A Modified Next-Generation Sequencing Methodology for Norovirus Genotyping

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Background

Norovirus (NoV) acute gastroenteritis (AGE) places a significant burden on the US healthcare system annually. Development of interventions including vaccines have been challenged by genomic diversity and scarcity of genomic data from NoV AGE cases. Improving next-generation sequencing (NGS) genotyping methods would allow for enhanced characterization of local NoV AGE cases and provide valuable data to inform vaccine design. We aimed to convert a standard single-amplicon Sanger sequencing approach to a NGS methodology to 1) increase Marshfield Clinic's NoV genotyping testing capability, and 2) improve NGS result turnaround times by aligning the NoV sequencing approach to other existing NGS workflows within the lab.

Methods

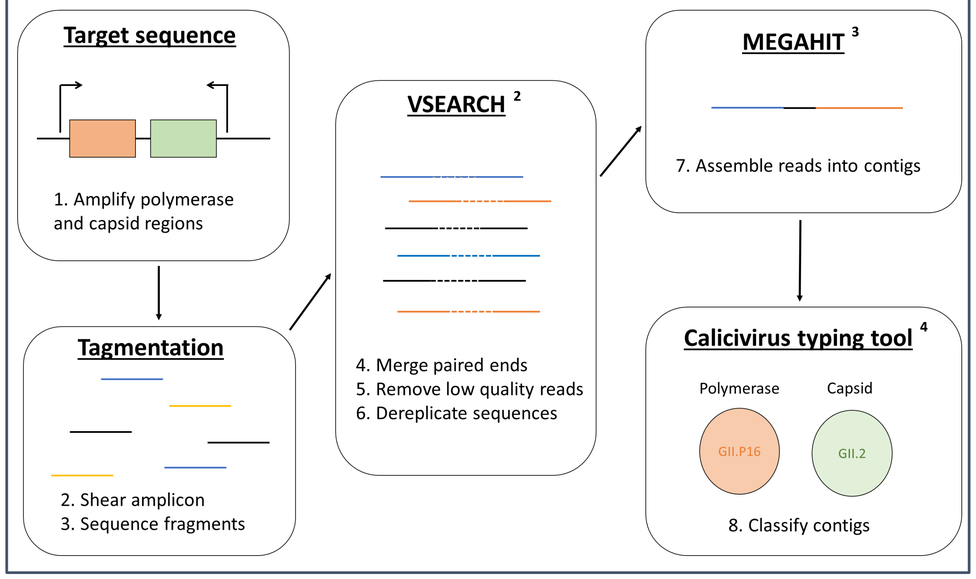
NoV AGE samples

- Validation: 40 previously genotyped NoV samples
- Prospective Testing: 115 NoV Group I and Group II positive samples identified from community and medically-attended surveillance studies during the 2024-2025 season

Laboratory methods

- RT-PCR followed by targeted Group I/Group II NoV polymerase-capsid (P:C) PCR gene amplification[1] **(Figure 1, Section 1)**
- Illumina DNA Prep NGS library prep and sequencing on an Illumina MiSeq instrument **(Figure 1, Sections 2 & 3)**
- In-house developed bioinformatics workflow **(Figure 1, Sections 4-8)**

Figure 1. Sequencing and Post-Run Analysis Workflow.



Results

- Validation study correctly identified all 40 previously genotyped samples with 100% accuracy **(Figure 2)**
  - ✓ 20 Group I NoV samples (6 unique genotypes)
  - ✓ 20 Group II NoV samples (12 unique genotypes)

- Testing of 115 prospectively collected samples generated 86 high confidence (successful capsid and polymerase type) NoV genotype calls and 8 lower confidence (successful capsid and untypeable polymerase) genotype calls. 12 samples failed to generate a genotype, and 9 samples were not selected for sequencing due to poor target amplification **(Figure 3)**

- NoV AGE samples were processed and loaded across 11 different MiSeq runs alongside SARS-CoV-2, influenza, and 16s samples to identify bacterial pathogens in ticks.

- Identified genotypes from prospective studies included **(Figure 4)**:
  - ✓ GII.17[P17] n = 72 (77%)
  - ✓ GII.4 Sydney[P16] n=13 (14%)
  - ✓ GII.6[P7] n=3 (3%)
  - ✓ GII.13[P16] n=2 (2%)
  - ✓ GI.3[P13] n=1 (1%)
  - ✓ GI.3[P3] n=1 (1%)
  - ✓ GI.5[P5] n=2 (1%)

Figure 2. Breakdown of Genotypes Tested in Validation Study

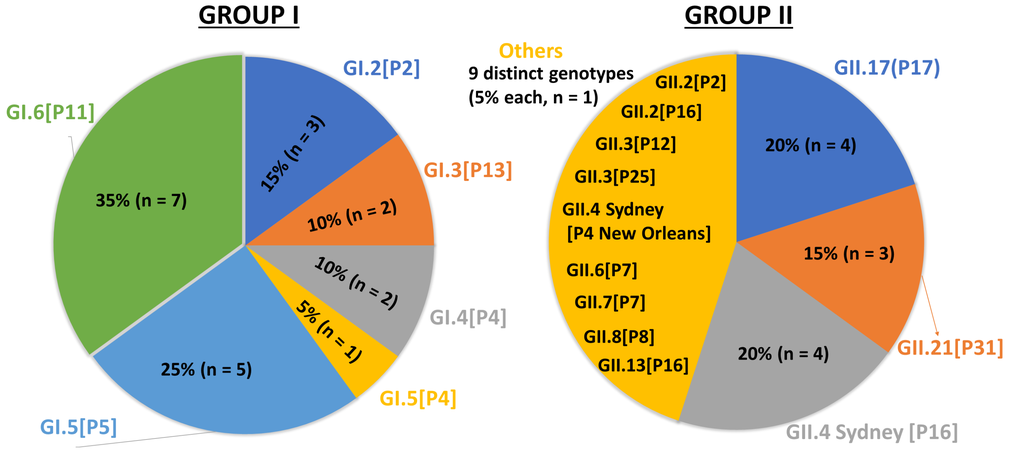


Figure 3. Representative image of the P:C NoV amplicon PCR reaction. 2% agarose gel with EtBr illustrating the heterogeneity in amplification between NoV samples (n = 20). The same samples were run on both the top and bottom wells, with the top lanes using an undiluted RNA template and the bottom lanes using a 1:10 RNA dilution for cDNA synthesis. **Blue boxes** mark failed samples, not sequenced; **Red boxes** mark samples with improved P:C PCR performance using the 1:10 RNA template; and the **Black box** denotes the water negative control. Green arrows indicate the 570/579 bp P:C amplicon.

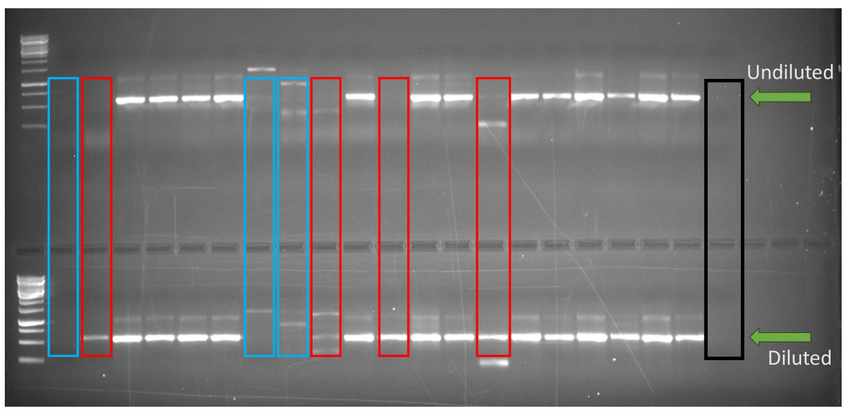
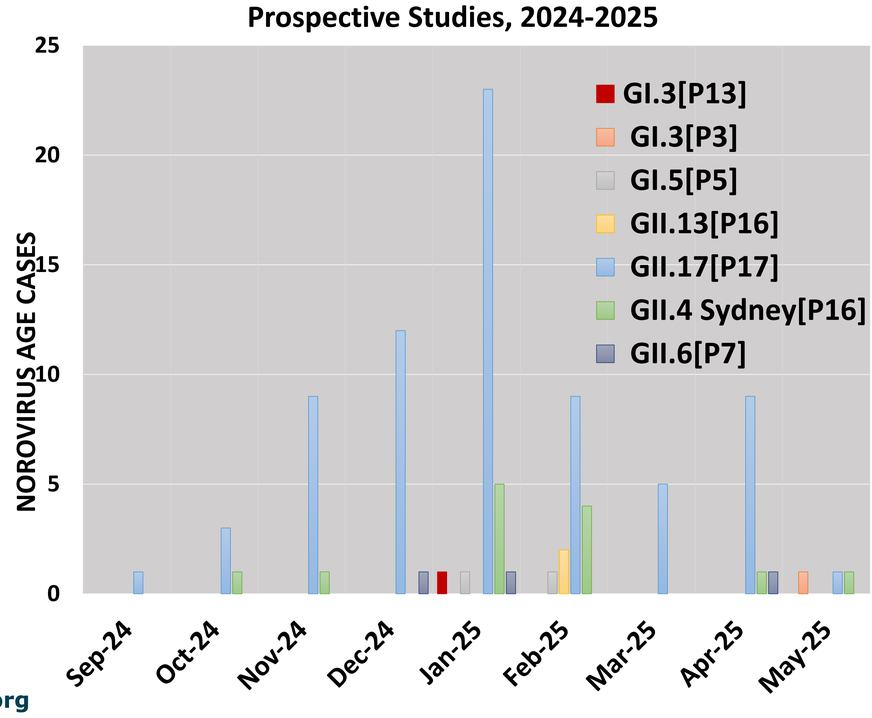


Figure 4. Human Norovirus Genotype Counts from Prospective Studies, 2024-2025



Conclusions

- Strong concordance was seen between established Sanger and new NGS NoV genotyping approaches.
- Labs with existing NGS workflows and instruments may adopt the NGS NoV amplicon methodology to increase sample volume testing through combining multiple sample types (e.g, influenza, SARS-CoV-2, NoV) on a single flow cell.
- Poor amplification of the polymerase:capsid target in previously identified NoV positive samples may be due to non-conserved nucleotides within the primer-binding sequence motif.

References

1. Chhabra P, Browne H, Huynh T, et al. Single-step RT-PCR assay for dual genotyping of GI and GII norovirus strains. J Clin Virol 2021.
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3. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 2015.
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