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

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IMPERIAL

Abstract

Enteroviruses, a highly widespread virus genera globally, are responsible for up to 80% of viral meningitis cases[1], posing a significant threat to public health. Between 2015 and 2017, 66 non-poliovirus enteroviruses serotypes were found across 24 European countries.[2] Early detection of high-risk serotypes is crucial for implementing control measures. One method of enterovirus surveillance in wastewater involves extracting viral RNA and using polymerase chain reaction (PCR) to amplify specific genomic regions for identification. PCR uses primers to target and replicate specific DNA segments, enabling subsequent sequencing[3]. Enteroviruses are taxonomically largely categorised into four species (A-D) based on genetic differences.[4] However, the VP1 region of the genome, which codes for a viral capsid protein, is unique to each serotype of enterovirus and is specifically sequenced for identification.[5] Two common primer sets (NCR_CRE and MM_EV) used to amplify a large part of the enterovirus genome have proved to have relatively low sensitivity when amplifying enteroviruses from wastewater.[6]

This project aims to test the performance of alternative primers targeting a shorter genomic region outside the VP1 coding area. While these new primers do not allow serotype identification, they could enable improved species detection. Using UK wastewater samples, the sensitivity of these new primers was compared to the NCR_CRE and MM_EV sets.

Results showed that NCR_CRE primers detected no enterovirus species, MM_EV primers identified two species, while the new primers detected three species with significantly more sequencing reads, implying greater sensitivity. Furthermore, the newer primers had significantly more sequencing reads which suggests the amplification process was more efficient.

In conclusion, these findings suggest that these new primers demonstrate improved detection sensitivity and could minimise false negatives when surveying enteroviruses in sewage. These primers are now in use in Ghana and Bangladesh for wastewater surveillance. Future research should investigate the primers' ability to detect a wide variety of virus isolate controls. Additionally, developing VP1-specific primers is necessary to obtain specific virus serotype information, which could better inform public health responses to enteroviral outbreaks.

Keywords: Enteroviruses, Polymerase Chain Reaction, Primers, VP1

Research Objectives & Questions

1. Are the For1_5NCR/Rev7_5NCR and For2_5NCR/Rev6_5NCR primer sets effective in amplifying enteroviral species?
2. Do the current 5'-NTR / MM_EVF2 and CRE / MM_EVF1 primer sets effectively amplify enteroviruses?

Introduction

Enteroviruses, a highly pervasive virus genera, cause illnesses ranging from mild gastroenteritis and respiratory illnesses to viral meningitis and hand, foot, and mouth disease.[7] These viruses belong to the *Picornaviridae* family, which also includes rhinoviruses and polioviruses. Enteroviruses are primarily transmitted through the fecal-hand-oral route, making them particularly harmful in regions lacking access to clean water and sanitation. In such developing countries, they account for 70-80% of viral meningitis cases.[1] Enteroviruses pose risks in developing countries as well; in the United States, EV-D68 caused over 1,300 confirmed cases in 2014 and tends to emerge every two years.[8]

It is vital to detect them quickly and accurately to prevent outbreaks and provide appropriate treatment to those infected. Early detection can help public health officials implement necessary control measures and reduce the spread of these viruses in communities through information dissemination across stakeholders such as proper hand washing technique (highlighting this post-defecation), early vaccination strategies and sewage management ensuring clean drinking water.[9]

The current gold-standard method of detection involves a cell culture-based approach. The virus is isolated and used to infect a susceptible cell line, allowing it to replicate in the cell and amplify. Once centrifuged, the viral RNA is extracted for reverse transcription-PCR (RT-PCR) and sequenced for its serotype (often using Sanger Sequencing). While this method of detection is highly sensitive and specific with high biological amplification of the virus, it comes at the cost of time (taking up to 8 days for diagnostics) and thus, ultimately, lives. By the time the results are available, the virus has spread further into the community.[10]

A promising new method is the direct detection and nanopore sequencing protocol.[3] This is a faster alternative that uses viral RNA directly from stool or sewage, amplifies it via RT-PCR, and sequences it using the Oxford Nanopore Technologies (ONT) MinION Mk1B sequencers. This advancement could greatly enhance public health strategies by facilitating timely identification and containment efforts.

Structure of enteroviruses

Enteroviruses contain positive-sense single-stranded RNA (+ssRNA) of approximately 7400 nucleotides in length within an icosahedral capsid (outer protein coat of the virus) made of four

polypeptides including VP1, VP2, VP3 and VP4. Their genome is positive-sense single-stranded RNA (+ssRNA), measuring approximately 7,400 nucleotides in length.

The viral genetic material is encased within an outer protective structure called a protein capsid. It exhibits an icosahedral geometric shape with 20 triangular faces. The capsid is composed of four main structural proteins, designated as VP1, VP2, VP3, and VP4. The VP1 protein has an important role in the replication cycle of enteroviruses and mutations in it can affect the virulence and the binding ability of the virus to target receptors. [11]

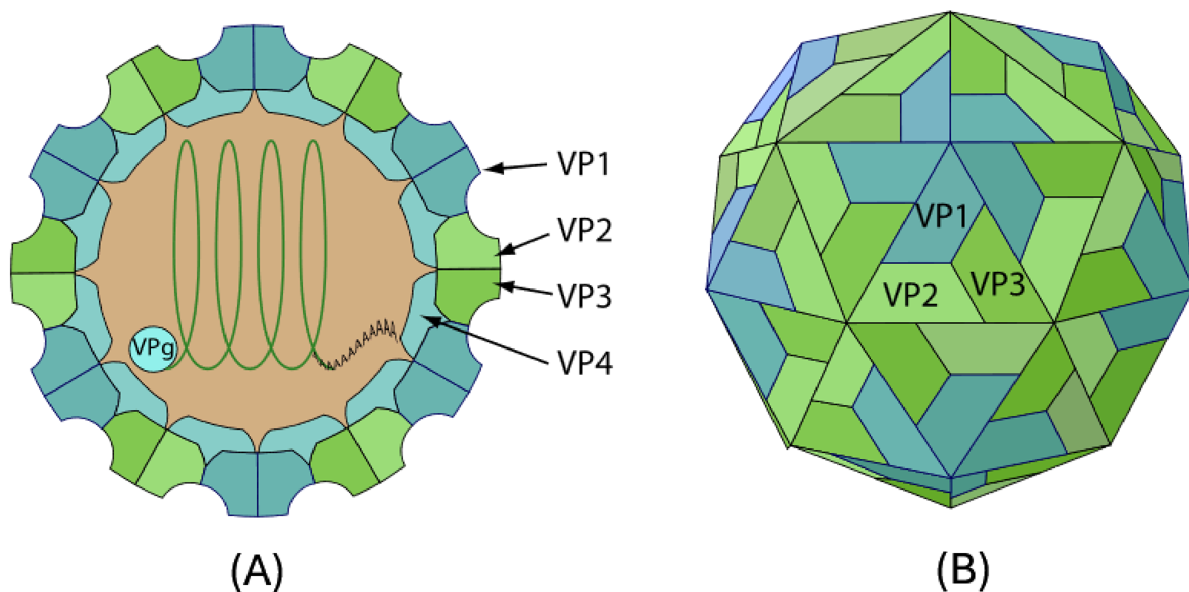

Figure 1: The tertiary structure of an Enterovirus. [1]

(A) The cross-section of an Enterovirus. The capsid is made of the polypeptides VP1, VP2, VP3 and VP4.

(B) The icosahedral arrangement of the polypeptides of the capsid.

The viral genome is split into three regions: P1, P2 and P3. The P1 region is responsible for encoding the physical structure of the protein and hence codes for the capsid proteins. Whereas the P2 and P3 regions encode the proteins associated with replication, e.g. enzymes such as the 3D polymerase for viral RNA synthesis. Nucleotide sequence analysis of the VP1 coding region allows the final identification of the serotype of the virus.[12]

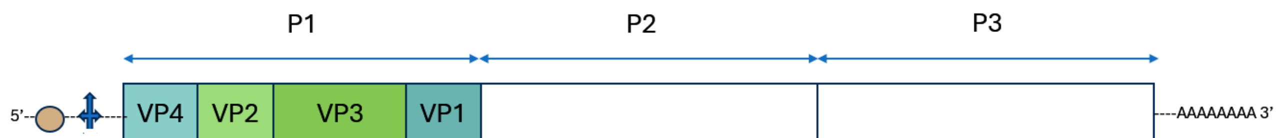

Figure 2: A linear positive sense ssRNA Enterovirus genome [diagram not to scale] adapted from https://viralzone.expasy.org/97

PCR and Primers[13]

Polymerase Chain Reaction (PCR) is a molecular biology technique that allows the amplification of small amounts of genetic material into volumes sufficient for comprehensive scientific analysis.

When working with viral genetic material, researchers must first convert RNA to DNA using a reverse transcriptase enzyme, a process known as reverse transcription (RT). This critical preparatory step ensures that the genetic material can be effectively amplified through the PCR process.

PCR amplifies a specific genomic region based on the ability of short DNA sequences (known as primers) to bind and act as molecular markers for enzymes to replicate the correct part of the DNA sequence.

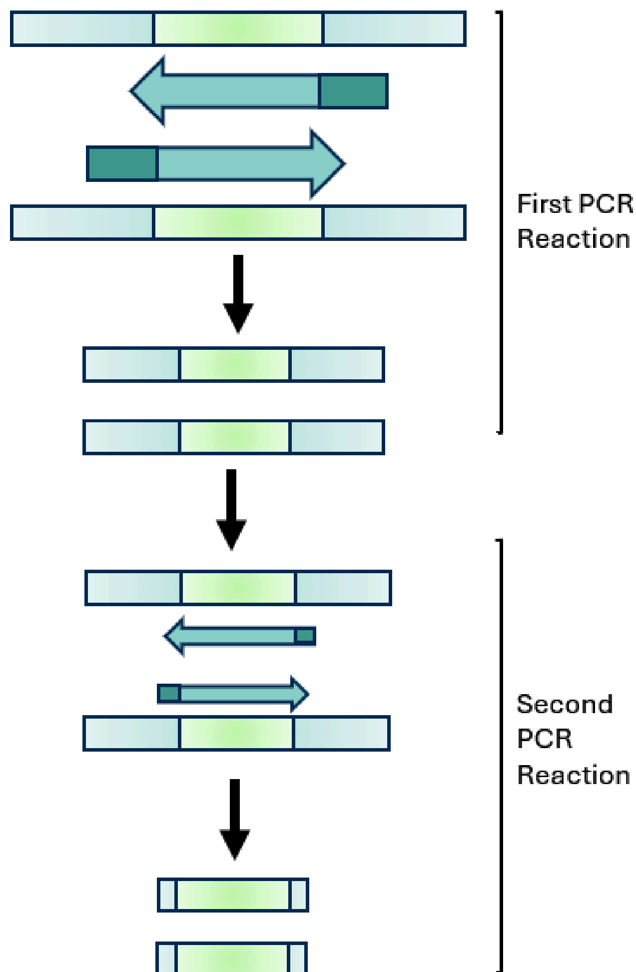

Figure 3: Diagrammatic representation of the steps of the polymerase chain reaction.

Nested PCR involves two consecutive polymerase chain reaction amplifications utilising two distinct sets of primers. The initial primary amplification employs an external primer pair targeting a broader genetic region. Subsequently, a second internal primer pair is strategically designed to amplify a more precise, nested subset within the initial amplification product.

Primers are normally chosen based on thermodynamic calculations, specificity to regions of interest and based on areas of 'conserved regions'.[14] This means primers should bind to an area of the genome that is common to as many enteroviruses as possible so as many serotypes can

be detected with these primers. The commonly used primer sets, NTR_CRE and MM_EV, are known as pan enteroviral primers because the primers amplify the capsid of most enteroviral genomes. The capsid includes the VP1 region, hence, viruses can be serotyped.[6] Amplifying the entire capsid can be difficult, however, especially with viral RNA concentrated from wastewater samples, and this can lower the sensitivity of detection of enteroviruses.

The newly designed For1_5NCR/Rev7_5NCR primer set and For2_5NCR/Rev6_5NCR primer set are in the non-coding region before the P1 region, where the 5′NTR (or 5’NCR) region allows the species (not as specific as the serotype) to be identified. Note that the For2_5NCR and Rev6_5NCR primers bind in a region inside the For1_5NCR/Rev7_5NCR primer set, hence forming a nested PCR. The shorter target length of the primers should give greater sensitivity of detection, and be more readily applicable for use in wastewater enterovirus surveillance.

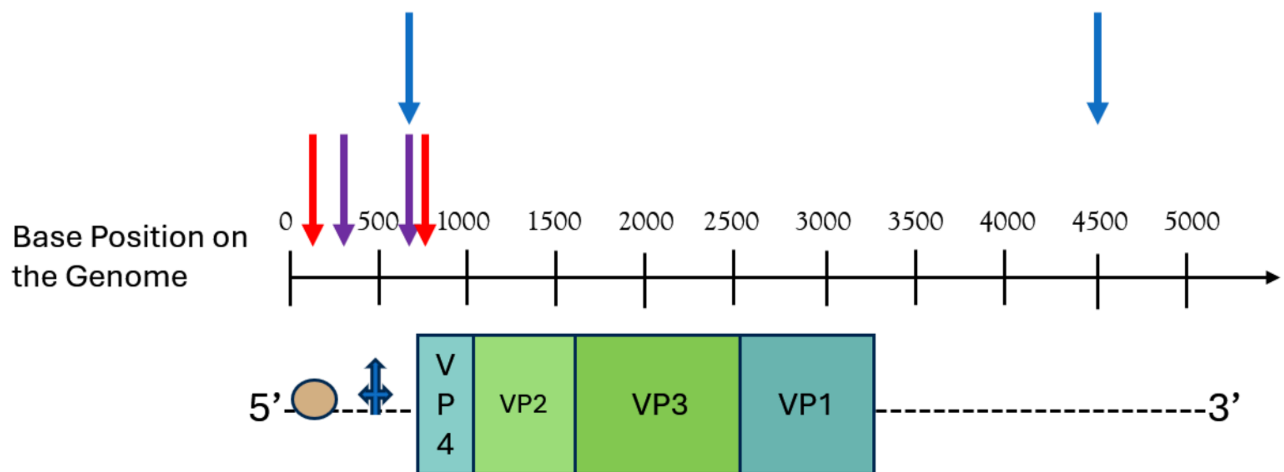

Figure 4: Primer positions on the Sabin AY184219 Reference (approximation).

Red = For1_5NCR and Rev7_5NCR

Purple = For2_5NCR and Rev6_5NCR

Blue = 5’-NTR / MM_EVF2 and CRE / MM_EVF1

Methods

Processing of Wastewater Samples - performed by Dr Ben Bellekom

Two 35ml samples of wastewater from Thames Water's Mogden wastewater treatment plant, along with corresponding negative sterile water controls, underwent a process to isolate viral RNA. The procedure involved decontamination, concentration, and extraction steps, culminating in a final product suspended in 100µl of elution buffer..

Genomic Information Amplification

Purified viral genomic RNA solution was first converted to cDNA with the reverse primer added and the forward primer was added for the PCR step. Hence for the reverse transcription (RT) reactions, each 5µl purified viral genomic RNA solution was used for RT using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity Polymerase, according to the manufacturer's instructions (total of 25µl of RT reaction solution). This was then incubated at 50°C for 30 minutes. Then the forward primer is added for the PCR step. PCR conditions consisted of a denaturing step at 94°C for 2 min and 42 cycles of thermal cycling at 94°C for 15 seconds, 55°C for 1 minute and 68°C for 5 minutes. After the cycles, there was a final extension step of 68°C for 10 minutes.

This process was carried out on two replicate samples, a positive control and a negative control.

Primer Sets		
Name	**Forward Primer (5' to 3' direction)**	**Reverse Primer (5' to 3' direction)**

NTR_CRE Set	TGGCGGAACCGACTACTTTGGGTG	TCAATACGGTGTTTGCTCTTGAACTG
MM_EV Set	CAGCGGAACCGACTACTTT	AATACGGCATTTGGACTTGAACTGT
For1_5NCR and Rev7_5NCR	CGGTACCYTTGTRCGCCTG	ATTGTCACCATAAGCAGCC
For2_5NCR and Rev6_5NCR	CAAGCACTTCTGTTWCCC	CCAAAGTAGTCGGTTCCGC

Table 1: Base sequences of each primer set.

Nested PCR is a technique that employs two successive rounds of PCR. As illustrated in figure 3, the first round of amplification utilised the For1_5NCR/Rev7_5NCR primer set to generate initial PCR products. Subsequently, the For2_5NCR/Rev6_5NCR primer set was used to amplify a specific region within these initial products, resulting in highly specific DNA amplification. The reaction was prepared using the DreamTaq PCR Master Mix (2X) from Thermo Fisher, following the manufacturer's prescribed protocol, with a total reaction volume of 25 µl.

Verified via Gel Electrophoresis

Gel electrophoresis separate DNA fragments by length using an electrical current in a 1% agarose gel. Fragments were visualised under UV light after staining with SYBR Safe dye. This technique confirmed PCR amplification by verifying the presence of DNA bands at the expected fragment lengths. However, this method only indicated the correct size of amplified fragments and could not definitively confirm the specific DNA sequence or distinguish between desired and unintended products. Expected fragment lengths are seen in table 2. The Agilent 2200 TapeStation System was also used instead for automated electrophoresis.

Primer Sets	
Name	**Expected Amplicon Lengths (base pairs)**
NTR_CRE Set	~3800
MM_EV Set	~3800
For1_5NCR and Rev7_5NCR	~535
For2_5NCR and Rev6_5NCR	~388

Table 2: Table of approximate PCR amplicon lengths.

Native barcoding protocol

Before sequencing the PCR products, they were cleaned, labelled and then put together in a single sample to input into the DNA sequencer. To clean the sample, AMPure XP beads (magnetic beads that attach to DNA-only products) were used and washed away with 80% ethanol. This was repeated three times to remove impurities from the PCR products.

During the sequencing process, equal quantities of PCR amplicons were crucial for each sample. Unequal amplification could lead to biased results, where samples with higher product concentrations would dominate the sequencing output, potentially obscuring the genetic information from other samples. To mitigate this issue, sample normalisation was performed, which involved standardising the concentration of PCR amplicons across all samples.

A unique barcode was added to each PCR amplicon tube and attached to the PCR amplicons, hence, when all samples were combined (known as a sequencing library) and sequenced, the correct sample could still be identified (known as multiplexing of samples). This was done using the Native Barcoding kit 96 (SQKNBD114.96). DNA sequencing was conducted using Oxford Nanopore Technologies' MinION Mk1B sequencers with R9.4.1 flow cells.

Bioinformatics

Nanopore data were drawn from the MinION sequencing runs. Data was read mapped via minimap2 and analysed on Geneious Prime© using an in-house database of the relevant genomic region.

Results

Electrophoresis reveals higher specificity of newly designed primer sets

To assess the amplification of genomic targets following PCR, we compared the observed amplicon lengths with the expected base pair (bp) sizes.

Electrophoresis results indicated that the 5’NTR_CRE primer set produced bands of the expected ~3.8 kilobase (kb) length in one wastewater replicate. However, non-specific binding was observed in both wastewater replicates and the negative control, despite no bands being expected in the negative control. Furthermore, the positive control did not display the expected band at 3.8 kb (Figure 5A).

For the MM_EV primer set, a faint band at ~3.8kb was detected. Multiple prominent bands of varying lengths were observed in one wastewater replicate and the negative control, indicating

substantial non-specific amplification. Similar to the 5’NTR_CRE set, the positive control did not produce the expected 3.8kb band (Figure 5B).

In contrast, the For2_5NCR/Rev6_5NCR primer set successfully amplified DNA bands of the expected 388bp length in both wastewater replicates (Figure 5C), demonstrating target specificity under the current PCR conditions.

Furthermore, the For1_5NCR/Rev7_5NCR primer sets produced DNA bands at the expected lengths of 535 base pairs (bp) in both the wastewater replicates and positive control. Only faint bands were observed in the negative controls (Figure 6).

Overall, these results showed that non-specific amplification and primer inefficiencies were significant for the 5’NTR_CRE and MM_EV primer sets, while the new primer sets (For2_5NCR/Rev6_5NCR and the For1_5NCR/Rev7_5NCR) demonstrated higher specificity and reliability.

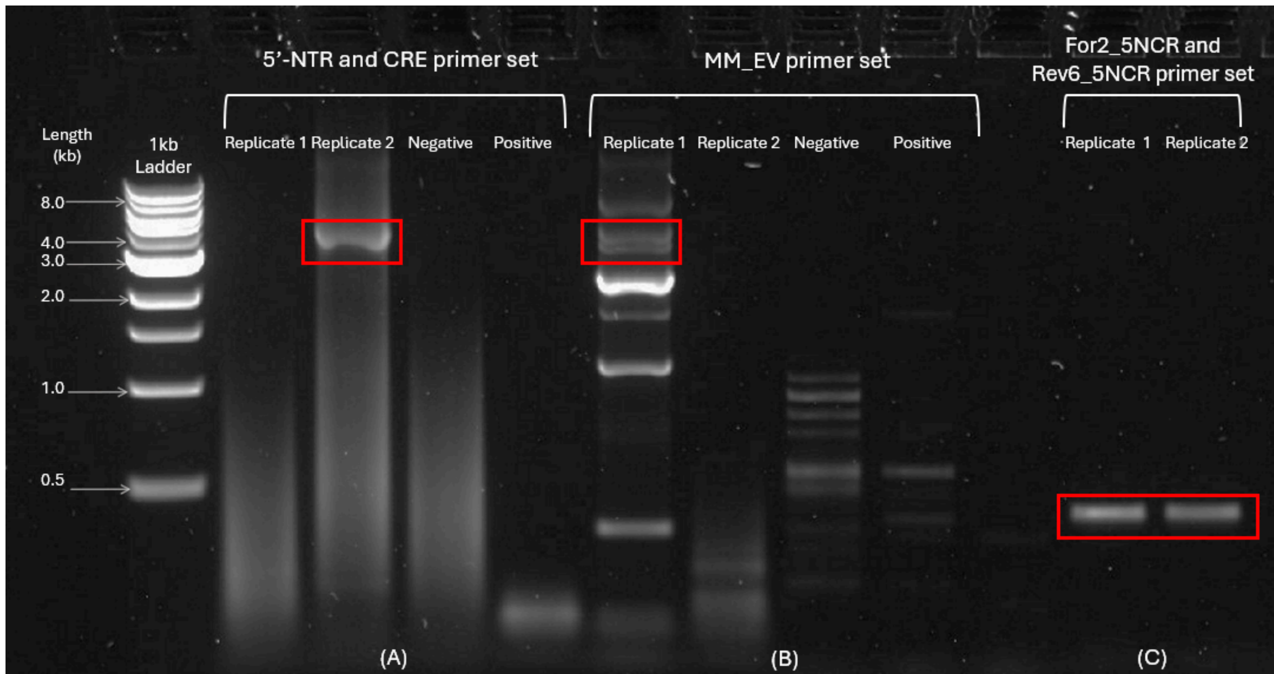

Figure 5: Agarose gel electrophoresis of PCR products with 1% agarose gel with 1kb ladder.

The red boxes indicate the expected bands.

(A) Lanes show amplification products using the NCR_CRE primer set.

(B) Lanes show amplification products using the MM_EV primers set.

(C) Lanes show amplification products using the For2_5NCR/Rev6_5NCR primers sets.

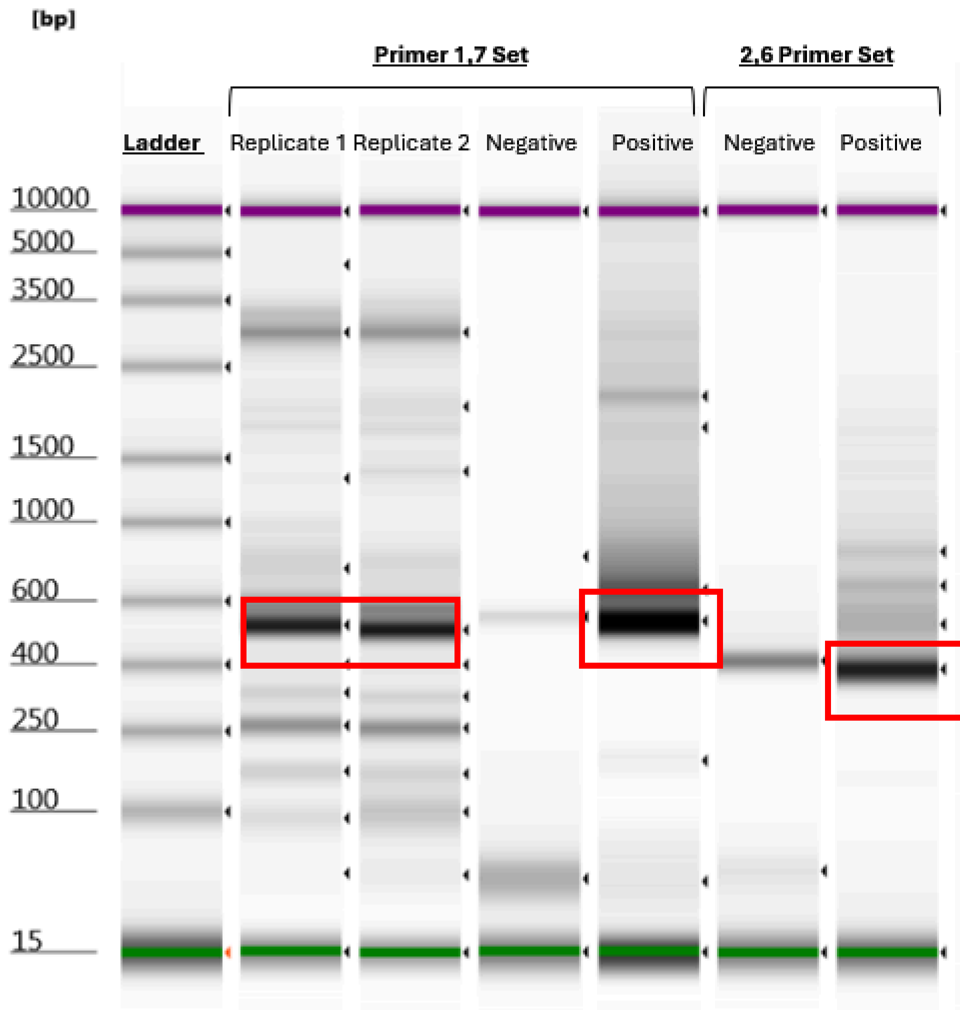

Figure 6: TapeStation electrophoresis of PCR products TapeStation analysis of PCR products with 1kb ladder. Lanes show amplification products from wastewater samples using For1_5NCR/Rev7_5NCR primers sets. The NCR_CRE and MM_EV also show amplification products with positive and negative controls. The red boxes indicate the expected bands.

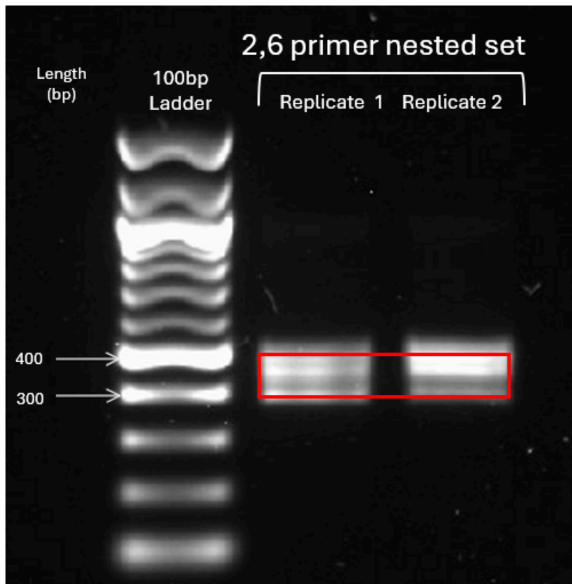

Figure 7: Agarose gel electrophoresis of nested PCR products with 1% agarose gel with 100bp ladder. Nested RT-PCR with For2_5NCR/Rev6_5NCR primers on For1_5NCR/Rev7_5NCR amplicons. The red boxes indicate the expected bands.

<u>Increased amplification efficiency observed with nested PCR approach</u>

Nested PCR was employed to enhance target DNA detection and improve amplification efficiency. The results (Figure 6) using the For1_5NCR/Rev7_5NCR and For2_5NCR/Rev6_5NCR primer sets showed amplifications at the expected 388bp DNA fragment length. Notably, the 388bp bands exhibited increased intensity, compared to the standard PCR results, indicating higher amplification efficiency associated with the nested PCR approach.

Positive Control					
Primer Sets / Species Type	5'NTR_CRE	MM-EV	For1_5NCR and Rev7_5NCR	For2_5NCR and Rev6_5NCR	For2_5NCR and Rev6_5NCR nested into For1_5NCR and Rev7_5NCR
A	0	0	0	0	0
B	0	0	0	0	0
C	21	6687	10221	57602	17038
D	0	0	0	0	0

Table 3: Following PCR product verification, the table shows the number of reads of each enteroviral species detected per primer set for the positive control.

Negative Control					
Primer Sets / Species Type	5'NTR_CRE	MM-EV	For1_5NCR and Rev7_5NCR	For2_5NCR and Rev6_5NCR	For2_5NCR and Rev6_5NCR nested into For1_5NCR and 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 4: Following PCR product verification, the table shows the number of reads of each enteroviral species detected per primer set for the negative control.

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

New primer sets demonstrate improved detection sensitivity for enteroviral species

To determine whether enteroviral species were present in the successfully amplified DNA, sequencing was performed on the PCR products.

In the positive control (Table 3), only species C was detected across all primer sets, with the exception of the 5'NTR_CRE primer set. Sequencing with the PanEV primers identified these species C enteroviruses as Coxsackievirus A20 and Coxsackievirus A13 serotypes, with Coxsackievirus A20 being the expected species. Notably, sequencing reads were higher with the new primer sets.

The negative control also exhibited similar sequencing results, suggesting potential contamination from the positive control (Table 4).

In the wastewater replicates (Table 5), the 5'NTR_CRE primer set did not generate any positive reads greater than 50, indicating a lack of sensitivity for detecting enteroviral species. Conversely,

the MM_EV primer set yielded positive reads for species A and B, while the newly developed primer sets in combination with nested PCR successfully detected species A, B, and C. These findings highlight notable differences in primer performance. The new primer sets and nested PCR protocol demonstrated increased sensitivity and an improved detection range compared to the commonly used 5’NTR_CRE and MM_EV primer sets.

Discussion

The detection of enteroviruses in wastewater is a critical component of public health surveillance, as it provides insights into community-level prevalence and diversity. Current detection methods face significant challenges due to the complex nature of wastewater matrices and limitations in the sensitivity and specificity of existing primer sets. Addressing these challenges is essential to improve enterovirus surveillance.

In 2018, Majumdar et al. study demonstrated efficacy of 5'-NTR-CRE and MM_EV primer sets for identifying D68 and C109 Strains in Scotland an Environmental Sample using Next Generation Sequencing, as opposed to the Oxford Nanopore MinION system.[15] A subsequent study in 2021 successfully utilised 5'-NTR-CRE and MM_EV primer sets for enterovirus species identification using Oxford Nanopore MinION system sequencing on clinical samples.[16]

However, recent laboratory anecdotal observations have indicated a progressive decline in the effectiveness of these primer sets. These observations are corroborated in our results when these primers were applied to wastewater surveillance, shedding light on their inadequacies in this specific context. This underscores the necessity of adapting and consistently validating molecular tools specifically for wastewater matrices. Environmental samples are inherently more complex than clinical specimens. Consequently, primers that perform well in clinical contexts may lack the sensitivity and specificity required for accurate surveillance in wastewater.

Here we present a protocol to directly detect enteroviral species using newly designed primers by Dr. Alexander Shaw to show more sensitive detection of enteroviral species than the 5'-NTR-CRE and MM_EV primer sets.

The nested PCR approach demonstrated superior performance, with the highest sequencing reads attributed to its two-stage amplification process. The short target region and nested approach enhanced sensitivity and specificity, resulting in increased target sequence yield and consequently higher read counts. However, variations in read counts may stem from incomplete normalisation or inconsistent sequencing depth, rather than solely reflecting differences in PCR efficiency.[17] We considered any reads above 50 as a positive indication of species presence.

Despite the promising approach, the project uncovered several methodological challenges. For the 5'-NTR-CRE and MM_EV primer sets, non-specific binding was observed during electrophoresis, and positive control detection failed, consistent with recent findings from other research laboratories. These discrepancies might be attributed to changes in reagent composition or complications arising from longer genomic target amplification.

The amplification process presents inherent limitations, particularly for longer genomic regions. Longer amplicons necessitate intact viral RNA for RT-PCR, but RNA's inherent instability complicates accurate amplification of extended sequences.[18,19] Additionally, longer target regions increase the likelihood of DNA depurination and damage during PCR.[20] While the 5'-NTR-CRE primers amplify approximately 3.8 kb of the genomic region, our new primers target a more concise ~600 bp segment.

A key limitation is highlighted in the positive controls. The use of Coxsackievirus A20, a species C[21] enterovirus, as a positive control raises methodological concerns, as it effectively validates primer functionality only for species C enteroviruses, despite detecting multiple species in

wastewater samples. Notably, negative controls across experiments showed contamination with the same positive control species, indicating potential cross-contamination issues.

Another significant limitation emerged in the detection (or rather, lack thereof) of species D enteroviruses. The absence of detection warrants further investigation to distinguish between a true biological absence and potential primer insensitivity. This challenge is characteristic of wastewater surveillance, where method detection quality critically influences data interpretation.

To address both these limitations, we recommend using previously confirmed samples from each enteroviral species as positive controls for comprehensive testing. This approach will help validate primer performance across different enteroviral species and improve the robustness of molecular detection methods.

Furthermore, wastewater composition exhibits significant geographical variability, rendering a United Kingdom Health Security Agency (UKHSA) approved protocol unsuitable for direct application in other global regions. This geographical heterogeneity necessitates comprehensive validation of molecular detection methods across diverse environmental contexts. Wastewater characteristics such as exposure to light, microbial composition, and environmental pollutants can substantially influence viral detection sensitivity and specificity.[22] Therefore, protocols developed for UK wastewater samples must undergo rigorous cross-validation and optimisation before being extrapolated to wastewater systems in different continents, climates, and urban or rural settings.

Moreover, the current study's methodology was exclusively evaluated using a single wastewater isolate, presenting a significant limitation in the research design. This narrow scope of sample testing restricts the generalisability of the findings and precludes comprehensive assessment of the detection method's performance across different sample types such as stool samples, cerebrospinal fluid and respiratory clinical samples. By broadening the sample diversity,

researchers can establish the method's reliability, sensitivity, and specificity across multiple sample types and research contexts, ultimately enhancing the protocol's scientific rigour.

Conclusion, Next Steps and Resources Needed

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.

The For2_5NCR/Rev6_5NCR and For1_5NCR/Rev7_5NCR primer sets have shown greater sensitivity by detecting more species (found three) than the 5’-NTR-CRE (found none) and MM-EV (found two) primer sets. However, further work must be done on isolates to show more accurate results and method validity.

The alternative primers bind outside the VP1 region, enabling only species-level detection, which represents a significant limitation in our current methodology. Future research must focus on developing VP1-specific primers to achieve precise serotype identification—a critical capability for comprehensive viral characterisation.

However, this objective presents substantial bioinformatics challenges. While numerous potential primer candidates can be theoretically designed (seen in Figure 8), the absence of robust in-silico PCR modelling tools creates a significant methodological barrier. Currently, no comprehensive computational framework exists to simulate the intricate thermodynamic interactions between primers and target sequences with sufficient accuracy to predict PCR reaction outcomes. The lack of advanced in-silico modelling means researchers are compelled to rely on traditional laboratory testing, which is time-consuming and resource-intensive. Resources should be invested in developing this tool to support primer design across all scientific fields requiring PCR.

This approach has the potential to accelerate the development of detection methods, lowering costs and improving the primer designing process to create primers to act specifically on the VP1 region alone.

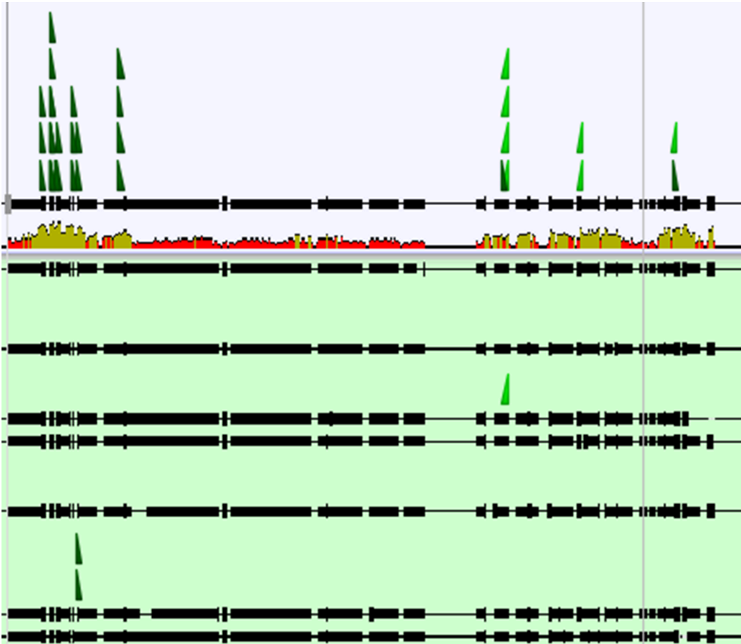

Figure 8: Screenshot of primer candidates for enteroviruses on Geneious Prime software

Ultimately, this project serves as the starting point of a comprehensive investigation into enteroviral detection methodologies, laying critical groundwork for future molecular epidemiological research while providing a temporary alternative primer set to an ongoing problem.

Potential Impacts

My research provides preliminary evidence of the higher sensitivity primer sets used for the diagnostics of enteroviruses at a time when the current standard primer sets are not performing adequately in field tests. The novel primers are already being used in Ghana and Bangladesh and

have significantly increased the detection rate of enteroviruses in the sewage. This protocol may be expanded to other areas to increase the scope of surveillance. This is aligned with the UN Sustainable Development Goals of Good health and well-being (SDG 3) and Clean water and sanitation (SDG 6).[23, 24]

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