Science of the Total Environment 954 (2024) 176365

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Development of new RT-PCR assays for the specific detection of BA.2.86 SARS-CoV-2 and its descendent sublineages

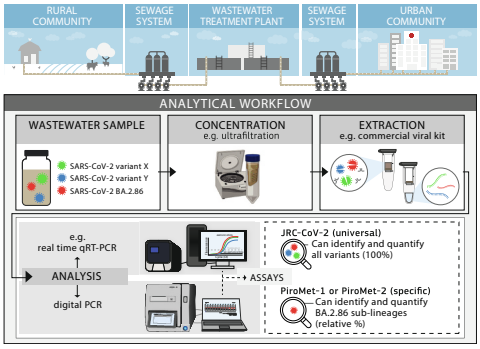
Katja Spiess [a,1], Mauro Petrillo [b,1], Valentina Paracchini [c], Gabriele Leoni [d], Ria Lassaunière [a], Charlotta Polacek [a], Ellinor Lindberg Marving [a], Nicolai Balle Larsen [a], Vithiagaran Gunalan [a], Aleksander Ring [a], Maireid Bull [a], Gerhard Buttinger [c], Carolina Veneri [e], Elisabetta Suffredini [f], Giuseppina La Rosa [e], Philippe Corbisier [c], Maddalena Querci [d], Morten Rasmussen [a,1], Antonio Marchini [c,*,1]

[a] Virus Research & Development, Department of Virus & Microbiological Special Diagnostics, Statens Serum Institut (SSI), Denmark
[b] Seidor Italy srl, Milano, Italy
[c] European Commission, Joint Research Centre (JRC), Geel, Belgium
[d] European Commission, Joint Research Centre (JRC), Ispra, Italy
[e] National Center for Water Safety (CeNSiA), Istituto Superiore di Sanità, Rome, Italy
[f] Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy

HIGHLIGHTS

- Two reverse transcription-PCR assays for the specific detection of BA.2.86 sublineages with high-level sensitivity.
- Evaluation of wastewater samples using digital RT-PCR demonstrated their effectiveness in environmental surveillance.
- In combination with OmMet and JRC-CoV-UCE.2 enables detection and quantification of BA.2.86 and other SARS-CoV-2 variants.
- These assays offer a rapid, cost-effective alternative to sequencing for SARS-CoV-2 monitoring in wastewater surveillance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Warish Ahmed

Keywords:
SARS-CoV-2
BA.2.86 sublineages
RT-PCR
Digital RT-PCR
Wastewater surveillance

ABSTRACT

The SARS-CoV-2 BA.2.86 variant, also known as Pirola, has acquired over 30 amino acid changes in the Spike protein, evolving into >150 sublineages within ten months of its emergence. Among these, the JN.1, has been rapidly increasing globally becoming the most prevalent variant. To facilitate the identification of BA.2.86 sublineages, we designed the PiroMet-1 and PiroMet-2 assays *in silico* and validated them using BA.2.86 viral RNA and clinical samples to ascertain analytical specificity and sensitivity. Both assays resulted very specific with limit of detection of about 1–2 RNA copies/μL. The assays were then applied in a digital RT-PCR format to wastewater samples, combined with the OmMet assay (which identifies Omicron sublineages except BA.2.86 and

* Corresponding author.
E-mail address: antonio.marchini@ec.europa.eu (A. Marchini).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.scitotenv.2024.176365
Received 8 May 2024; Received in revised form 14 August 2024; Accepted 16 September 2024
Available online 17 September 2024

its descendants) and the JRC-UCE.2 assay (which can universally recognize all SARS-CoV-2 variants). When used together with the OmMet and JRC-CoV-UCE.2 assays, the PiroMet assays accurately quantified BA.2.86 sublineages in wastewater samples. Our findings support the integration of these assays into routine SARS-CoV-2 wastewater surveillance as a timely and cost-effective complement to sequencing for monitoring the prevalence and spread of BA.2.86 sublineages within communities.

1. Introduction

At the beginning of August 2023, multiple countries worldwide simultaneously detected a new, highly mutated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lineage (Lasrado et al., 2023). This lineage was designated as BA.2.86 (nicknamed "Pirola"). On August 17, 2023, the World Health Organisation (WHO) classified BA.2.86 as a "variant under monitoring" (World Health Organization, 2023a).

All BA.2.86 sequences at the time were reconstituted from sequencing data from clinical samples. However, immediately after its discovery, subsequent analysis of sequencing data from wastewater surveillance samples from Thailand (Wannigama et al., 2023) and Germany (Bartel et al., 2024), uncovered BA.2.86 signatures. These findings indicate that the variant was already circulating before receiving official designation, underscoring the importance of comprehensive surveillance in promptly identifying emerging viral strains.

After August 2023, the proportion of SARS-CoV-2 BA.2.86 infections slowly but constantly increased. BA.2.86 branched into different sublineages including recombinant viruses (Centers for Disease Control and Prevention, 2023), an additional indication of its constant circulation and evolution.

On 21 November 2023, WHO classified BA.2.86 as a "Variant of Interest" (VOI) (World Health Organization, 2023b). This classification was attributed to (i) its unexpectedly high number of genomic mutations with >30 coding mutations within the S region encoding for the Spike protein, including at least 18 unique mutations and novel combinations that were never observed before, and (ii) its "simultaneous appearance" in multiple countries resembling the pattern seen with the emergence of the Omicron variant at the end of 2021 (World Health Organization, 2023b).

In December 2023, the count of identified BA.2.86 sublineages increased to over 20, for instance, BA.2.86.1 to BA.2.86.4, JQ.1, and JN.1 to JN.10, collectively denoted as BA.2.86* (BA.2.86×) sublineage hereafter. According to CoV-Spectrum (C. Chen et al., 2021), at the beginning of December 2023, in Denmark and UK, >70 % and 50 % of all SARS-CoV-2 deposited sequences were classified as BA.2.86×, respectively. Among these sublineages, JN.1 (characterised by the L455S additional mutation in the receptor binding domain of S protein when compared to its predecessor BA.2.86) rapidly became predominant in countries like France and Denmark, surpassing both the ancestor BA.2.86 and the other so-called FLip (L455F + F456L) variant (Yang et al., 2023). JN.1 continued to be reported in multiple countries and rapidly increased globally, and on 19 December 2023, WHO classified it as a separate VOI from the parent lineage BA.2.86 (World Health Organization, 2023c).

Wastewater surveillance involves the systematic analysis of sewage to detect and track the presence of various contaminants, pathogens, and substances, providing valuable insights into community health and the broader environmental landscape (Schmidt, 2020). This approach has proven successful in monitoring the presence of SARS-CoV-2 (and other pathogens), effectively detecting and tracking the virus within communities in high correlation with clinical data (Kaya et al., 2022; Smith et al., 2022; Lamba et al., 2023). Moreover, there is evidence indicating that the detection of the virus in wastewater samples precedes its detection at the clinical level (Espinosa-Gongora et al., 2023; Rajput et al., 2023; Marchini et al., 2023). Therefore, in addition to offering timely and reliable information on the circulation of the virus in a specific community, it serves as a valuable sentinel tool for early outbreak identification.

Nevertheless, the use of varied analytical methods, protocols, and controls in WWS presents challenges in guaranteeing comparability across results obtained from different laboratories (Ahmed et al., 2022).

Next-generation sequencing (NGS) has been a pivotal technology in understanding and fighting SARS-CoV-2 by enabling rapid and detailed analysis of the virus's genome, including in wastewater surveillance. Despite its significant contributions, several limitations affect its efficacy in variant detection within wastewater samples. High costs, technical complexity, specialised equipment and personnel can hinder widespread implementation, particularly in resource-limited settings. The pooled nature of wastewater samples introduces challenges such as mixed viral populations with some variants being underrepresented compared to others. Amplification and sequencing biases may lead to inaccurate variant representation, while low sequencing depth can miss rare variants. Low viral loads and fragmented viral RNA in wastewater samples can further compromise data quality and result in gaps in sequence coverage. Standardizing procedures, improving bioinformatics workflows, and benchmarking are essential to mitigate these limitations and enhance the reliability of NGS data in wastewater analysis.

Quantitative polymerase chain reaction PCR (qRT-PCR) is a complementary technique respect to NGS and it is considered the gold standard for detecting and quantifying viral RNA. While NGS provides comprehensive insights into the entire viral genome, enabling the identification of novel variants and detailed genetic analysis, qRT-PCR can be used to validate NGS findings and quantify specific variants with high sensitivity and specificity. Together, these techniques offer a robust toolkit for comprehensive viral surveillance and research.

Digital PCR (dPCR) is characterised by its ability to accurately and sensitively quantify nucleic acids, eliminating the need for a standard curve through its innovative method of dividing samples into many individual reactions (Whale et al., 2022; Quan et al., 2018). This technique offers several advantages, including single-molecule resolution, robustness to PCR inhibitors, and improved accuracy and reproducibility (Quan et al., 2018; Pinheiro et al., 2012). In addition, its high sensitivity allows the detection of rare targets, while its multiplexing capabilities allow the simultaneous analysis of multiple targets, positioning digital PCR as an indispensable tool in various fields, including molecular biology, diagnostics and clinical and environmental research (Hou et al., 2023). dPCR has been widely used to quantify DNA and RNA viruses, including SARS-CoV-2 (Whale et al., 2022; Kuypers and Jerome, 2017; Vasudevan et al., 2021; Heijnen et al., 2021).

Timely and accurate data on the virus's transmission patterns are crucial for formulating informed strategies and implementing targeted interventions to safeguard public health. At the time of the advent of Omicron, the European Commission's Joint Research Centre (JRC) quickly responded to the emergency with the development of a novel RT-PCR method specific for the detection of Omicron, called OmMet (Corbisier et al., 2022). A few months later we complemented OmMet, with a novel duplex RT-PCR method called JRC-CoV-UCE (including JRC-CoV-UCE.1 and JRC-CoV-UCE.2), which targets ultra-conserved elements into the SARS-CoV-2 genome *i.e.*, genomic regions not prone to mutate. We demonstrated that JRC-CoV-2-UCE detected all SARS-CoV-2 variants in clinical and wastewater samples (Marchini et al., 2023). As this method relies on conserved elements, it is anticipated to also detect future emerging variants, such as BA.2.86.

Given the continuous evolution of the virus, there is a potential risk that acquired mutations may fall within regions recognized by the assay

Table 1
PiroMet-1 and PiroMet-2 forward primers, reverse primers, probes and target amplicon sequences.

Name	Code	Oligo	Length (bp)	GC (%)	Tm (°C)	Position (NC_045512.2)
PiroMet-1	Piro1-Fow	5′-GGGTAACAAACCTTGTAAAGGTAAA-3′	26	36.0	58.0	22,987–23, 128
	Piro1-Rev	5′-TGCTCGGTGCATGTAGAAGTTC-3′	21	47.6	58.6	Size: 139 bp
	Piro1-P	5′-FAM (or VIC)-GGTTTCCGACCCACTTATGGTGTTGGTC-QSY-3′	28	53.6	61.3	
Amplicon-1	CGGTAACAAACCTTGTAAAGGTAAAGGTCCTAATTGTTACTTTCCTTTACAATCATATGGTTTCCGACCCACTTATGGTTGGT					
	CACCAACCATACAGAGTAGTAGTACTTTCTTTTGAACTTCTACATGCACCAGCA					
PiroMet-2	Piro2-Fow	5′-AGGAAGTCTAARCTCAAACCTTTGA-3′	26	34.6–38.5	59.6–61.1	22, 931–23,141
	Piro2-Rev	5′-GTCCACAAACAGTTGCTGGTG-3′	21	52.4	60.2	Size: 208 bp
	Piro2-P	5′-FAM (or VIC)-AGGTAAAGGTCCTAATTG-MGB-3′	18	38.9	62.8	
Amplicon-2	AGGAAGTCTAAACTCAAACCTTTGAGAGAGATATTTCAACTGAAATCTATCAGGCGGTAACAAACCTTGTAAAGGTAAAGGTCCTAATTGTTACTTTCCTTTA					
	CAATCATATGGTTTCCGACCCACTTATGGTGTTGGTCACCAACCATACAGAG					
	TAGTAGTACTTTCTTTTGAACTTCTACATGCACCAGCAACTGTTTGTGGAC					

primers and probe, affecting assay performance. Therefore, regular assessment is crucial to ensure the constant effectiveness of the SARS-CoV-2 diagnostic assays. We have recently described an *in silico* method that may alert a decay in assay performance (Marchini et al., 2023). By using this tool, we predicted a failure of OmMet in the detection of BA.2.86 sequences. After confirmation of OmMet's inability to detect BA.2.86, in the present study, we introduced PiroMet-1 and PiroMet-2, two new RT-PCR-based detection assays designed to specifically identify BA.2.86 sequences. We validated the specificity and efficacy of PiroMet-1 and PiroMet-2 in clinical samples. In addition, we have verified that both PiroMet-1 and PiroMet-2 perform well on wastewater samples.

Finally, in this study, we introduce a rapid and cost-effective strategy to distinguish BA.2.86* from other variants in wastewater samples. This involves combining JRC-CoV-UCE either with PiroMet-1 or PiroMet-2 and JRC-CoV-UCE with OmMet. We propose this RT-PCR strategy as an alternative or complementary approach to sequencing for tracking the spread of BA.2.86 sublineages in defined communities.

2. Materials and methods

2.1. In silico design of the RT-PCR assays

PiroMet-1 and PiroMet-2 were independently developed by the JRC and the Statens Serum Institut (SSI), respectively. Both assays (Table 1) were developed by applying allele-specific RT-qPCR strategy, a highly sensitive and time-saving method for single nucleotide polymorphism genotyping (Yan et al., 2021). This strategy relies on the use of allele-specific oligonucleotides (primers or probes) capable of discriminating single nucleotide variants, and recently it has been successfully adopted for identification of SARS-CoV-2 mutations in a pooled sample such as wastewater (Graber et al., 2021), and to differentiate viral subvariants in clinical settings (Li et al., 2024). The details of the design of the two methods are as follows.

2.1.1. PiroMet-1

As of January 30, 2024, 16,892 complete genomic sequences labelled as BA.2.86 or BA.2.86.1 had been deposited in GISAID (Elbe and Buckland-Merrett, 2017). These sequences were used as input data for the generation of representative Pango lineages (Rambaut et al., 2020) consensus sequences (Marchini et al., 2023) by running an in-house developed script that parses data retrieved from the Broad Institute COVID CG (A.T. Chen et al., 2021) application programming interface (used consensus threshold of 0.8). Sequences corresponding to OmMet target regions were extracted and aligned using the MAFFT online service (Katoh et al., 2019), and manually inspected with the Integrative Genomics Viewer (Robinson et al., 2011) to confirm variants using the NCBI sequence NC_045512.2 as reference (Wu et al., 2020). Following the anticipation of OmMet's failure in identifying the BA.2.86 lineage branches, due to mismatches in its forward primer (Fig. 1A), we developed PiroMet-1. The forward primer and the probe were designed, considering the BA.2.86 signatures identified by the aforementioned analysis. The OmMet reverse primer was used, as previously described (Corbisier et al., 2022). The sequences of the PiroMet-1 primers/probe, together with the amplicon generated, and its position within the viral genome are reported in Table 1 (Corbisier et al., 2022).

2.1.2. PiroMet-2

SARS-CoV-2 sequences were retrieved from positive patient samples identified through the national surveillance program in Denmark. Sequences were aligned and BA.2.86 signature mutations in the spike protein (Δ483/E484K) were identified for the PiroMet-2 PCR.

Complete, high quality SARS-CoV-2 genomes with $\geq$ 90 % ATCG-count and $\leq$ 5 ambiguous sites were retrieved from positive patient samples identified through the national surveillance program in Denmark.

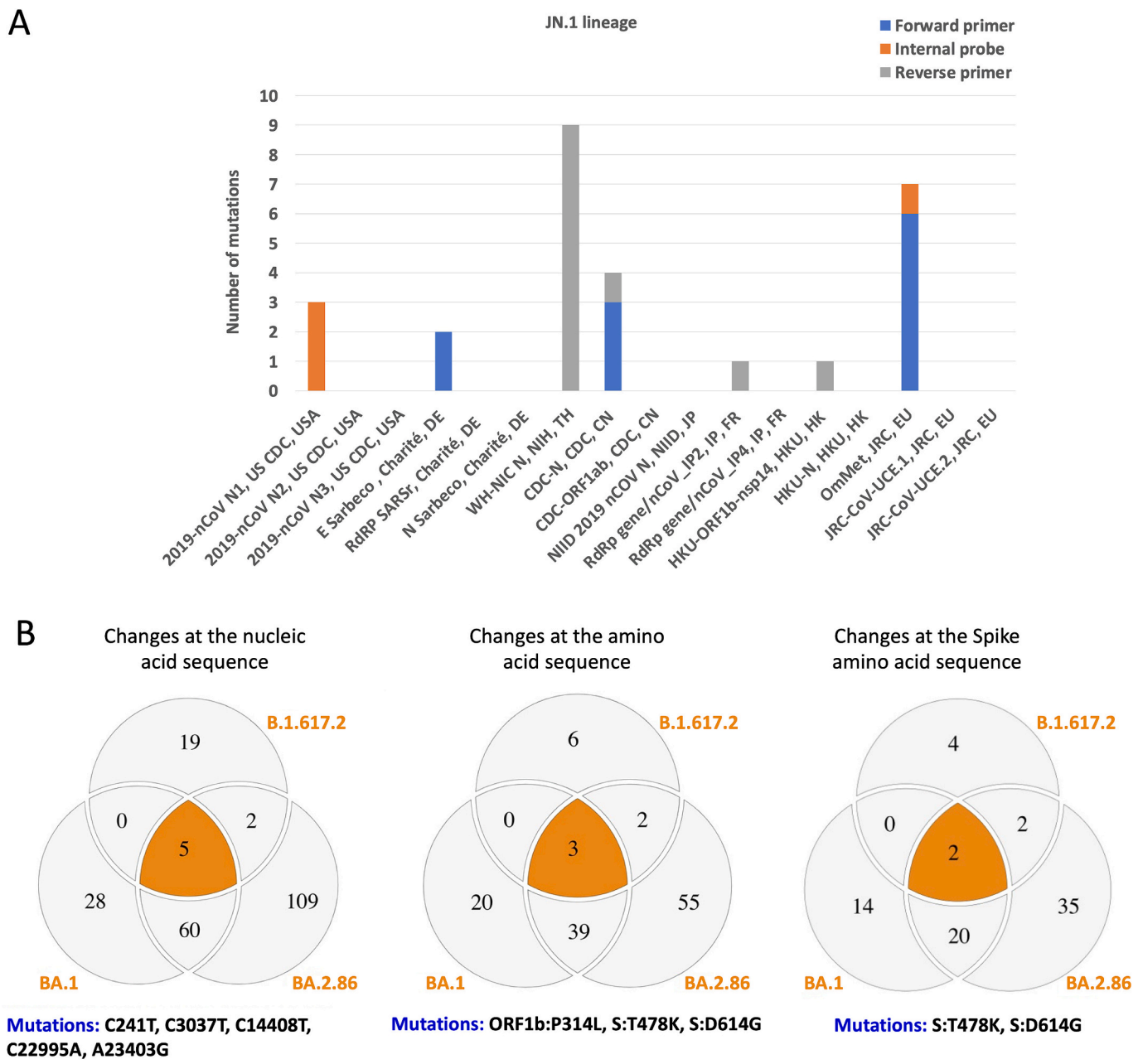


Fig. 1. BA.2.86 sublineages divergence from other SARS-CoV-2 lineages may impact on the performance of SARS-CoV-2 diagnostic assays. A. Mutations in JN.1 targeted regions of primers/probe of WHO- recommended RT-PCR assays and methods developed by JRC OmMet and JRC-CoV-UCE.1 and JRC-CoV-UCE.2. JN.1 consensus sequence was obtained as previously described (Marchini et al., 2023). Alignment was carried out with 1116 JN.1 complete and high-quality sequences deposited in GISAID. Bars represent the number of non-redundant mismatches/gaps present in the forward primer (blue), reverse primer (orange) and probe (gray) of the various assays. B. The Venn diagrams show changes at the nucleic acid (left) or amino acid (middle) or Spike amino acid (right) sequence levels found in lineages B.1.617.2, BA.1, and BA.2.86 that gave rise to the Delta, Omicron, and Pirola lineage branches, respectively. The diagrams indicate the number of distinct changes that characterize each lineage in comparison with the Wuhan reference sequence and any changes shared among two lineages. Changes common to all three lineages are highlighted in orange. Diagrams were obtained using covSPECTRUM (C. Chen et al., 2021), which was set to consider changes as belonging to a lineage if at least 90 % of the samples of the lineage had the change. Deletions are also included in the counts.

Genomes were aligned and mutations relative to the Wuhan-Hu-1 reference genome (Genbank Accn: NC_045512.2) were derived using the Nextclade tool (Aksamentov et al., 2021) and a custom R script was used to collate mutations over the study period. From here, the signature mutations (Δ483/E484K) for the PiroMet-2 PCR were identified as potential PCR targets.

The primers for the PiroMet-2 were modified for specific detection of BA.2.86 by degeneration (Table 1), but are located as described previously (Spiess et al., 2023). To gain specificity for BA.2.86 the probe of the PiroMet-2 PCR covered the Δ483 deletion in addition to the E484K amino acid substitution.

2.2. *In silico PCR simulation and sequence alignment*

Consensus sequences were used to evaluate *in silico* the detectability of the various assays, using the *thermonucleotideBLAST* software (Gans and Wolinsky, 2008) with the following parameters: *-e 20 -E 20 -l 200*. Sequence alignments were produced by using MAFFT online service (Katoh et al., 2019) and alignments' representations were obtained by running the *showseq* tool of EMBOSS package (Rice et al., 2000). Among

the sequences deposited in GISAID and assigned to BA.2.86, we noted that in 6.3 % of them the 3 base pair deletion corresponding to the amino acid deletion of valine 483 residue on the spike protein (S:V483del), a distinctive signature of Pirola, was inaccurately represented as NNN. For those sequences with available raw datasets (as fastq files) in GISAID, we verified manually that the three NNN erroneously replaced the base deletion in the target region (Fig. S1). For sequences lacking raw data, we postulated a similar scenario. Consequently, any predictions of PCR efficiency decay attributed to the NNN in that region were deemed invalid.

We also carried out *in silico* control analyses to exclude cross-reactivity with other coronaviruses (including those causing common cold, *i.e.* HCoV-OC43, HCoV-229E, HCoV-NL63). We used the sequence of the oligonucleotides and amplicons reported in Table 1 as queries on the online NCBI BLAST server (Altschul et al., 1990) to look for similarities with publicly available sequences of *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. BLAST was run with very relaxed parameters (*i.e.*, blastn as algorithm, word size set to 7 and no mask of regions of low compositional complexity). *In silico* PCR was then performed to exclude that PiroMet-1 and PiroMet-2 assays would unspecifically amplify some regions within the genome of these CoV.

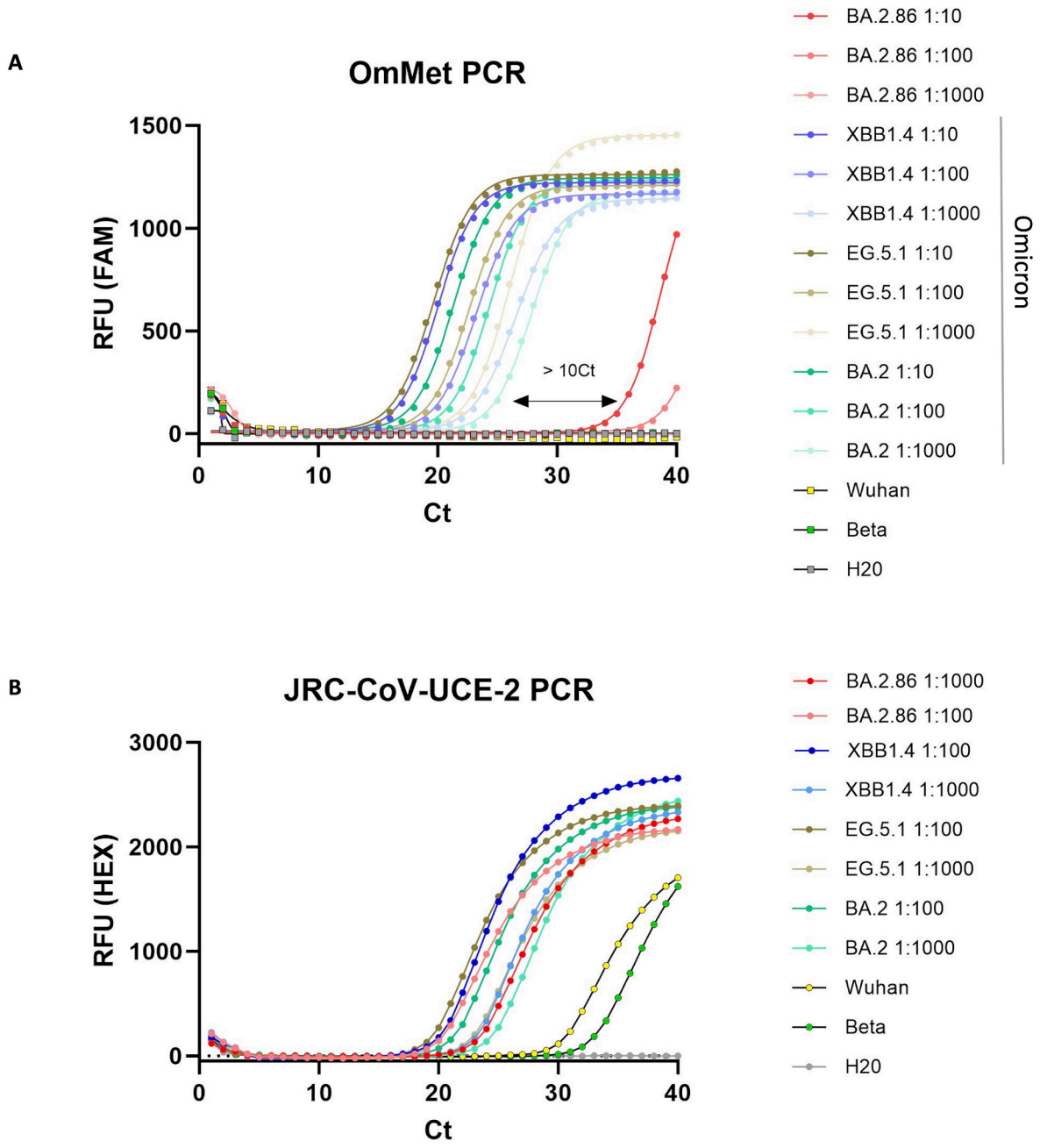


Fig. 2. OmMet failed to detect the BA.2.86 lineage in contrast with JRC-CoV-UCE.2. A. Nucleic acid isolates from cells infected with different SARS-CoV-2 variants tested by the OmMet PCR (Omicron variants specific). Late Ct detectable for high concentrated BA.2.86 cell culture isolate (>Ct 35), with >10 Ct difference to cells infected with Omicron variants. B. Cell culture isolates from cells infected with different SARS-CoV-2 variants detected by the JRC-CoV-UCE.2 assay (general SARS-CoV-2). Synthetic controls of the Wuhan-Hu-1 strain and the B.1.351 (Beta) variant were included as positive controls (1:1000 dilution of the original concentration), and PCR grade water as negative control.

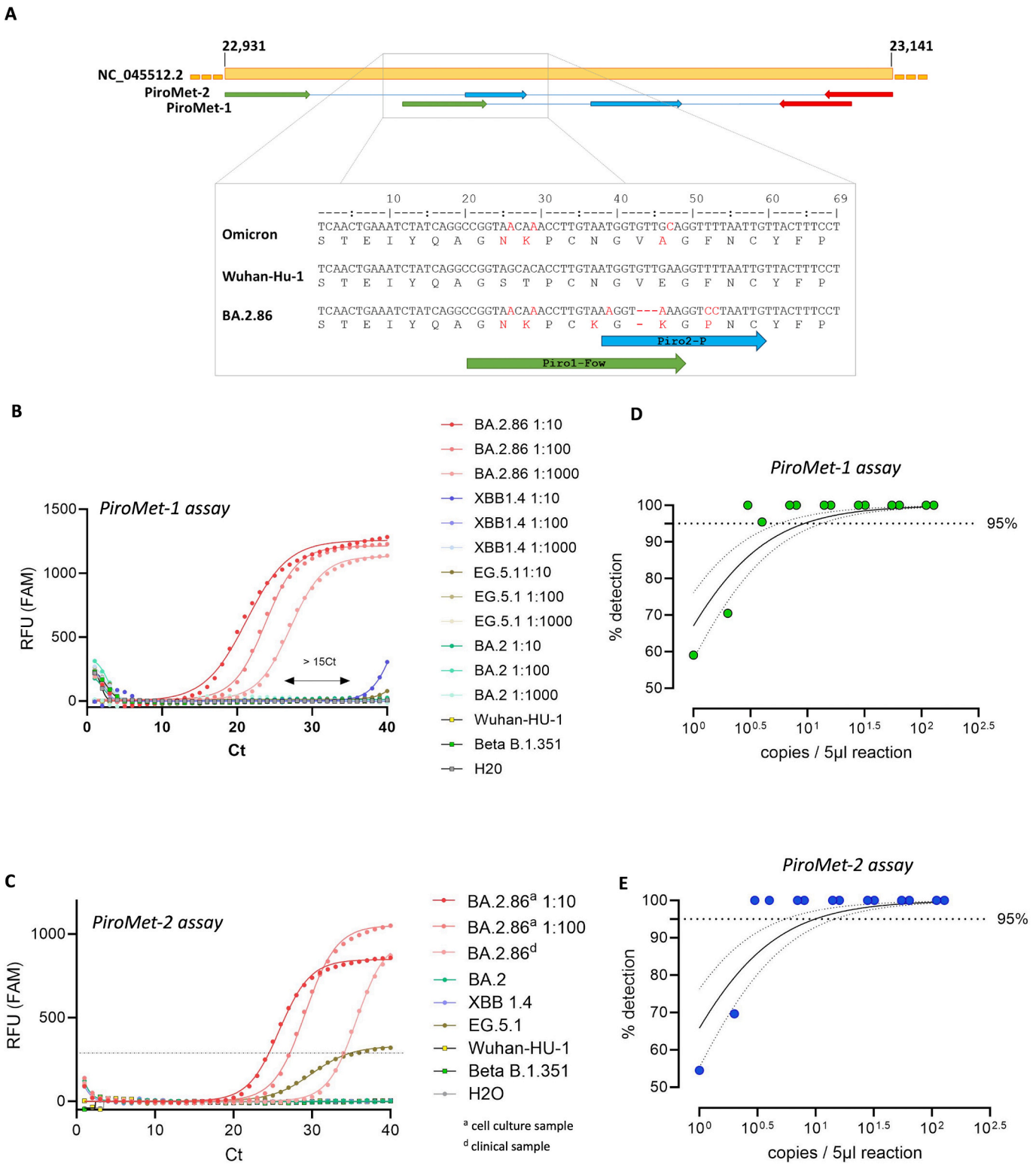


Fig. 3. Development of PiroMet-1 and PiroMet-2 assays. A. Visual comparison of PiroMet-1 and PiroMet-2 together with the alignment of the corresponding target regions in BA.2.86. The mutations of BA.2.86 incorporated in the primers/probe of the two assays are highlighted in red. For each method, the forward, probe and reverse oligonucleotides are represented as green, blue, and red arrows, respectively. B and C. Method specificity. PiroMet-1 and PiroMet-2 PCRs were tested for specificity using cell culture samples and synthetic RNA controls of SARS-CoV-2 variants. D and E. Limit of detection calculation. Limit of detection for the PiroMet-1- and PiroMet-2 assays were determined by qPCR.

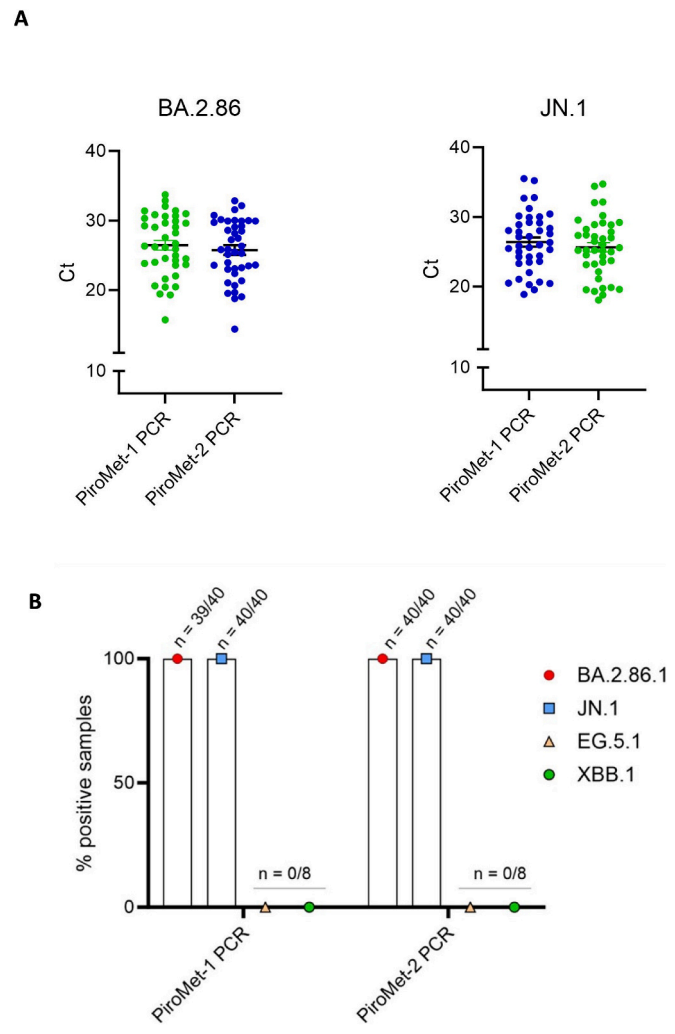


Fig. 4. BA.2.86 sublineages are specifically detected by the PiroMet-1 and PiroMet-2 assays in clinical samples. A. Detection of BA.2.86 (upper panel) and JN.1 (lower panel) sublineages in positive clinical samples using the PiroMet-1 and PiroMet-2 assays. B. Assay specificity. Specificity of PiroMet-1 and PiroMet-2 assays was tested with clinical samples known to be positive by whole genome sequencing for the four different SARS-CoV-2 lineages.

2.3. Samples, sample processing, and RNA extraction procedures

2.3.1. Cell culture isolates and clinical samples

SARS-CoV-2 isolates were propagated in Vero E6 cells containing Dulbecco's Modified Eagle Medium with 5 % Fetal Calf Serum, 1 % Penicillin/Streptomycin, and 10 mM HEPES buffer. The virus was harvested from the infected cell culture by a − 80 °C freeze/thaw cycle to lyse infected cells and release any cell-associated virus. The passage 2 viral stocks used in this study had 50 % tissue culture infectious dose titres per mL of 2.89×10^5–5.15×10^6. The accession numbers for the individual strains are as follows: OY747656 for the BA.2 strain SARS-CoV-2/Hu/DK/SSI-H68/2023; OY747657 for the XBB.1.4 strain SARS-CoV-2/Hu/DK/SSI-H116/2023; OY747654 for the EG.5.1 strain SARS-CoV-2/Hu/DK/SSI-H121/2023; and OY747653 for the BA.2.86 strain SARS-CoV-2/Hu/DK/SSI-H135/2023. Clinical cases positive for human coronavirus were sampled and diagnosed at Danish hospitals from 14 to 24 November 2023 subsequently confirmed by NGS at SSI (Rasmussen et al., 2023). Total nucleic acid from SARS-CoV-2 patient- and cell culture isolates was extracted with the MagNA Pure96 extraction robot using the MagNA Pure 96 DNA kit (Roche). Extraction was performed according to the manufacturer's protocol with 200 μL input and 100 μL elution volumes.

2.3.2. Wastewater samples

A total of 18 wastewater samples (up to five per month; Supplementary Table 1), collected from different locations in Italy between September and December 2023 as a part of the monthly surveys for variant identification (see https://www.iss.it/cov19-acque-reflue), were tested. The selected samples included all those for which the presence of either Omicron EG.5 (13 samples) or JN.1 (5 samples) variants had already been proved by sequencing analysis. This panel was therefore intended to provide a manageable and yet representative sample set for the validation of our real-time RT-PCR assay on naturally contaminated wastewater samples. The sampling methodology, viral concentration, and nucleic acid isolation of these samples were conducted following a standardised protocol (La Rosa et al., 2021) as delineated in prior studies (La Rosa et al., 2022). In summary, samples were heat-inactivated at 56 °C for 30 min, then concentrated using a polyethylene glycol (PEG)-based technique. The RNA was isolated using magnetic silica beads and further purified with the OneStep PCR Inhibitor Removal Kit (Zymo Research) before preservation at −80 °C for molecular analysis. The protocol used for variant analysis was a long-nested PCR assay of the spike protein (approximately 1,330 base pairs), followed by Sanger sequencing.

2.4. RT-PCR testing

The digital RT-PCR conditions of the OmMet and JRC-CoV-UCE assays have been previously described (Marchini et al., 2023; Corbisier et al., 2022). The PiroMet-1 and PiroMet-2 were tested on a Bio-Rad CFX real-time PCR system. Master mixes contained: 12.5 μL of Luna universal probe one-step RT-qPCR reaction buffer (New England BioLabs Inc.). 1.25 of Luna Warm Start RT-qPCRs enzyme mix, primer and probes at a final concentration of 0.4 μM and 0.2 μM, respectively, DNase/RNase-free water, and 5 μL of the template for a total volume of 25 μL. Cycling conditions were as follows: reverse transcription at 55 °C for 10 min, followed by an initial denaturation step at 95 °C for 3 min, and 45 cycles of amplification consisting of 15 s at 95 °C and 30 s at 62 °C (PiroMet-1) or 58 °C (PiroMet-2). For the wastewater analysis, the PiroMet PCRs were multiplexed together with the OmMet or JRC-CoV-UCE PCRs, here an annealing temperature of 60 °C was chosen.

PCR curves were evaluated using Bio-Rad CFx software and C_T and end relative fluorescent units (RFU) were exported as .CSV files. Files were imported into a laboratory database for data analysis.

2.5. Annealing temperature optimization

Clinical samples and cell culture isolates positive for SARS-CoV-2, including BA.2.86, were tested at different annealing temperatures to determine the optimal temperature following the protocol described under 2.4. Four different annealing temperatures ranging from 58.0 to 62.0 °C were tested.

2.6. Analytical sensitivity

To calculate the limit of detection for the PiroMet-1 and PiroMet-2 PCRs, cell culture propagated BA.2.86 was purified and quantified by digital PCR on a naica system (Stilla Technologies) using E Sarbeco primer-probe set (Corman et al., 2020). Each reaction consisted of 1× qScript XLT 1-Step Toughmix (QuantaBio), 0.8 μM primers, 0.3 μM probe and 100 nM disodium fluorescein (VWR). Three series of two-fold dilutions and a no template control were analysed in duplicate on a Ruby (Stilla) chip with the following cycling conditions: 50 °C for 10 min, 95 °C for 1 min, 45 cycles of 95 °C 10 s and 58 °C for 40 s. Images were captured according to manufactures recommendations and quantified with Crystal Miner v4 software (Stilla).

For determination of the limit of detection, the digital PCR quantified

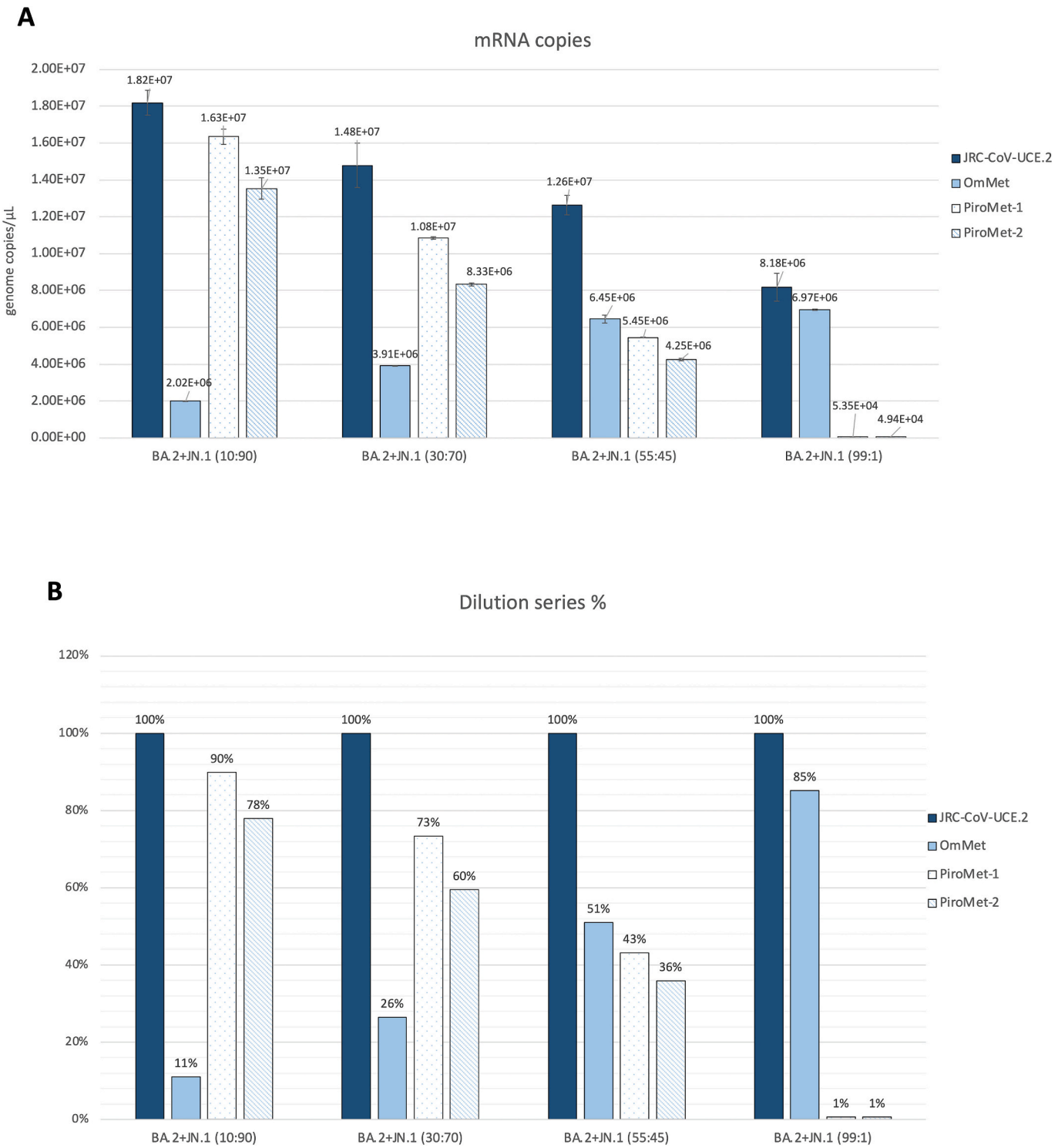


Fig. 5. Specificity analysis of duplex PCRs using mixed SARS-CoV-2 RNA targets extracted from cell culture. A and B. RNAs from Omicron BA.2 and/or JN.1 (Pirola) sublineages were mixed in different proportions and analysed for the OmMet, PiroMet-1 or PiroMet-2 assays, all in duplex with the JRC-CoV-UCE.2 assay. A. Concentrations in genome copies/µL of SARS-CoV-2 targets in mixed RNA samples. B. Relative amount of SARS-CoV-2 specific targets over the total amount of SARS-CoV-2. The total viral content is given by the JRC-CoV-UCE.2 universal assay (dark blue bar).

RNA was diluted in two-fold dilutions from 128 to 1 copy/5 µL reaction. 11 technical replicates of each concentration were analysed with both PiroMet-1 and PiroMet-2 using conditions described above. The experiments were repeated 3 times for each assay. The fractions of positive samples for a total of 33 repeats was calculated for both PiroMet-1 and PiroMet-2. The limit of detection (LoD) was calculated using non-linear log *vs.* response least squares fit (Hill slope constrained to 1) in GraphPad Prism version 10 (GraphPad).

2.7. Assay validation

Prior to validation with clinical samples, the following positive detection criteria were defined, based on assay performance with cultured virus. For the PiroMet-1 PCR, detection was based on C_T values

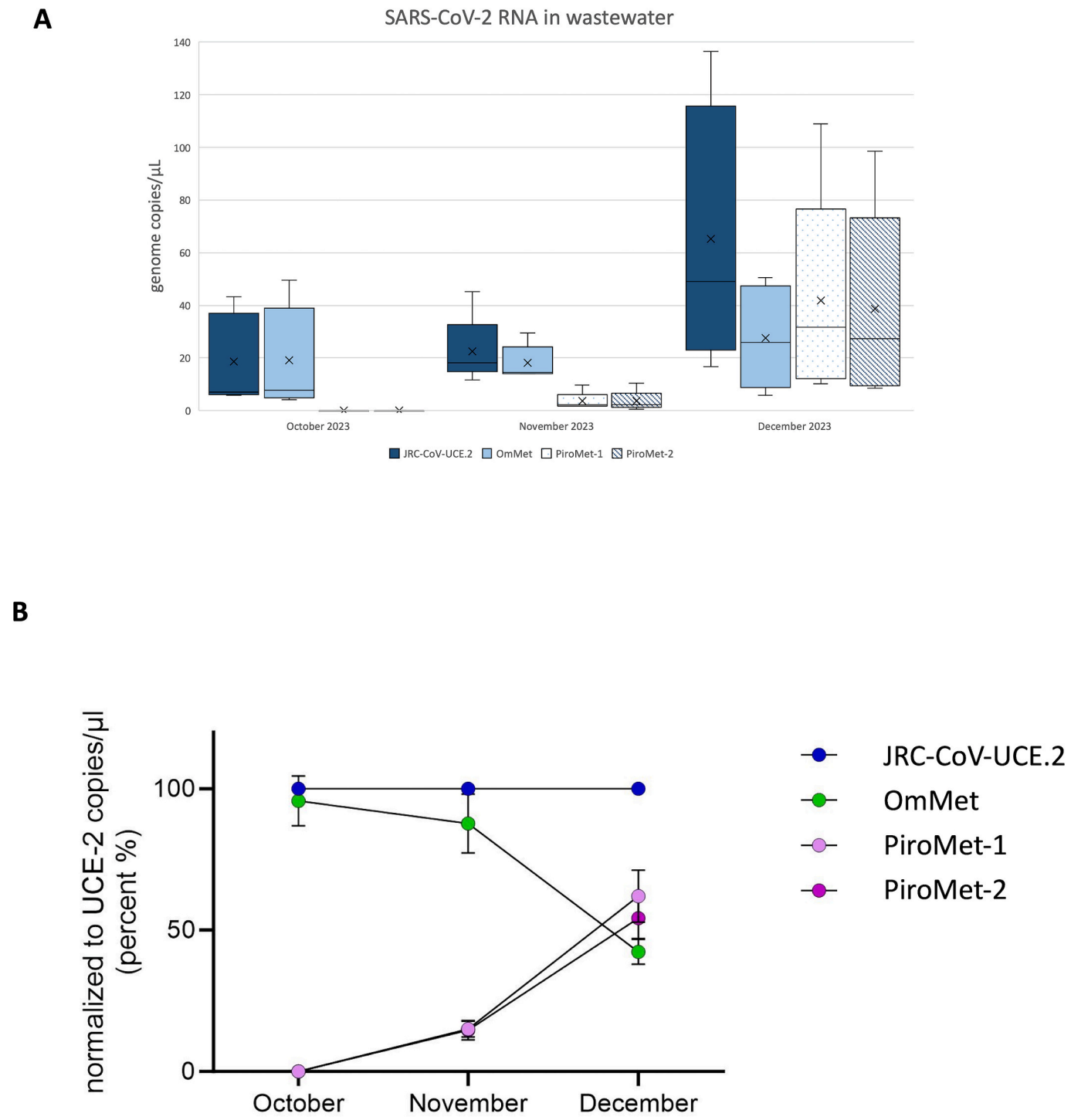


Fig. 6. Concentration of SARS-CoV-2 variants in wastewater samples from Italy. A. Box plot illustrating the amount of SARS-CoV-2 (genomes copies/µL) and its variants in wastewater samples between October and December 2023. The lines of the box represent the 25th, 50th and 75th percentile (bottom, middle, and top lines, respectively); whiskers show the range from minimum to maximum values. B. Line chart illustrating the relative percentage of variant RNA copies detected with RT-PCR OmMet, PiroMet-1, PiroMet-2 assays over the total SARS-CoV-2 RNA copies detected with the JRC-CoV-UCE.2 universal assay.

(C_T values cut-off between 12 and 36) and for the PiroMet-2 PCR a C_T value <38 and an End RFU of >30 % of the BA.2.86 positive run control.

The specificity of the PiroMet-1 and PiroMet-2 assays was confirmed by re-analysing SARS-CoV-2 positive clinical samples for which sub-lineage calls by whole genome sequencing were available. 40 BA.2.86.1, 40 JN.1, 8 EG.5 and 8 XBB.1.16 samples were selected and analysed as described above.

2.8. Quantification of viral load by digital RT-PCR on wastewater samples

Digital RT-PCR analysis was conducted using the QIAcuity Four 5-plex digital PCR system (Qiagen), using the 4× One-Step Advanced Probe Master Mix (Qiagen). Each reaction (40 µL per well) consisted of 1× QIAcuity One-Step Viral PCR Master Mix, 1× One-Step Advanced RT Mix (Reverse Transcription), 0.4 µM of each forward and reverse

primers, 0.15 μM (PiroMet-1, PiroMet-2, OmMet) and 0.05 μM (JRC-CoV-UCE.2) of probe, RNase-Free Water up to 36 μL and 4 μL of extracted RNA. Three duplex assays were performed: JRC-CoV-UCE.2/OmMet, JRC-CoV-UCE.2/PiroMet-1, and JRC-CoV-UCE.2/PiroMet-2. For each sample, two technical replicates were performed. Cycling conditions were as follows: reverse transcription for 40 min at 50 °C, enzyme activation for 2 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. The measurements were performed using a 24-well nanoplate with approximately 26,000 partitions.

Partitions were imaged with 500 ms exposure time, with gain set to 6 for both channels (green and yellow). The QIAcuity Software Suite version 2.5.0.0 was used to determine sample thresholds using positive and no-template control wells according to the manual global threshold approach. Samples were considered positive if they exhibited at least three positive partitions in one of the two replicates. Lastly, the results were normalised to correct for the day-to-day and nanoplate-to-nanoplate variability (the average of the positive control was used for normalization).

3. Results

3.1. OmMet assay fails to detect BA.2.86 lineages in contrast to JRC-CoV-UCE.2 assay

The BA.2.86 lineage and its sublineages, including the JN.1 lineage prevalent as of March 2024, have undergone extensive genomic evolution characterised by numerous mutations, including point mutations, deletions, and insertions, as illustrated in Fig. 1B (left panel). Specifically, when compared with the original Wuhan-Hu-1 (Index) lineage and the Omicron BA.1 lineage, a significant divergence is evident with 176 and 111 nucleotide mutations, respectively. At the amino acid level, these mutations translate in 99 and 57 unique changes (Fig. 1B middle panel), respectively. Among these unique changes, 59 and 37 occurred in the amino acid sequence of the Spike protein in comparison to Wuhan-Hu-1 and Omicron BA.1, respectively.

We generated seven consensus viral genomic sequences of the BA.2.86 lineage and its main branches (BA.2.86.1, BA.2.86.2, BA.2.86.3, JN.1, JN.2 and JN.3), from a total of 45,059 GISAID sequences assigned to either BA.2.86* or JN* sublineages (75 % of the whole branch as of January 30, 2024). To evaluate the sequence similarity with currently used methods, we compared the consensus sequences with the primer sets of the 14 WHO recommended RT-PCR assays, and with the two assays previously developed by our team, namely the OmMet and JRC-CoV-UCE duplex assay (JRC-CoV-UCE.1 and JRC-CoV-UCE.2). 100 % nucleotide sequence identity between primers/probe and corresponding viral sequences was found for the JRC-CoV-UCE.2 assay (Marchini et al., 2023) and eight WHO recommended assays, namely the 2019-nCoV N2 and 2019-nCoV N3 (Centers for Disease Control and Prevention, 2020a, 2020b), the RdRP SARSr and N Sarbeco (Corman et al., 2020), the CDC ORF1 ab (Chinese Center for Disease Control and Prevention, 2020), RdRp gene/nCoV IP4 (Institut Pasteur, Paris, n.d.), NIID 2019 nCOV N (Shirato et al., 2020) and HKU-N (HKU, HK) (Chu et al., 2020). A single nucleotide mismatch was found in the reverse primers of the nCoV IP2 (Institut Pasteur, Paris, n.d.) and HKU-ORF1b-nsp14 assays (Chu et al., 2020), two mismatches in the forward primer of E Sarbeco (Corman et al., 2020), three mismatches in the internal probe of the 2019-nCoV N1 (Centers for Disease Control and Prevention, 2020a, 2020b) and in the forward primer of CDC-N assay (Chinese Center for Disease Control and Prevention, 2020) which also included another mismatch in its reverse primer. The WH-NICN assay (Department of Medical Sciences, Ministry of Public Health, n.d.) developed in 2020, presented nine mismatches all within its reverse primer, most likely precluding the ability of this assay to recognize the BA.2.86 lineages and branches. We also identified five mismatches within the forward primer and one mismatch within the probe of the OmMet assay (Corbisier et al., 2022). This assay was developed by the Joint Research Centre (JRC) at the time of the appearance of Omicron and was proven to be able to detect all Omicron variants before BA.2.86.

Based on the high number of mismatches found in the primers of the OmMet assay, we first ran *in silico* PCR simulations using the consensus sequences as a template which predicted the failure of OmMet assay to detect BA.2.86 sequences (data not shown). These results prompted us to verify the performance of OmMet assay. To this end, we used 10-fold serially diluted RNA extracted from cell culture isolates of BA.2.86 and other Omicron variants, and synthetic viral RNA of the original Wuhan-Hu-1 and Beta lineages. In agreement with the *in silico* PCR simulations, the RT-PCR analysis confirmed inability of the OmMet assay to efficiently detect BA.2.86, with a notable difference of >10 Ct values compared to other Omicron sublineages when using the same quantity of RNA input (Fig. 2A). Concurrently, the JRC-CoV-UCE.2 assay, was able to amplify BA.2.86, confirming its ability to amplify all SARS-CoV-2 variants (Fig. 2B), as foreseen in (Yang et al., 2023) and from our alignment.

3.2. Development of PiroMet-1 and PiroMet-2 assays

Building upon the identified primer/probe mismatches, we refined the OmMet assay by introducing modifications to both the forward primer and the probe, resulting in a lineage-specific set of primers tailored to BA.2.86* (Table 1). We called the new RT-PCR assay, PiroMet-1 (**Piro**la **Met**hod). In addition, we developed a second assay, PiroMet-2, built upon the same BA.2.86 signatures as PiroMet-1. However, PiroMet-2 targets BA.2.86 sublineages through the probe rather than the forward primer (Fig. 3A) and produces an amplicon slightly larger (69 base pairs) than that generated by PiroMet-1 (Table 1).

After revealing the inability of the OmMet assay to recognize the BA.2.86 lineage, we conducted an RT-PCR simulation to evaluate the specificity of PiroMet-1 and PiroMet-2 *in silico*, using the same set of consensus sequences previously employed in the OmMet analysis as templates. *In silico* PCR simulation predicted excellent matching and a high specificity and efficiency for both PiroMet-1 and PiroMet-2 assays, with the theoretical detection of 100 % of BA.2.86 sublineages registered in GISAID at the time of writing.

Furthermore, BLAST alignment and *in silico* PCR analysis did not identify any similarities between PiroMet-1 and PiroMet-2′ amplicons and oligonucleotides with the genome of other coronaviruses belonging to the genera *Alphacoronavirus*, *Betacoronavirus* (with the exception of SARS-CoV-2), *Gammacoronavirus* and *Deltacoronavirus* including human coronaviruses causing mild upper respiratory infectious in humans. We thus concluded that, at least according to the current knowledge of deposited and available sequences, no PCR amplification is possible on nucleic acids from other than SARS-CoV-2 coronaviruses.

We first ran a temperature gradient optimisation to find the optimal annealing temperature for the two assays using clinical samples and cell culture isolates. A temperature of 58 °C or 62 °C was selected for PiroMet-1 and PiroMet-2, respectively. We then evaluated the specificity of the two assays using clinical samples tested positive for different SARS-CoV-2 variants (Fig. 3B and C). Both PiroMet-1 and PiroMet-2 demonstrated high specificity for BA.2.86. While PiroMet-1 produced a signal close to background levels for the higher concentrated sample of the XBB1.4.1 sublineage (more than a 15 Ct difference compared to BA.2.86), a low signal was observed with PiroMet-2 for the EG.5.1 sublineage, but below the detection criteria >30 % of the positive control. Moreover, given that EG.5.1 has nearly disappeared, accounting for <0.5 % of deposited sequences from April to July 2024 our *in silico* analysis on GISAID deposed sequences, we concluded that both assays are highly specific for BA.2.86 sublineages with PiroMet-1 being exclusive for these sublineages.

We then calculated the analytical limit of detection for both assays using serial dilutions of the BA.2.86 RNA. The PiroMet-1 and PiroMet-2 assays have detection limits of 9 copies per 5 μL reaction (95 % confidence interval: 6 to 13 copies) and 10 copies per 5 μL reaction (95 %

confidence interval: 6 to 15 copies), respectively. Therefore, the limit of detection for both assays is close to the theoretical minimum of approximately 3 copies per reaction, which corresponds to about 1.2–2 copies per μL. These results indicate that both PiroMet-1 and PiroMet-2 can detect BA.2.86 sublineages with high-level sensitivity. (Fig. 3D and E). Additionally, we examined if PiroMet-1 would maintain its performance using the OmMet probe, given that the two probes differ by only the first nucleotide. The PiroMet-1 performs with similar efficiency with the probe from the OmMet method, showing no discrepancies in performance between the two probes (Fig. S2).

We further evaluated the capability and specificity of PiroMet-1 and PiroMet-2 in identifying BA.2.86 sublineages within clinical specimens. To this end, we analysed 40 clinical samples known to contain BA.2.86 and another 40 samples positive for the JN.1 variant, each with varying viral concentrations (Fig. 4A). As negative controls, we included clinical samples positive for EG.5.1 ($n = 8$) and XBB.1 (n = 8) Omicron sublineages as determined by SARS-CoV-2 whole genome sequencing. Consistent with prior findings, PiroMet-1 and PiroMet-2 confirmed their high specificity on clinical samples, successfully detecting BA.2.86 sublineages in all examined samples. In contrast, both assays were unable to detect the EG.5.1 and XBB.1 variants (Fig. 4).

3.3. Development of a strategy for the monitoring of BA2.86* in wastewater samples

Based on our results, we devised a targeted approach for wastewater surveillance to determine the prevalence of BA.2.86 in a community over a specific period. This strategy aimed to detect and quantify BA.2.86* among various SARS-CoV-2 lineages in wastewater samples, offering a quick and cost-effective alternative to sequencing. It consists of two duplex digital RT-PCR assays in which we combine the JRC-CoV-UCE.2 assay, capable of quantifying all SARS-CoV-2 (100 %) in a sample with either OmMet or with one of the PiroMet assays. The duplex assay including the OmMet aims to measure the relative fraction of Omicron sublineages excluding BA.2.86*. In contrast, the duplex with PiroMet, is designed to specifically quantify the fraction of BA.2.86 sublineages relative to the total SARS-CoV-2 virus in the sample. Firstly, we performed proof-of-concept digital RT-PCR experiments testing both duplex assays using viral mRNAs of BA.2 and JN.1 sublineages, isolated from cell cultures, individually or mixed in various ratios. These trials validated our hypothesis that the assays could function in a duplex format. Notably, PiroMet assays in combination with JRC-CoV-UCE.2 successfully identified low levels of JN.1 against a predominant background of BA.2. (Fig. 5).

Secondly, we applied our strategy to 18 RNAs extracted from wastewater samples, collected in Italy in the period September–December 2023 (three samples in September, five samples for each month in October, November and December), and preliminarily screened by nested PCR and Sanger sequencing to identify the dominant SARS-CoV-2 sublineages (Supplementary Table 1). In these samples, BA.2.86 lineage specific mutations were detected only in December 2023, whereas in the previous months, only the XBB.1.5/XBB.1.9 associated mutations were identified.

We analysed these wastewater samples with digital RT-PCR, employing JRC-CoV-UCE.2/OmMet, JRC-CoV-UCE.2/PiroMet-1, and JRC-CoV-UCE.2/PiroMet-2 duplex assays. The results showed a low prevalence of total SARS-CoV-2 in September, which was supposedly under the quantification limit (data not shown). The total viral concentration rose during October and November, with a marked increase in December (Fig. 6). Notably, BA.2.86* was absent in the October samples but began to emerge in November and increased in December, alongside a decrease in other Omicron sublineages. Interestingly, despite Sanger sequencing failed to detect BA.2.86* in November within a background rich in EG.5, the duplex assays incorporating PiroMet were able to detect the presence of BA.2.86* even when it was a minor fraction of the Omicron sublineages. However, the expected positive percentage agreement (PPA) and negative percentage agreement (NPA) with the Sanger sequencing were both high. PiroMet 1 and PiroMet2 consistently detected the presence of BA.2.86, matching Sanger sequencing results in five out of five December samples (PPA 100 %), and did not produce false positive results in the eight BA.2.86 negative samples collected in September and October, containing the Omicron EG.5 sublineage. These results reflect the assays' capability to specifically and accurately identify BA.2.86 sublineages. This highlights the benefits of the incorporation of this strategy in wastewater surveillance, particularly when next-generation sequencing is often replaced with Sanger sequencing due to budgetary constraints.

4. Discussion

Nearly ten months from its first detection in July/August 2023, BA.2.86 lineage has diversified into >150 sublineages, with one in particular, JN.1, becoming the dominant lineage worldwide (Kaku et al., 2024a). When compared to previous primary lineages, like B.1.617.2 (Delta) and BA.1 (Omicron), BA.2.86 exhibits a significant array of additional mutations. The high number of changes observed in BA.2.86*, particularly in the Spike encoding region (as well as in other parts of the genome), underscores the ability of the virus to evolve, leading to the development of quite distinct lineages.

Overall, these mutations may affect the detection of the virus by diagnostic assays, as previously reported by us and others for other variants (Yang et al., 2023; Vasudevan et al., 2021; Heijnen et al., 2021; Corbisier et al., 2022; Yan et al., 2021). It is therefore important to validate sensitivity and specificity of the various SARS-CoV-2 diagnostic assays, many of them developed during the first year of the pandemic for variants no longer in circulation, against the emerging BA.2.86 sublineage and future variants.

In this study, we present two novel independent RT-PCR-based methods, PiroMet-1 and PiroMet-2, specifically designed to detect BA.2.86*. To the best of our knowledge, these represent the first BA.2.86 specific RT-PCR assays described to date. Unlike existing RT-PCR strategies that may depend on the failure of amplifying a mutated S-gene target, the two assays developed here enable direct specific identification of BA.2.86* and its related sublineages without detecting prior Omicron lineages. The assays display high sensitivity, even in samples in which BA.2.86* is present in low abundance and in a background of other environmental RNAs including RNAs from other SARS-CoV-2 variants. When combined with other assays such as JRC-CoV-UCEs, each PiroMet assay can provide insights into the relative quantities of BA.2.86* in samples containing different SARS-CoV-2 lineages, such as wastewater samples.

The principal difference between the two assays lies in their response to BA.2.86 negative samples, yet containing other SARS-CoV-2 sublineages. PiroMet-1 does not generate any BA.2.86 amplicon due to the annealing failure of the forward primer. In contrast, PiroMet-2 can produce an amplicon, but will not produce any fluorescent signal due to the inability of the BA.2.86-specific probe to bind. This distinction allows laboratories to select the method that best suits their diagnostic setup. For instance, they could use either PiroMet assay in combination with the JRC-CoV-UCE.2 assay (Marchini et al., 2023), or an equivalent assay to detect all SARS-CoV-2 variants. According to our *in silico* analyses, the methods presented here are also able to recognize the new variants KP.*, including KP.2 (data not shown), which has been recently estimated to potentially become the new predominant lineage worldwide (Kaku et al., 2024b). Our *in silico* PCR analyses have predicted that most of the WHO-recommended assays remain effective in identifying BA.2.86* sequences.

In the process of designing our assays *in silico*, we observed that the distinctive 3 bp deletion (corresponding to S:V483del), at the basis of the development of PiroMet-1 and PiroMet-2, is not always consistently reported in the final assembled sequences, and became evident only after manual inspection of the raw data. The "misdetection" of the

nucleic acid deletion (and consequently of the deleted amino acid) could be attributed to alignment errors between target and reference genomes with a high degree of similarity in adjacent codons. We have encountered difficulties in retrieving raw datasets belonging to the BA.2.86 lineage, as many laboratories do not deposit raw sequence data, but only final assembled genomic sequences. This can limit the confidence in the characterisation of a novel emerging lineage such as BA.2.86 with its related variants, which is crucial for surveillance purposes. Hence, we recall and strongly recommend the use of standardised, harmonised FAIR bioinformatics workflows to enhance reliability and reproducibility in variant detection (Goble et al., 2023; de Visser et al., 2023). This, along with the FAIR+E principles for data sharing (Neves et al., 2023), is critical for the effective execution of genomic surveillance and alignment with WHO guidelines (World Health Organization, 2022).

An alternative/complementary approach to PCR-based methods, applied in wastewater surveillance is the use of next-generation sequencing (NGS) followed by complex bioinformatics analysis. While NGS excels in detecting rare variants and providing extensive genomic insights (Yousif et al., 2023; Lipponen et al., 2024), it demands significant resources, expertise and infrastructure, rendering it less accessible for continuous surveillance (Akande et al., 2023). Furthermore, with an eye towards cost-effectiveness, there has been a tendency to use the more economical Sanger sequencing (Veneri et al., 2024; Frydman et al., 2024). While Sanger sequencing offers certain advantages, it notably falls short in accurately detecting minority variants within samples that contain a mix of different variants (Iaconelli et al., 2017; Suffredini et al., 2018).

Our strategy with mutation-specific digital RT-PCR assays, particularly for analysing SARS-CoV-2 RNA in wastewater, enables an efficient, rapid, and reliable monitoring of variants circulating within a community. It consists of two duplex digital RT-PCR assays in which we combine the JRC-CoV-UCE.2 assay, capable of quantifying all SARS-CoV-2 present in a sample, with lineage-specific detection methods such as OmMet or with PiroMet-1. This strategy can be adopted when it is not feasible to perform NGS. Furthermore, the strategy can be easily adapted by considering new lineage-specific assays to evaluate the spread of new emerging lineages. For example, in a BA.2.86* dominant background, a method able to specifically detect a new variant different from any BA.2.86* can be designed, *in silico* tested, validated on real samples, and coupled in a duplex with PiroMet-1 or JRC-CoV-UCE.2 methods. A decline in performance or failure of detection with PiroMet-1 but not with JRC-CoV-UCE.2 is indicative of the emergence of a new potential variant, as it may indicate that the virus is acquiring new mutations in the primer/probe regions of PiroMet-1. This decay in performance may suggest the emergence of a potentially new variant, thereby enhancing our ability to detect and respond to viral evolution. Conversely, if mutations occur outside the regions recognized by the primers/probes, PiroMet-1 will continue to recognize the new subvariants but will not distinguish them from previous ones. This is exemplified by the recent JN.1 subvariants KP.2 and KP.3 which are becoming dominant in US and rising in other part of the world, including Europe. These subvariants feature an additional deletion (S:S31del) outside the primers/probe regions of PiroMet-1 and PiroMet-2 and are therefore predicted by *in silico* PCR to be still detected by the two assays. This underscores the need for ongoing surveillance and sequencing to monitor and understand SARS-CoV-2 genomic changes.

The target region of OmMet, PiroMet-1 and PiroMet-2 is particularly suitable for designing lineage-specific methods, as it corresponds to the receptor-binding motif present in the S1 domain of the spike protein (Omotoso et al., 2021; Ashwaq et al., 2021). This motif is a known hotspot of mutations featuring the turnaround of dominant variants, as already observed in the Delta-Omicron replacement and more recently in the Omicron evolution to BA.2.86*. As the virus continues to evolve, these assays could be instrumental in detecting the emergence of new variants, particularly if BA.2.86* levels decline while the overall SARS-CoV-2 concentration remains stable. This capability can alert the scientific community and public health officials, potentially enhancing surveillance efforts and informing science-based decision-making processes.

5. Conclusions

We have developed and validated PiroMet-1 and PiroMet-2 (d)RT-PCR based assays for the rapid identification and monitoring of the SARS-CoV-2 BA.2.86 variant and its sublineages. These assays have demonstrated high specificity and sensitivity, effectively detecting as low as 1–2 copies per microliter. By integrating these assays with the OmMet and JRC-CoV-UCE.2 assays, we were able to accurately quantify the presence of BA.2.86 alongside other variants in wastewater samples. The adoption of these digital RT-PCR assays offers a rapid, cost-effective alternative to sequencing, enhancing our ability to monitor the spread of SARS-CoV-2 variants through routine wastewater surveillance. Hence, the two assays extend our surveillance capabilities to monitor currently circulating SARS-CoV-2 variants, as well as alert for their further evolution.

Ethics

Exemption for review by the ethical committee system and informed consent were given by the Committee on Biomedical Research Ethics—Capital Region in accordance with Danish law on assay development projects.

CRediT authorship contribution statement

Katja Spiess: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Mauro Petrillo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Valentina Paracchini:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Gabriele Leoni:** Writing – review & editing, Methodology. **Ria Lassaunière:** Writing – review & editing, Formal analysis. **Charlotta Polacek:** Writing – review & editing, Formal analysis. **Ellinor Lindberg Marving:** Writing – review & editing, Formal analysis. **Nicolai Balle Larsen:** Writing – review & editing, Formal analysis. **Vithiagaran Gunalan:** Writing – review & editing, Formal analysis. **Aleksander Ring:** Writing – review & editing, Formal analysis. **Maireid Bull:** Writing – review & editing, Formal analysis. **Gerhard Buttinger:** Writing – review & editing, Formal analysis. **Carolina Veneri:** Writing – review & editing, Resources, Formal analysis. **Elisabetta Suffredini:** Writing – review & editing, Resources, Formal analysis. **Giuseppina La Rosa:** Writing – review & editing, Resources, Formal analysis. **Philippe Corbisier:** Writing – review & editing. **Maddalena Querci:** Writing – review & editing. **Morten Rasmussen:** Writing – review & editing, Supervision, Conceptualization. **Antonio Marchini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

We extend our gratitude to Dennis Jelsbak Schmidt, Susanne Lopez Rasmussen and Birgit Knudsen from the Statens Serum Institute, Denmark for their assistance and technical support. We thank Veronique Vanherck for her assistance with the graphical abstract. We would like also to express our gratitude to Bernd Gawlik and the EU4S DEEP team for scientific discussion. We gratefully acknowledge the Authors and the Originating laboratories where the clinical specimen or virus isolate was first obtained and the Submitting laboratories, where sequence data have been generated and submitted to GISAID, on which this research is based. The work described in this manuscript was co-financed through the DURABLE project. The DURABLE project has been co-founded by the European Union, under the EUHealth Programme (EU4H), Project no. 11102733. Views and opinions expressed are however reflect those of the authors only and do not necessarily reflect those of the European Union nor the granting authority can be held responsible for them.

We gratefully acknowledge the COVID CG team of the BROAD INSTITUTE for helping us in setting up the query to the COVID CG application programming interface (API). We are also very thankful to the SARI network laboratory for providing us the extracted RNAs from wastewater samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.176365.

References

Ahmed, W., Simpson, S.L., Bertsch, P.M., Bibby, K., Bivins, A., Blackall, L.L., Bofill-Mas, S., Bosch, A., Brandao, J., Choi, P.M., Ciesielski, M., Donner, E., D'Souza, N., Farnleitner, A.H., Gerrity, D., Gonzalez, R., Griffith, J.F., Gyawali, P., Haas, C.N., Hamilton, K.A., Hapuarachchi, H.C., Harwood, V.J., Haque, R., Jackson, G., Khan, S. J., Khan, W., Kitajima, M., Korajkic, A., La Rosa, G., Layton, B.A., Lipp, E., McLellan, S.L., McMinn, B., Medema, G., Metcalfe, S., Meijer, W.G., Mueller, J.F., Murphy, H., Naughton, C.C., Noble, R.T., Payyappat, S., Petterson, S., Pitkanen, T., Rajal, V.B., Reyneke, B., Roman Jr., F.A., Rose, J.B., Rusinol, M., Sadowsky, M.J., Sala-Comorera, L., Setoh, Y.X., Sherchan, S.P., Sirikanchana, K., Smith, W., Steele, J. A., Sabburg, R., Symonds, E.M., Thai, P., Thomas, K.V., Tynan, J., Toze, S., Thompson, J., Whiteley, A.S., Wong, J.C.C., Sano, D., Wuertz, S., Xagoraraki, I., Zhang, Q., Zimmer-Faust, A.G., Shanks, O.C., 2022. Minimizing errors in RT-PCR detection and quantification of SARS-CoV-2 RNA for wastewater surveillance. Sci. Total Environ. 805, 149877.

Akande, O.W., Carter, L.L., Abubakar, A., Achilla, R., Barakat, A., Gumede, N., Guseinova, A., Inbanathan, F.Y., Kato, M., Koua, E., Leite, J., Marklewitz, M., Mendez-Rico, J., Monamele, C., Musul, B., Nahapetyan, K., Naidoo, D., Ochola, R., Ozel, M., Raftery, P., Vicari, A., Wijesinghe, P.R., Zwetyenga, J., Safreed-Harmon, K., Barnadas, C., Mulders, M., Pereyaslov, D.I., Sacks, J.A., Warren, T., Cognat, S., Briand, S., Samaan, G., 2023. Strengthening pathogen genomic surveillance for health emergencies: insights from the World Health Organization's regional initiatives. Front. Public Health 11, 1146730.

Aksamentov, I., Roemer, C., Hodcroft, E.B., Neher, R.A., 2021. Nextclade: clade assignment, mutation calling and quality control for viral genomes. Journal of Open Source Software 6 (67), 3773. https://doi.org/10.21105/joss.03773.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Ashwaq, O., Manickavasagam, P., Haque, S.M., 2021. V483A: an emerging mutation hotspot of SARS-CoV-2. Future Virol. https://doi.org/10.2217/fvl-2020-0384.

Bartel, A., Grau, J.H., Bitzegeio, J., Werber, D., Linzner, N., Schumacher, V., Garske, S., Liere, K., Hackenbeck, T., Rupp, S.I., Sagebiel, D., Bockelmann, U., Meixner, M., 2024. Timely monitoring of SARS-CoV-2 RNA fragments in wastewater shows the emergence of JN.1 (BA.2.86.1.1, Clade 23I) in Berlin, Germany. Viruses 16, 1.

Centers for Disease Control and Prevention, 2020a. Cdc 2019-novel coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel: instructions for use. Available online: https://www.fda.gov/media/134922/download.

Centers for Disease Control and Prevention, 2020b. Coronavirus research use only 2019-novel coronavirus (2019-nCoV) real-time RT-PCR primers and probes. Available online: https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html.

Centers for Disease Control and Prevention, 2023. Update on SARS-CoV-2 variant BA.2.86 being tracked by CDC November 27, 2023. https://www.cdc.gov/respiratory-viruses/whats-new/covid-19-variant-update-2023-11-27.html.

Chen, C., Nadeau, S., Yared, M., Voinov, P., Xie, N., Roemer, C., Stadler, T., 2021a. CoV-Spectrum: analysis of globally shared SARS-CoV-2 data to identify and characterize new variants. Bioinformatics 38 (6), 1735–1737.

Chen, A.T., Altschuler, K., Zhan, S.H., Chan, Y.A., Deverman, B.E., 2021b. COVID-19 CG enables SARS-CoV-2 mutation and lineage tracking by locations and dates of interest. Elife 10.

Chinese Center for Disease Control and Prevention, 2020. Technical guidelines for COVID-19 laboratory testing. China CDC Wkly. 2, 332–336.

Chu, D.K.W., Pan, Y., Cheng, S.M.S., Hui, K.P.Y., Krishnan, P., Liu, Y., Ng, D.Y.M., Wan, C.K.C., Yang, P., Wang, Q., Peiris, M., Poon, L.L.M., 2020. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. Clin. Chem. 66 (4), 549–555.

Corbisier, P., Petrillo, M., Marchini, A., Querci, M., Buttinger, G., Bekliz, M., Spiess, K., Polacek, C., Fomsgaard, A., Van den Eede, G., 2022. A qualitative RT-PCR assay for the specific identification of the SARS-CoV-2 B.1.1.529 (omicron) variant of concern. J. Clin. Virol. 152, 105191.

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brunink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 25 (3).

Department of Medical Sciences, Ministry of Public Health. Diagnostic detection of novel coronavirus 2019 by real time RT-PCR. Available online. https://www.who.int/docs/default-source/coronaviruse/conventional-rt-pcr-followed-by-sequencing-for-detection-of-ncov-rirl-nat-inst-health-t.pdf?sfvrsn=42271c6d_4.

Elbe, S., Buckland-Merrett, G., 2017. Data, disease and diplomacy: GISAID's innovative contribution to global health. Global Chall. 1 (1), 33–46.

Espinosa-Gongora, C., Berg, C., Rehn, M., Varg, J.E., Dillner, L., Latorre-Margalef, N., Szekely, A.J., Andersson, E., Movert, E., 2023. Early detection of the emerging SARS-CoV-2 BA.2.86 lineage through integrated genomic surveillance of wastewater and COVID-19 cases in Sweden, weeks 31 to 38 2023. Euro Surveill. 28, 46.

Frydman, C., Miño, S., Iglesias, N.G., Carballeda, J.M., Simari, M., Pisano, M.B., Dus Santos, M.J., Mozgovoj, M., 2024. Wastewater surveillance of enteric viruses in eastern Argentina: high rates of detection and first report of NoV GI.5 and GII.20. Environmental. Advances 15, 100501.

Gans, J.D., Wolinsky, M., 2008. Improved assay-dependent searching of nucleic acid sequence databases. Nucleic Acids Res. 36 (12), e74.

Goble, C., Soiland-Reyes, S., Bacall, F., Owen, S., Pireddu, L., Leo, S., 2023. EOSC-Life Implementation of a Mechanism for Publishing and Sharing Workflows Across Instances of the Environment. Zenodo. https://doi.org/10.5281/zenodo.7886545.

Graber, T.E., Mercier, E., Bhatnagar, K., Fuzzen, M., D'Aoust, P.M., Hoang, H.D., Tian, X., Towhid, S.T., Plaza-Diaz, J., Eid, W., Alain, T., Butler, A., Goodridge, L., Servos, M., Delatolla, R., 2021. Near real-time determination of B.1.1.7 in proportion to total SARS-CoV-2 viral load in wastewater using an allele-specific primer extension PCR strategy. Water Res. 205, 117681.

Heijnen, L., Elsinga, G., de Graaf, M., Molenkamp, R., Koopmans, M.P.G., Medema, G., 2021. Droplet digital RT-PCR to detect SARS-CoV-2 signature mutations of variants of concern in wastewater. Sci. Total Environ. 799, 149456.

Hou, Y., Chen, S., Zheng, Y., Zheng, X., Lin, J.-M., 2023. Droplet-based digital PCR (ddPCR) and its applications. TrAC Trends Anal. Chem. 158, 116897.

Iaconelli, M., Valdazo-González, B., Equestre, M., Ciccaglione, A.R., Marcantonio, C., Della Libera, S., La Rosa, G., 2017. Molecular characterization of human adenoviruses in urban wastewaters using next generation and Sanger sequencing. Water Res. 121, 240–247.

Institut Pasteur, Paris. Protocol: real-time RT-PCR assays for the detection of SARS-CoV-2. Available online: https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2.

Kaku, Y.; Okumura, K.; Padilla-Blanco, M.; Kosugi, Y.; Uriu, K.; Hinay, A. A., Jr.; Chen, L.; Plianchaisuk, A.; Kobiyama, K.; Ishii, K. J.; Genotype to Phenotype Japan, C.; Zahradnik, J.; Ito, J.; Sato, K., Virological characteristics of the SARS-CoV-2 JN.1 variant. Lancet Infect. Dis. 2024, 24, (2), e82.

Kaku, Y., Uriu, K., Kosugi, Y., Okumura, K., Yamasoba, D., Uwamino, Y., Kuramochi, J., Sadamasu, K., Yoshimura, K., Asakura, H., Nagashima, M., , Genotype to Phenotype Japan (G2P-Japan) Consortium, Ito, J., Sato, K., 2024b. Virological characteristics of the SARS-CoV-2 KP.2 variant. Lancet Infect. Dis. 24 (7), e416.

Katoh, K., Rozewicki, J., Yamada, K.D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. 20 (4), 1160–1166.

Kaya, D., Falender, R., Radniecki, T., Geniza, M., Cieslak, P., Kelly, C., Lininger, N., Sutton, M., 2022. Correlation between clinical and wastewater SARS-CoV-2 genomic surveillance, Oregon, USA. Emerg. Infect. Dis. 28 (9), 1906–1908.

Kuypers, J., Jerome, K.R., 2017. Applications of digital PCR for clinical microbiology. J. Clin. Microbiol. 55 (6), 1621–1628.

La Rosa, G., Bonadonna, L., Suffredini, E., 2021. Protocollo della Sorveglianza di SARS-CoV-2 in reflui urbani (SARI) - rev. 3 (Rev. 3). Zenodo. https://doi.org/10.5281/zenodo.5758725.

La Rosa, G.; Iaconelli, M.; Veneri, C.; Mancini, P.; Bonanno Ferraro, G.; Brandtner, D.; Lucentini, L.; Bonadonna, L.; Rossi, M.; Grigioni, M.; network, S.; Suffredini, E., The rapid spread of SARS-COV-2 Omicron variant in Italy reflected early through wastewater surveillance. Sci. Total Environ. 2022, 837, 155767.

Lamba, S., Ganesan, S., Daroch, N., Paul, K., Joshi, S.G., Sreenivas, D., Nataraj, A., Srikantaiah, V., Mishra, R., Ramakrishnan, U., Ishtiaq, F., 2023. SARS-CoV-2 infection dynamics and genomic surveillance to detect variants in wastewater - a longitudinal study in Bengaluru. India. Lancet Reg Health Southeast Asia 11, 100151.

Lasrado, N.; Collier, A.-r. Y.; Hachmann, N. P.; Miller, J.; Rowe, M.; Schonberg, E. D.; Rodrigues, S. L.; LaPiana, A.; Patio, R. C.; Anand, T.; Fisher, J.; Mazurek, C. R.; Guan,

R.; Wagh, K.; Theiler, J.; Korber, B. T.; Barouch, D. H., Neutralization escape by SARS-CoV-2 Omicron subvariant BA.2.86. Vaccine 2023, 41, (47), 6904–6909.
Li, J., Cheng, R., Bian, Z., Niu, J., Xia, J., Mao, G., Liu, H., Wu, C., Hao, C., 2024. Development of multiplex allele-specific RT-qPCR assays for differentiation of SARS-CoV-2 Omicron subvariants. Appl. Microbiol. Biotechnol. 108 (1), 35.
Lipponen, A., Kolehmainen, A., Oikarinen, S., Hokajarvi, A.M., Lehto, K.M., Heikinheimo, A., Halkilahti, J., Juutinen, A., Luomala, O., Smura, T., Liitsola, K., Blomqvist, S., Savolainen-Kopra, C., Pitkanen, T., WastPan Study, G, 2024. Detection of SARS-COV-2 variants and their proportions in wastewater samples using next-generation sequencing in Finland. Sci. Rep. 14 (1), 7751.
Marchini, A., Petrillo, M., Parrish, A., Buttinger, G., Tavazzi, S., Querci, M., Betsou, F., Elsinga, G., Medema, G., Abdelrahman, T., Gawlik, B., Corbisier, P., 2023. New RT-PCR assay for the detection of current and future SARS-CoV-2 variants. Viruses 15, 1.
Neves, A., Cuesta, I., Hjerde, E., Klemetsen, T., Salgado, D., van Helden, J., Rahman, N., Fatima, N., Karathanasis, N., Zmora, P., Akerstrom, W.N., Grellscheid, S.N., Waheed, Z., Blomberg, N., 2023. FAIR+E pathogen data for surveillance and research: lessons from COVID-19. Front. Public Health 11, 1289945.
Omotoso, O.E., Babalola, A.D., Matareek, A., 2021. Mutational hotspots and conserved domains of SARS-CoV-2 genome in African population. Beni Suef Univ J Basic Appl Sci 10 (1), 11.
Pinheiro, L.B., Coleman, V.A., Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal. Chem. 84 (2), 1003–1011.
Quan, P.L., Sauzade, M., Brouzes, E., 2018. dPCR: a technology review. Sensors (Basel) 18, 4.
Rajput, V., Pramanik, R., Malik, V., Yadav, R., Samson, R., Kadam, P., Bhalerao, U., Tupekar, M., Deshpande, D., Shah, P., Shashidhara, L.S., Boargaonkar, R., Patil, D., Kale, S., Bhalerao, A., Jain, N., Kamble, S., Dastager, S., Karmodiya, K., Dharne, M., 2023. Genomic surveillance reveals early detection and transition of delta to omicron lineages of SARS-CoV-2 variants in wastewater treatment plants of Pune. India. Environ Sci Pollut Res Int 30 (56), 118976–118988.
Rambaut, A., Holmes, E.C., O'Toole, A., Hill, V., McCrone, J.T., Ruis, C., du Plessis, L., Pybus, O.G., 2020. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat. Microbiol. 5 (11), 1403–1407.
Rasmussen, M., Møller, F.T., Gunalan, V., Baig, S., Bennedbæk, M., Christiansen, L.E., Cohen, A.S., Ellegaard, K., Fomsgaard, A., Franck, K.T., Larsen, N.B., Larsen, T.G., Lassaunière, R., Polacek, C., Qvesel, A.G., Sieber, R.N., Rasmussen, L.D., Stegger, M., Spiess, K., Tang, M.E., Vestergaard, L.S., Andersen, T.E., Hoegh, S.V., Pedersen, R.M., Skov, M.N., Steinke, K., Sydenham, T.V., Hoppe, M., Nielsen, L., Krause, T.G., Ullum, H., Jokelainen, P., 2023. First cases of SARS-CoV-2 BA.2.86 in Denmark, 2023. Euro Surveill. 28, 36.
Rice, P., Longden, I., Bleasby, A., 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. 16 (6), 276–277.
Robinson JT; Thorvaldsdóttir H; Winckler W; Guttman M; Lander ES; Getz G; JP, M., Integrative genomics viewer. Nat. Biotechnol. 2011, Jan;29(1):24–6.
Schmidt, C., 2020. Watcher in the wastewater. Nat. Biotechnol. 38 (8), 917–920.
Shirato, K., Nao, N., Katano, H., Takayama, I., Saito, S., Kato, F., Katoh, H., Sakata, M., Nakatsu, Y., Mori, Y., Kageyama, T., Matsuyama, S., Takeda, M., 2020. Development of genetic diagnostic methods for detection for novel coronavirus 2019(nCoV-2019) in Japan. Jpn. J. Infect. Dis. 73 (4), 304–307.
Smith, T., Holm, R.H., Yeager, R., Moore, J.B.t., Rouchka, E.C., Sokoloski, K.J., Elliott, E. M., Talley, D., Arora, V., Moyer, S., Bhatnagar, A., 2022. Combining community wastewater genomic surveillance with state clinical surveillance: a framework for SARS-CoV-2 public health practice. Food Environ Virol 14 (4), 410–416.
Spiess, K., Gunalan, V., Marving, E., Nielsen, S.H., Jorgensen, M.G.P., Fomsgaard, A.S., Nielsen, L., Alfaro-Nunez, A., Karst, S.M., Danish, C.-G.C., Mortensen, S., Rasmussen, M., Lassauniere, R., Rosenstierne, M.W., Polacek, C., Fonager, J., Cohen, A.S., Nielsen, C., Fomsgaard, A., 2023. Rapid and flexible RT-qPCR surveillance platforms to detect SARS-CoV-2 mutations. Microbiol Spectr 11 (1), e0359122.
Suffredini, E., Iaconelli, M., Equestre, M., Valdazo-González, B., Ciccaglione, A.R., Marcantonio, C., Della Libera, S., Bignami, F., La Rosa, G., 2018. Genetic diversity among genogroup II noroviruses and progressive emergence of GII.17 in wastewaters in Italy (2011-2016) revealed by next-generation and sanger sequencing. Food Environ Virol 10 (2), 141–150.
Vasudevan, H.N., Xu, P., Servellita, V., Miller, S., Liu, L., Gopez, A., Chiu, C.Y., Abate, A. R., 2021. Digital droplet PCR accurately quantifies SARS-CoV-2 viral load from crude lysate without nucleic acid purification. Sci. Rep. 11 (1), 780.
Veneri, C., Brandtner, D., Mancini, P., Bonanno Ferraro, G., Iaconelli, M., Del Giudice, C., Ciccaglione, A.R., Bruni, R., Equestre, M., Marcantonio, C., Suffredini, E., La Rosa, G., 2024. Detection and full genomic sequencing of rare hepatitis E virus genotype 4d in Italian wastewater, undetected by clinical surveillance. Sci. Total Environ. 913, 169698.
de Visser, C., Johansson, L.F., Kulkarni, P., Mei, H., Neerincx, P., Joeri van der Velde, K., Horvatovich, P., van Gool, A.J., Swertz, M.A., Hoen, P.A.C., Niehues, A., 2023. Ten quick tips for building FAIR workflows. PLoS Comput. Biol. 19 (9), e1011369.
Wannigama, D.L., Amarasiri, M., Phattharapornjaroen, P., Hurst, C., Modchang, C., Chadsuthi, S., Anupong, S., Miyanaga, K., Cui, L., Fernandez, S., Huang, A.T., Ounjai, P., Tacharoenmuang, R., Ragupathi, N.K.D., Sano, D., Furukawa, T., Sei, K., Leelahavanichkul, A., Kanjanabuch, T., Higgins, P.G., Nanbo, A., Kicic, A., Singer, A. C., Chatsuwan, T., Trowsdale, S., Khatib, A., Shibuya, K., Abe, S., Ishikawa, H., Hongsing, P., Pathogen Hunters Research, T, 2023. Tracing the new SARS-CoV-2 variant BA.2.86 in the community through wastewater surveillance in Bangkok, Thailand. Lancet Infect. Dis. 23 (11), e464–e466.
Whale, A.S., von der Heide, E.K., Kohlenberg, M., Brinckmann, A., Baedker, S., Karalay, O., Fernandez-Gonzalez, A., Busby, E.J., Bustin, S.A., Hauser, H., Missel, A., O'Sullivan, D.M., Huggett, J.F., Pfaffl, M.W., Nolan, T., 2022. Digital PCR can augment the interpretation of RT-qPCR Cq values for SARS-CoV-2 diagnostics. Methods 201, 5–14.
World Health Organization, 2022. Global genomic surveillance strategy for pathogens with pandemic and epidemic potential 2022–2032. https://www.who.int/initiatives/genomic-surveillance-strategy.
World Health Organization, 2023a. Weekly Epidemiological Update on COVID-19 - 17 August 2023 (Edition 156).
World Health Organization, 2023b. Updated risk evaluation for EG.5 and its sublineages, 21 November 2023. https://www.who.int/docs/default-source/coronaviruse/21112023_ba.2.86_ire.pdf.
World Health Organization, 2023c. Initial risk evaluation of JN.1, 19 December 2023. https://www.who.int/docs/default-source/coronaviruse/18122023_jn.1_ire_clean.pdf.
Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579 (7798), 265–269.
Yan, M.T., Yang, S.S., Tseng, M.H., Cheng, C.J., Tsai, J.D., Sung, C.C., Hsu, Y.J., Lin, S.H., 2021. Allele-specific RT-PCR for the rapid detection of recurrent SLC12A3 mutations for Gitelman syndrome. NPJ Genom. Med. 6 (1), 68.
Yang, S., Yu, Y., Xu, Y., Jian, F., Song, W., Yisimayi, A., Wang, P., Wang, J., Liu, J., Yu, L., Niu, X., Wang, J., Wang, Y., Shao, F., Jin, R., Wang, Y., Yunlong, 2023. Fast evolution of SARS-CoV-2 BA.2.86 to JN.1 under heavy immune pressure. Lancet Infect 24 (2), e70–e72.
Yousif, M., Rachida, S., Taukobong, S., Ndlovu, N., Iwu-Jaja, C., Howard, W., Moonsamy, S., Mhlambi, N., Gwala, S., Levy, J.I., Andersen, K.G., Scheepers, C., von Gottberg, A., Wolter, N., Bhiman, J.N., Amoako, D.G., Ismail, A., Suchard, M., McCarthy, K., 2023. SARS-CoV-2 genomic surveillance in wastewater as a model for monitoring evolution of endemic viruses. Nat. Commun. 14 (1), 6325.