IMPERIAL

Waste Not, Want Not: Optimising Wastewater Surveillance of Human Enteroviruses.

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01 Introduction

- Enteroviruses, one of the most prevalent genera of viruses globally, can pose a significant threat, causing up to 80% of viral meningitis cases worldwide.[1] Early detection of dangerous serotypes in wastewater is crucial for implementing control measures.
- They are RNA viruses with an outer protein coat made of four polypeptides including VP1, VP2, VP3 and VP4.[2]
- The VP1 region is typically used to identify the serotype of the virus.[2]
- Polymerase chain reaction (PCR) assays can be used to amplify target regions in the genome for identification via sequencing. The PCR specificity relies on short DNA fragments known as primers.
- Two common primer sets (NCR_CRE and MM_EV) used to amplify a large part of the enterovirus genome (which includes the VP1 region) have performed poorly in several wastewater surveillance labs worldwide.[3]
- This project aims to compare the efficacy of alternative primers in detecting enteroviruses For1_5NCR/Rev7_5NCR and For2_5NCR/Rev6_5NCR (designed by Dr. Alexander Shaw) against current primers using identical samples. These primers do not target the VP1 region and hence, would only identify the species of enteroviruses.

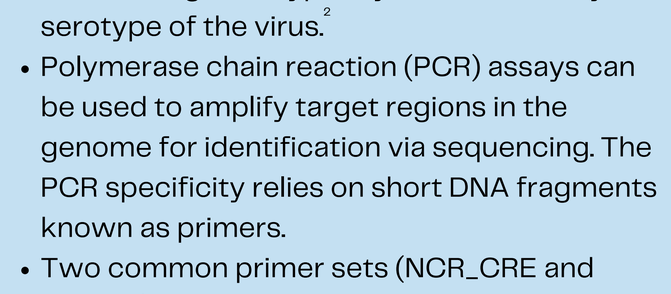

Figure 1: Primer positions on the Sabin AY184219 Reference (approximation).
Red = For1_5NCR and Rev7_5NCR
Purple = For2_5NCR and Rev6_5NCR
Blue = 5'-NTR / MM_EVF2 and CRE / MM_EVF1

02 Method

Viral RNA Extraction from wastewater using the MagMax Viral RNA isolation kit

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Nested Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Then, verification using Gel Electrophoresis and TapeStation©

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Amplicon clean-up using the AMPure XP beads. End-prep & dA-tailing, then ligation of barcodes using the Native Barcoding kit 96 (SQK-NBD114.96)

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Preparation of, and sequencing amplicons on the ONT© MinION Flow Cell. Data was read-mapped via minimap2 and analysed on Geneious Prime©.

03 Results

RT-PCR was performed on positive/negative controls and wastewater replicates. Amplicon sizes were verified via gel electrophoresis (see Figure 2 and 4) and TapeStation© (see Figure 3). After which, the PCR products were sequenced. An arbitrary threshold of over 50 reads was interpreted as positive detection. The resulting data (see Table 1 and 2) shows the average number of reads of each enteroviral species detected per primer set for wastewater amplicon replicates and the negative control.

1. 5'-NTR-CRE primers: No species detected.
2. MM_EV primers: Detected species A and B.
3. For1_5NCR/Rev7_5NCR, For2_5NCR/Rev6_5NCR, and nested primers: High read counts for species A, B, and C.
4. Negative control: Positive for species C with new primers.

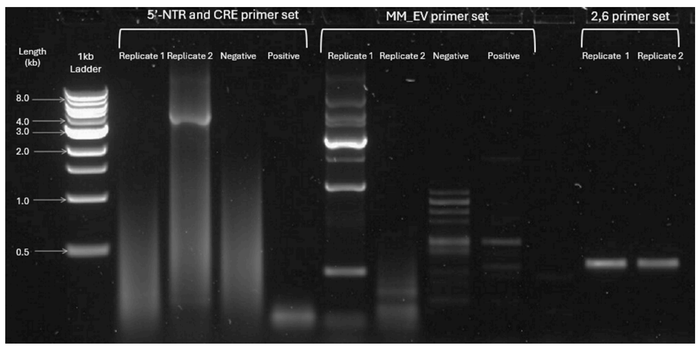

Figure 2: Agarose gel electrophoresis of PCR products with 1% agarose gel with 1kb ladder. Lanes show amplification products from wastewater samples using NCR_CRE, MM_EV, and For2_5NCR/Rev6_5NCR primers sets. The NCR_CRE and MM_EV also show amplification products with positive and negative controls.

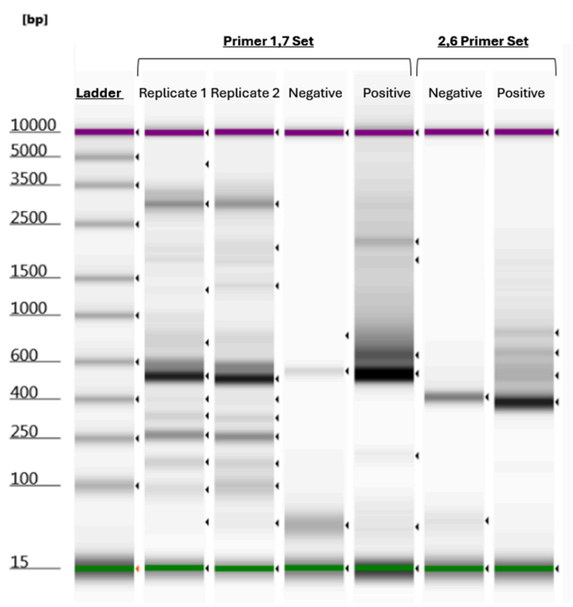

Figure 3: TapeStation analysis of PCR products with 1kb ladder. Lanes show amplification products from wastewater samples using For1_5NCR/Rev7_5NCR primers sets. The NCR_CRE and MM_EV also show amplification products with positive and negative controls.

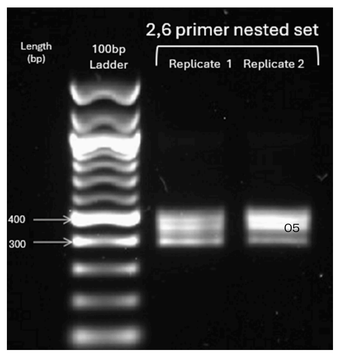

Figure 4: Agarose gel electrophoresis of PCR products with 1% agarose gel with 100bp ladder. Nested RT-PCR with For2_5NCR/Rev6_5NCR primers on For1_5NCR/Rev7_5NCR amplicons.

Primer Sets / Species Type	5'NTR_CRE	MM_EV	For1_5NCR and Rev7_5NCR	For2_5NCR and Rev6_5NCR	For2_5NCR/Rev6_5NCR Nested into For1_5NCR/Rev7_5NCR
A	8	155	690	560	10866
B	1	190	1074	1018	2217
C	16	9	8472	42939	12884
D	0	0	1	8	26

Table 1: Following PCR product verification, the table shows the average number of reads of each enteroviral species detected per primer set for wastewater amplicon replicates.

Primer Sets / Species Type	5'NTR_CRE	MM_EV	For1_5NCR and Rev7_5NCR	For2_5NCR and Rev6_5NCR	For2_5NCR/Rev6_5NCR Nested into For1_5NCR/Rev7_5NCR
A	0	0	0	1	2
B	0	0	1	0	0
C	0	9	700	37684	28125
D	0	0	0	0	0

Table 2: Following PCR product verification, the table shows the average number of reads of each enteroviral species detected per primer set for the negative control.

04 Discussion

- The Majumdar et al. study utilised 5'-NTR-CRE and MM_EV primer sets for enterovirus species identification.[3] However, this project revealed non-specific binding and failed detection in positive controls, corroborating recent observations from other laboratories. This discrepancy may be attributed to alterations in reagent composition.
- Figure 1 illustrates that 5'-NTR-CRE amplifies a ~3.8kb genomic region, while the new primers target a ~600bp segment. Longer amplicons necessitate intact viral RNA for RT-PCR, but RNA's inherent instability complicates accurate amplification of extended sequences. Additionally, longer target regions increase the likelihood of DNA depurination and damage during PCR.[4]
- Observed differences in read counts may result from incomplete normalisation or variations in MinION sequencing depth, rather than solely differential PCR efficiency.
- As expected, nested PCR yielded the highest sequencing reads due to its two-step amplification process. The short target region and nested approach enhanced sensitivity and specificity, resulting in increased target sequence yield and consequently higher read counts.
- Negative controls across all experiments exhibited contamination with the positive control species, suggesting potential carry-over.
- The use of Coxsackievirus A20 as a positive control has implications for method validity. The absence of species D enterovirus detection warrants further investigation to determine whether it reflects a true absence or insufficient primer sensitivity for this species.

05 Conclusion and Next Steps

- This experiment shows that the current primers used for gold-standard diagnostics are less sensitive and alternative primers provide a method to detect enteroviral species diversity in sewage.
- Further work must be done on isolates to show more accurate results and method validity.
- Consider further work to explain why the NTR-CRE and MM-EV primers may not be working.
- The alternative primers bind outside the VP1 region, allowing only species detection. Future work should develop VP1-specific primers for serotype identification.

06 Acknowledgements and References

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