immunity and protection against challenge virus replication in hamsters. PLoS Pathogens, 20(5), e1012198. https://doi.org/10.1371/journal.ppat.1012198

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