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Comparison of gene targets and sampling regimes for SARS-CoV-2 quantification for wastewater epidemiology in UK prisons

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ABSTRACT

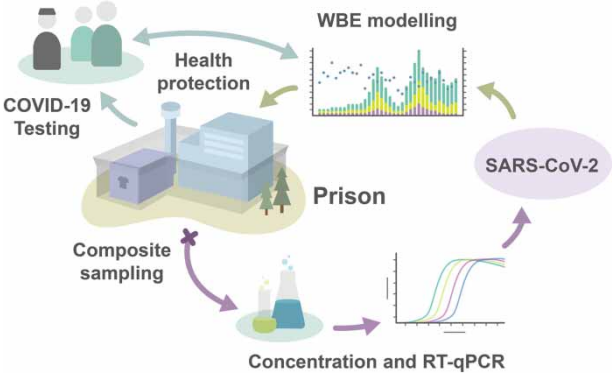
Prisons are high-risk settings for infectious disease transmission, due to their enclosed and semi-enclosed environments. The proximity between prisoners and staff, and the diversity of prisons reduces the effectiveness of non-pharmaceutical interventions, such as social distancing. Therefore, alternative health monitoring methods, such as wastewater-based epidemiology (WBE), are needed to track pathogens, including SARS-CoV-2. This pilot study assessed WBE to quantify SARS-CoV-2 prevalence in prison wastewater to determine its utility within a health protection system for residents. The study analysed 266 samples from six prisons in England over a 12-week period for nucleoprotein 1 (*N*1 gene) and envelope protein (*E* gene) using quantitative reverse transcriptase-polymerase chain reaction. Both gene assays successfully detected SARS-CoV-2 fragments in wastewater samples, with both genes significantly correlating with COVID-19 case numbers across the prisons ($p < 0.01$). However, in 25% of the SARS-positive samples, only one gene target was detected, suggesting that both genes be used to reduce false-negative results. No significant differences were observed between 14- and 2-h composite samples, although 2-h samples showed greater signal variance. Population normalisation did not improve correlations between the *N*1 and E genes and COVID-19 case data. Overall, WBE shows considerable promise for health protection in prison settings.

Key words: COVID-19, health monitoring, prisons, RT-qPCR, SARS-CoV-2, wastewater-based epidemiology

HIGHLIGHTS

- Wastewater epidemiology is effective in flagging COVID-19 cases in prison settings.
- SARS-CoV-2 levels were quantified using *N*1 and *E* gene targets.
- Both targets should be used in routine analysis to reduce false negatives.
- COVID-19 case numbers significantly correlated with both wastewater gene targets across all samples.
- Each prison is different – local activities are important when designing sampling programmes.

GRAPHICAL ABSTRACT



INTRODUCTION

COVID-19 and its global pandemic have been a public health emergency of international concern since 2020. The rate of transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19, is dependent upon the specific variant but is generally driven by the physical proximity of individuals and relative ventilation (Jones *et al.* 2020). Therefore, many countries implemented strategies that reduce opportunities for transmission, including social distancing, travel bans, community lockdowns, remote working, school closures, and mass testing. However, the implementation of such strategies was more limited in enclosed or semi-enclosed settings, which are often restricted by environmental and other factors. As a result, such locations are more prone to infectious disease outbreaks (Finnie *et al.* 2012; Bays *et al.* 2021). Examples of semi-enclosed settings are care homes, schools, cruise ships, and herein, prisons.

However, prisons should be considered distinct from most semi-enclosed settings. Unlike schools and care homes, prisons are designed to be *total* institutions (Goffman 1957), where staff and users often spend more than 95% of a typical day indoors (O'Mara n.d.). Due to their social design, the relative utility of non-pharmaceutical interventions (NPIs) to reduce disease spread can be limited. Environmental factors, such as elevated human population densities and inadequate ventilation (SAGE 2021), and personal factors, such as deprivation of liberty, illiteracy, and compliance, often limit the value and utility of diagnostic testing in reducing transmission (O'Moore 2020; Lambert & Wilkinson 2021; SAGE 2021). In particular, the regular movement and contact between new, transferred, and released prisoners, staff, social and legal visitors, and their diverse health profiles make prisons a uniquely challenging environment to monitor infectious diseases. As medical anthropologist, Farmer (2004) wrote, 'better habitats for epidemics of airborne disease could hardly be found' (p. 121). Therefore, rapid outbreaks are common due to their contained design and limited forewarning. As such, prison settings are distinct from the wider community, often distinct from each other, and require innovative methods of identifying and responding to the presence of infectious diseases (SAGE 2021).

During the initial waves of the pandemic, the UK Government implemented NPIs in prisons across England and Wales, which variably included symptomatic testing, the restriction of prisoners to their cells, mask mandates, and social distancing (SAGE 2021). However, such measures were not wholly successful in reducing the scale and duration of COVD-19 outbreaks in prisons (SAGE 2021). In the first wave, 7.6 per 100,000 prisoners contracted COVID-19 compared to 4.9 per 100,000 in the general population (Ministry of Justice 2021). Over the 12 months to February 2021, prisoners were more likely to become infected, be hospitalised, and die from COVID-19 than their counterparts in the wider community (Braithwaite *et al.* 2021; Hassard *et al.* 2022).

Infection in prisoners was associated with more severe clinical presentations when compared to patients from the community, such as higher rates of intensive care admissions (Altibi *et al.* 2021) and mortality (Braithwaite *et al.* 2021). As of February 2023, official statistics indicate that 215 prisoners died from COVID-19-related causes since the onset of the pandemic, and nearly 49,000 prisoners tested positive across all establishments (Ministry of Justice 2023). These data suggest that prisons need alternative approaches for health surveillance, including earlier warning systems and infection control to protect the health of prisoners.

Wastewater-based epidemiology (WBE) has been used as a public health surveillance tool since it was first promoted in 2001 (Daughton 2001), including monitoring bacterial (Yan *et al.* 2018) and viral (Klapsa *et al.* 2022; Tedcastle *et al.* 2022) disease outbreaks, and illicit drug use (Zuccato *et al.* 2005). Following the onset of the COVID-19 pandemic, WBE was successfully implemented to detect SARS-CoV-2 in wastewater in numerous countries worldwide, including the UK (Morvan *et al.* 2022). Many of these programmes monitor wastewater at a community level and have shown that WBE can be successful for larger human population groups, such as monitoring at community wastewater treatment plants (WWTPs). However, as prison populations are generally smaller than WWTP catchments with lower volumetric flowrates, SARS-CoV-2 signals and chemical and biological markers are much more variable over time due to less mass-averaging (Gerrity *et al.* 2022; Wade *et al.* 2022). This study aimed to investigate how wastewater sampling and analysis methods need to be adapted to 'near-source' prison settings, determine whether SARS-CoV-2 RNA can be detected and quantified in prison wastewater, and how WBE might enhance other approaches in an integrated health protection system.

MATERIALS AND METHODS

Sample collection

Six prisons in England (referred to as Prison 1–6) were selected for sampling in a single geographic region. Based on site surveys, untreated wastewater from a single sewer line that exited each site was collected 4 days per week for a 12-week period (Monday to Thursday from January to April 2021). Overnight 14-h composite samples were collected using an autosampler (every 15 min) from 19:00 to 09:00. If a successful composite sample was not obtained, a single grab sample was collected instead. Additional samples were collected at Prison 5 to determine whether 2-h samples, collected at a higher frequency (i.e., 07:00 to 09:00 every 5 min), might provide more representative samples by obtaining the morning 'first flush'. Samples were collected in sterile 1 L bottles and transported on ice to the laboratory, where they were stored at 4 °C and processed within 4 h.

Wastewater parameters, including pH, electrical conductivity (EC), and absorbance at 600 nm, were measured *in situ* using a multiparameter probe (HQ30D – Hach, USA) and a spectrophotometer (DR3900 – Hach, USA). Total suspended solids (TSS) and volatile suspended solids were determined according to Standard methods 2540D and 2540E (Rice *et al.* 2012) by filtering a defined water volume through a membrane Whatman GF/F glass-fibre filter. Ammonia concentrations were measured using a kit (LCK 303 – Hach, USA) following manufacturer's instructions.

Sample concentration

The virus was concentrated from wastewater samples in duplicate (2 × 100 ml) using a polyethylene glycol (PEG) precipitation method. Briefly, to each 100 ml sample, 10^7 PFU of the bacteriophage ϕ6 (Phi6) was added as a process control and incubated at room temperature with agitation for 5 min at 250 rpm. Large particles were removed by centrifugation at 3,984 × g for 30 min at 4 °C. To the supernatant 10% (w/v) molecular grade PEG 8000 and 2% (w/v) sodium chloride was added, and samples were mixed on a rotary mixer for 15 min at 40 rpm at room temperature. Samples were then centrifuged for 90 min at 12,000 × g at 4 °C with a low brake. The supernatant was discarded, and samples were centrifuged for a further 5 min at 12,000 × g at 4 °C with a low brake. The supernatant was discarded.

Viral nucleic acid extraction

The concentrated pellet was resuspended in 800 µl of TRIzol™ Reagent (Invitrogen, USA), vortexed for 15 s at 2,500 rpm, and then transferred to a microcentrifuge tube. 200 µl of chloroform was added, and the samples were thoroughly mixed by shaking and incubated for 3 min at room temperature.

Samples were transferred to a Qiagen MaXtract High Density gel tube (Qiagen, Germany) and centrifuged at 12,000 × g at 4 °C for 15 min. The upper liquid phase, which contained the semi-purified RNA, was transferred to a new tube containing 600 µl of ethanol (96–100%). This mixture was briefly vortexed and then purified using the NucleoSpin RNA Virus kit (Macherey Nagel, Germany), without the lysis step, following manufacturer's instructions. RNA purity was verified using a DS-11 spectrophotometer/fluorometer (DeNovix Inc., USA) and stored at −80 °C.

Detection and quantification of SARS-CoV-2 RNA using quantitative reverse transcriptase-polymerase chain reaction

SARS-CoV-2 and Phi6 were quantified using a one-step quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) using the BioRad CFX96 System (BioRad, USA). All primers and probes used in this study are shown in Table 1.

Table 1 | Primer–probe sets used in this study for RT-qPCR assays

Target	Primer	Sequence (5′–3′)	Amplicon size (bp)	Annealing (°C)	Reference
N1	F	GACCCCAAAATCAGCGAAAT	73	57	CDC (2020)
	R	TCTGGTTACTGCCAGTTGAATCTG			
	Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1			
E	F	ACAGGTACGTTAATAGTTAATAGCGT	113	60	Medema *et al.* (2020)
	R	ATATTGCAGCAGTACGCACACA			
	Probe	TxRed-ACACTAGCCATCCTTACTGCGCTTCG-BHQ2			
gfp	F	TCGGTTATGGTGTTCAATGC	147	57	This study
	R	GACTTCAGCACGTGTCTTGTAG			
	Probe	Cyanine55-ATAACCTTCAGGCATGGCACTCT-BHQ3			
phi6	F	TGGCGGCGGTCAAGAGC	100	60	Gendron *et al.* (2010)
	R	GGATGATTCTCCAGAAGCTGCTG			
	Probe	TxRed-CGGTCGTCGCAGGTCTGACACTCGC-BHQ2			

No template controls, in which the template RNA was replaced with sterile water, were carried out in duplicate on all RT-qPCR plates to ensure no contamination or non-specific amplification was present. To determine potential inhibition, an additional reaction was performed for each sample by adding 10^3 gene copies of synthetic single-stranded RNA of the green fluorescent protein gene (*gfp*). Inhibition was considered minimal when the difference in the *gfp* Ct value (ΔCt) was less than 1 Ct between the spiked sample and the positive control.

For SARS-CoV-2 nucleoprotein 1 (*N1* gene) and the envelope protein (*E* gene) (Corman *et al.* 2020), amplification reaction mixtures (final volume 20 µl) contained 4 µl of template RNA, 1 µl of gfp (10^3), 10 µl of 2 × SsoAdvanced™ Universal Probes Supermix (BioRad, USA), 400 nM of forward and reverse primer for *N1* and *gfp*, and 200 nM of probe. For SARS-CoV-2 *E* gene, amplification reaction mixtures (final volume 20 µl) contained 4 µl of template RNA, 10 µl of 2 × SsoAdvanced™ Universal Probes Supermix (BioRad, USA), 400 nM of forward and reverse primer for Phi6 and *E*, and 200 nM of probe. The thermal cycling protocol was as follows: 10 min at 50 °C for cDNA synthesis, 3 min of initial denaturation at 95 °C, followed by 45 cycles of 5 s denaturation at 95 °C and 30 s annealing/extension (temperatures are shown in Table 1).

Standard curves for each set of primers were constructed using plasmid clones of the target sequences, which were loaded in triplicate for each qPCR run. The amplification of test samples was run in parallel. 'Neat' and 1:10 dilutions of each sample were always measured using the optimum qPCR conditions that are determined for each primer set. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using 2-fold dilution series covering a range of 1–500 gene copies (gc) from each standard and analysed eight times (R script available at https://github.com/cmerkes/qPCR_LOD_Calc).

The LOD was defined as the lowest concentration that can be reliably detected, i.e., where at least 95% of standard reactions were expected to amplify. The LOQ was defined as the lowest standard that can be quantified with a coefficient of variation less than 0.35 (Klymus *et al.* 2020). Recovery efficiency was determined by calculating the total gene copy number of the bacteriophage Phi6 in each sample after processing as a percentage of the quantity of Phi6 spiked into the sample before processing.

RESULT AND DISCUSSION

Sampling strategies

A total of 266 samples were collected from Prisons 1 to 6 during the 12-week sampling period. Population sizes ranged from 200 to 1,300 prisoners (Supplementary Information (SI) Table S1), with Prison 4 having the oldest population (15%, >60 years of age) and Prison 6 the youngest (100%, <50 years of age). Overnight 14-h composite samples were collected at all prisons because fewer staff and no visitors were onsite during this period, thus reducing non-prisoner sewage contributions to the collected samples. Overnight sampling also was chosen to reduce non-faecal contributions to the wastewater that could affect the recovery of the virus, such as surfactants and other chemicals from daily cleaning or laundry activities. Many cleaning, laundry, and personal products include surfactants that can negatively impact the recovery of SARS-CoV-2 and other virus from wastewater (Kevill *et al.* 2022; Robinson *et al.* 2022). In addition to overnight sampling, 2-h sampling was also used at Prison 5 to assess how a shorter window might influence *N1* and *E* gc/L signals.

Although overnight 14-h composite sampling was used across prisons, Figure 1 shows that 2-h morning sampling at Prison 5 (designated P5-A and P5-B, respectively) provided comparable SARS-CoV-2 data for both gene targets. The signal variance was slightly greater for the shorter sampling window, but this comparison suggests that short sampling windows are potentially feasible for monitoring.

We had been concerned that morning activities, such as intense laundry use, would inhibit or skew SARS-CoV-2 signal detection, but this was not the case at Prison 5. However, it should be noted that different prisons are managed and operated very differently; therefore, local operating and management factors should be considered to identify the 'optimal' sampling regime for any given facility.

Of the 266 sampling events, there were 10 composite samples that could not be sampled due to either autosampler failure or 'ragging', i.e., the blockage of the sampling tubing with larger obstructive items such as bed sheets. In these instances, grab samples were collected to maintain the data series for prison populations. The sample size of grab samples was insufficient for comparative statistics, but it was observed that when a grab and composite sample was collected from the same prison within a 24-h period (e.g., a morning grab sample followed by an overnight composite sample beginning at 7 pm in the evening), SARS-CoV-2 concentrations detected in the grab samples were lower compared to the parallel composite samples. In some cases, SARS-CoV-2 gene targets were not detectable in the grab samples but were detected in composites. This emphasises the importance of performing composite sampling in near-source settings, such as prisons, because they reduce the likelihood of false-negative results.

All prison sites were monitored 4 days per week in this study, but as a matter of curiosity, we sub-sampled the four-sampling-per-week data to just two samplings per week (Monday and Thursday only) to determine if less frequent sampling might still provide data of strategic value. A significant positive correlation between the 2-day and 4-day means was observed for both *N1* ($r^2 = 0.91$, $p < 0.01$) and *E* ($r^2 = 0.96$, $p < 0.01$) gene signals, showing that sampling twice per week produces similar trends in gene data (SI Figure S1). An additional bootstrapping analysis was run using the 2- and 4-day means, and

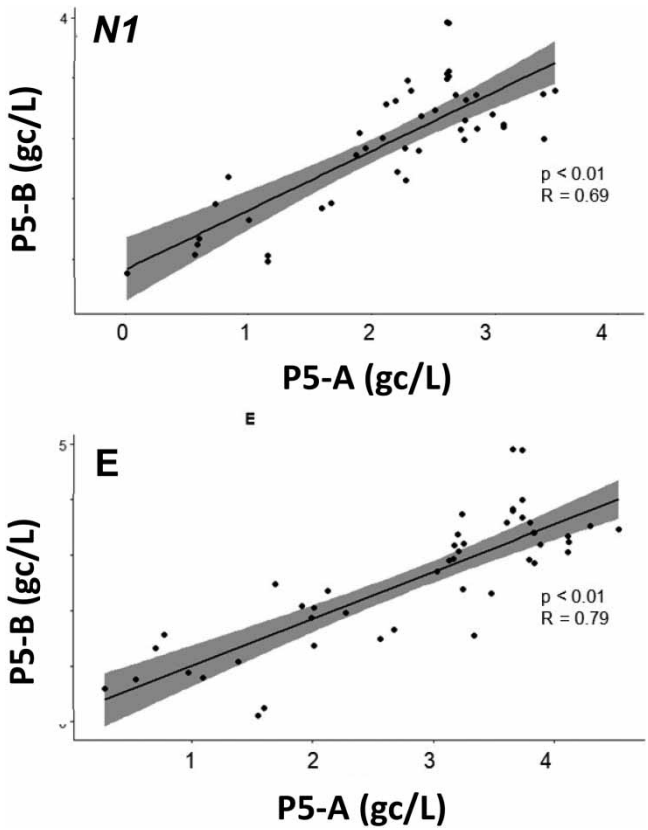


Figure 1 | Comparison of *E* gene and *N1* SARS-CoV-2 wastewater data, collected at Prison 5 in the two different sampling windows, P5-A (14-h, overnight composite, 19:00–09:00) and P5-B (2-h, 07:00–09:00).

although there was some skew within the data, the information provided from sampling on 2 and 4 days was comparable and statistically the same ($p < 0.01$). This suggests that twice-weekly overnight sampling is able to provide significant data on overall signal prevalence, but we suspect less frequent sampling would impact the ability of WBE to be used as an early warning system for SARS-CoV-2 cases, resulting in a less immediate response to potential outbreaks.

Variability of wastewater characteristics

In addition to SARS-CoV-2 quantification, wastewater physicochemical characteristics were measured, including pH, EC, TSS, and ammonia (SI Figure S1), to assess how wastewater chemistry differed among prisons and whether any anomalies in prison wastewater exist that might impact SARS-CoV-2 quantification in the analysis. As examples, higher concentrations of SARS-CoV-2 were associated with wastewaters with pH between 7.1 and 7.4 (Amoah *et al.* 2022), whereas virus detection was reduced under more alkaline pHs (Varbanov *et al.* 2021). Furthermore, wastewater biosolid levels can influence virus recovery, including SARS-CoV-2, with as much as 23% of total SARS-CoV-2 RNA being lost via adsorption to solids during processing (Forés *et al.* 2021). Given that physicochemical parameters were quite highly variable within and across prisons, such factors might be especially important here.

The mean wastewater pH, EC, TSS, and ammonia levels (ranges in brackets) across the prisons were 7.74 (6.0–10.25), 859 μS/cm (398–1,888 μS/cm), 266 mg/L (8–10,388 mg/L), and 48.7 mg/L (11.2–118 mg/L), respectively (see SI Figure S1). These means are not too atypical of community wastewater (Henze *et al.* 2002), although the ranges are very large, exemplifying how variable wastewater can be from near-source settings compared with sampling a WWTP. It is noteworthy, however, the elevated mean and wide range in measured ammonia levels because ammonia has previously been used for population normalisation in WBE (Been *et al.* 2014), including for SARS-CoV-2 (Cluzel *et al.* 2022; Wade *et al.* 2022).

Here, measured ammonia levels ranged from 11.2 to 118 mg/L, and each prison was different (see SI Figure S2). 'Typical' community wastewater ammonia levels are between 30 and 40 mg/L (Henze *et al.* 2002), which were observed at five of the six prisons. However, the mean wastewater ammonia level in Prison 5 was over 90 mg/L and the mean pH was 8.55, which are both high, suggesting very different activities and management practices that affect wastewater were occurring at that prison. Given the statistical consistency of the SARS-CoV-2 data from across prisons tested (see later), such wastewater variability is not impacting the overall value of WBE for prison monitoring, but it does suggest that a parameter like ammonia is unsuitable for prison wastewater normalisation (see later). More specific faecal biomarkers, such as Pepper mild mottle virus (PMMoV) and crAssphage (Bivins *et al.* 2020; Wu *et al.* 2020; Holm *et al.* 2022), might be more suitable for population normalisation in prison WBE.

Detection of SARS-CoV-2 using the *N1* and *E* gene targets

Concentrations of SARS-CoV-2 were quantified in all wastewater samples collected using 14-h overnight sampling over the duration of the project for both *N1* and *E* genes. An additional RT-qPCR was performed for all samples using a spiked addition of gfp RNA as a PCR template to determine if there were any inhibitors present that might impact the SARS-CoV-2 concentrations detected, potentially leading to false-negative results, possibly resulting from variable wastewater characteristics. For all 266 samples, the mean Ct difference between the sample and control was 0.18 Ct, with a standard deviation of 0.20 Ct, indicating that the method used to process and extract samples resulted in minimal inhibitors present in the RNA. Furthermore, all SARS-CoV-2-positive samples were tested using a Phi6 process control, although recovery percentages were variable (<0.1–7%), which can result from the instability of the bacteriophage marker in wastewater matrices. Previous studies also noted low Phi6 recovery (Alamin *et al.* 2022; Farkas *et al.* 2023), suggesting that it may not be an appropriate control for WBE and alternative controls should be considered.

Daily SARS-CoV-2 concentrations, expressed as $\log_{10}$ of gc/L, are summarised for samples from Prison 1 to 6 in Figure 2. In the absence of a successful composite sample, grab sample data are included. As evidenced by both *N1* and *E* data, SARS-CoV-2 levels in prison wastewater samples were elevated early in our sampling programme. As sampling proceeded, wastewater SARS-CoV-2 levels declined to the point where most samples tested negative, and positives were incidental across the prisons. This decline in SARS-CoV-2 signals was seen across the UK in wider monitoring (Wade *et al.* 2022).

However, three prisons, 1, 3, and 5 experienced 'outbreaks' of COVID-19 close to when our sampling began in January 2021. During these periods, the concentrations of both targets increased in all three prisons, reaching a peak of 3 or 4 weeks into our study, although the signals subsequently declined (see SI Figure S3 for typical patterns). Early in the outbreak, SARS-CoV-2 concentrations increased quickly, suggesting increasing viral load and infections in the local prison populations,

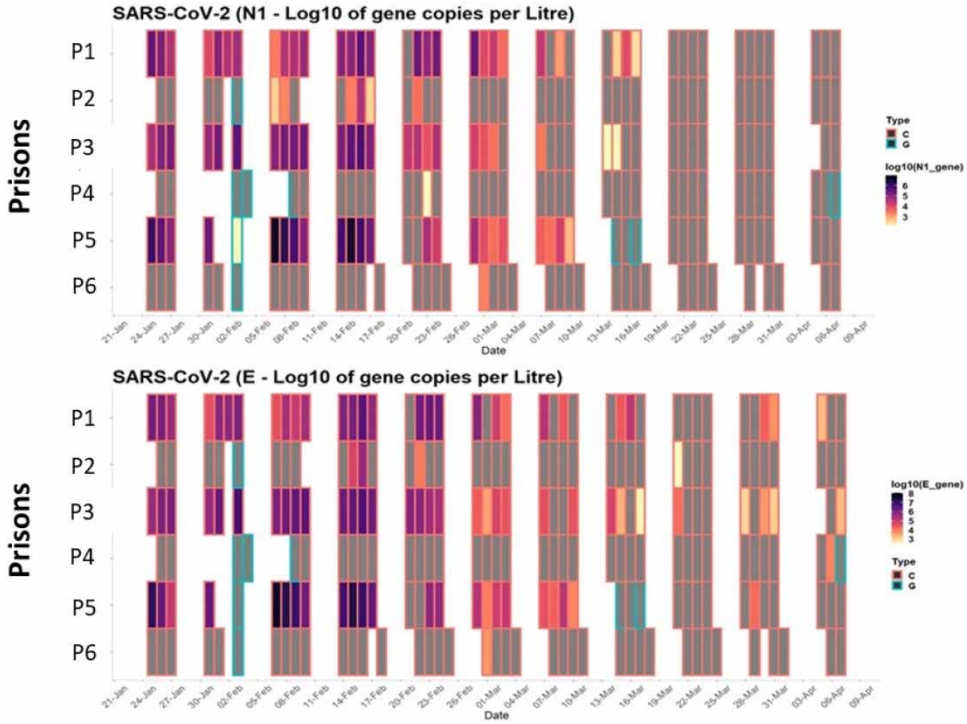


Figure 2 | Heatmap of SARS-CoV-2 concentrations using *N1* (upper) and *E* gene (lower) targets across the six prisons for the duration of the study. Composite samples (outlined in orange, labelled as 'C') were primarily collected. In the absence of composite samples, grab samples (outlined in blue, labelled as 'G') were taken and included.

but they then declined quickly, possibly due to the introduction of public health interventions, such as COVID-19 case isolation, increased mask-wearing, or reduced susceptibility in the target population. SARS-CoV-2 signals were only detected in sporadic samples in the remaining three prisons (Prisons 2, 4 and 6), suggesting a low local incidence of COVID-19 during the sampling period, i.e., no outbreaks were apparent.

Comparison and assessment of the *N1* and *E* gene targets for prison applications

A comparison of the SARS-CoV-2 quantification data for all samples highlights the importance of analysing more than one target to avoid false-negative results. About 75% of the samples in which SARS-CoV-2 was detected tested positive for both *N1* and *E* genes, but in 25% of the positive samples, the signal was only detected for one target gene, with a negative result being returned for the second target (Figure 3). Contradictory data generally occurred in samples that contained lower concentrations of SARS-CoV-2, levels close to the LOD of the assay. Specifically, for samples that had positives for both genetic targets, the mean detected *E* gene concentration was 3.6×10^6 gc/L, whereas in samples where only the *E* gene was detected, the mean *E* gene concentration was 8.4×10^3 gc/L.

Due to the potential for the rapid spread of infection in prisons (SAGE 2021), it is important that even at low concentrations, the virus is detected to act as an early warning surveillance system for outbreaks, allowing the prison facility to implement public health measures to reduce the transmission and spread. Therefore, it is recommended that a minimum of two SARS-CoV-2 genetic targets be measured in near-source WBE in prisons to reduce the probability of false negatives.

Comparison of wastewater *N1* and *E* gene signals and COVID-19 case data

At the time of this study, no mass testing was being performed at the six prisons sampled in this project. Testing was being performed on symptomatic users when symptoms were apparent (and their close contacts) and there was voluntary staff testing. Due to this testing policy, many positive COVID-19 cases will not have been detected, particularly asymptomatic cases, through the point of care testing. The lack of mass testing data limited our ability to assess population normalisation options

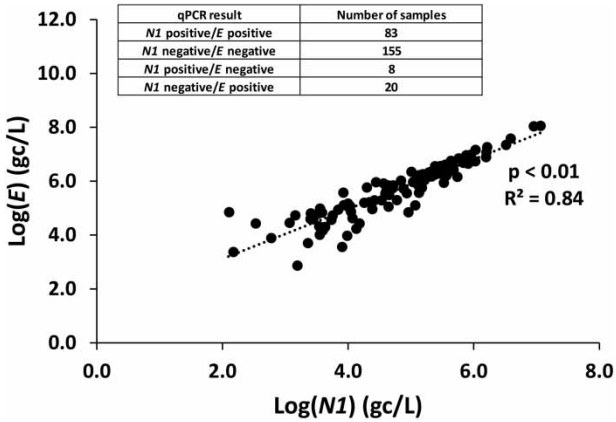


Figure 3 | SARS-CoV-2 *E* gene versus *N1* concentrations. Table summarising *N1* and *E* gene results for all samples tested.

due to a shortage of case data. However, non-normalised gene copy data were more than adequate to show that wastewater SARS-CoV-2 and COVID-19 data significantly correlated (Figure 4), which were the main goals of the pilot study.

Specifically, comparisons were made between the available positive COVID-19 case data and SARS-CoV-2 concentrations detected in wastewater samples. Weekly case data and averaged weekly SARS-CoV-2 levels detected in wastewater (a 'week' is defined as the first sampling day, Monday, and the subsequent 6 days) were compared and are provided in SI Table S2. In Prisons 1, 3, and 5, higher SARS-CoV-2 wastewater concentrations positively trended with higher case numbers detected in those prisons, which is consistent with COVID-19 outbreaks at those prisons (e.g., for Prison 5 in SI Figure S2). Higher case

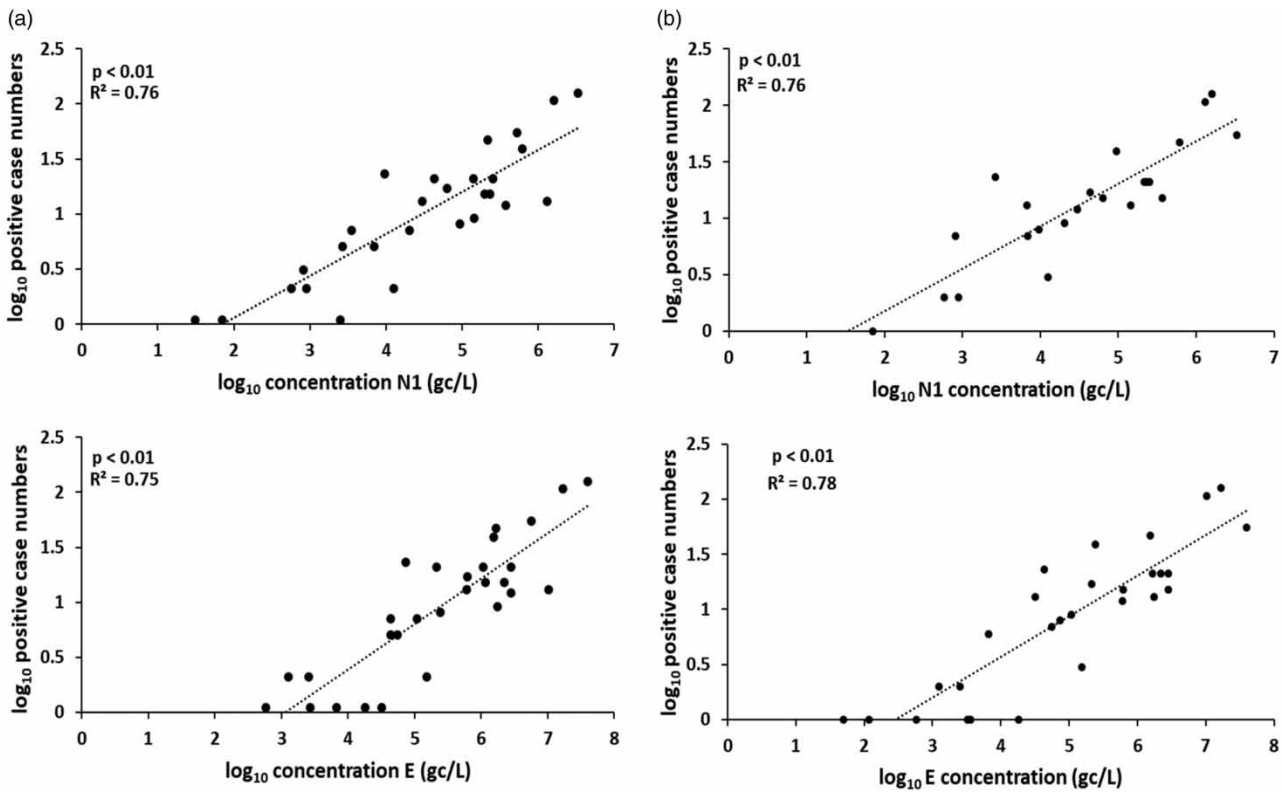


Figure 4 | Relationship between positive COVID-19 case numbers and wastewater concentrations of SARS-CoV-2 *N1* and *E* genes. (a) Log of *N1* and *E* gene concentrations compared to the log of case data from the same 7-day period. (b) Log of *N1* and *E* gene concentrations compared to the log of case data from the subsequent 7-day period, i.e., with a 1-week lag in case data.

numbers at the start of the sampling period, particularly in weeks 2 and 3, coincided with the highest concentrations of SARS-CoV-2 found wastewater samples. Conversely, positive case numbers were consistently lower in some prisons with parallel low or non-detectable levels of SARS-CoV-2 in their wastewater.

Scatterplots of wastewater *N1* and *E* gene concentrations versus COVID-19 case numbers per week for samples from across all six prisons are shown in Figure 4(a). Data show that even with suboptimal clinical case testing conditions, a significant positive correlation is seen between wastewater gene levels and COVID-19 case data for both *N1* ($r^2 = 0.76$, $p < 0.01$) and *E* gene ($r^2 = 0.75$, $p < 0.01$) signals. Shedding of the SARS-CoV-2 fragments in faeces can occur from 2 to 5 days before symptoms appear (Jones *et al.* 2020). Therefore, scatterplot analysis was repeated assuming a 1-week lag between wastewater detection and COVID-19 case appearance (Figure 4(b)), and one also finds a similar significant positive correlation between *N1* ($r^2 = 0.76$, $p < 0.01$) and *E* gene ($r^2 = 0.78$, $p < 0.01$) signals and cases.

It should be noted that correlations also were performed using *N1* and *E* gene concentrations normalised to prison population size and ammonia levels, respectively, but in both cases, r^2 values with COVID-19 case numbers declined with data normalisation. For example, comparable r^2 values for non-normalised versus normalised *N1* and *E* gene copy data with case data were 0.76 versus 0.72 for *N1* and 0.75 versus 0.59 for *E* gene. As such, non-normalised data were used to demonstrate trends.

The above results show the potential value of WBE as a passive health surveillance tool for populations in confined settings, such as prisons. Despite suboptimal case testing here (i.e., no clinical mass testing), wastewater *N1* and *E* concentration data mirrored COVID-19 case data across samples taken throughout the prison study, implying that WBE monitoring can help and even substitute for individual testing if testing data are not available. This is especially important because testing of individuals can be very challenging in prisons. Therefore, wastewater monitoring can provide a health protection 'safety net' in such settings, i.e., where unpredictable personal behaviour and other factors restrict systematic clinical testing.

Clinical testing remains the 'gold standard' for qualitative assessments of infection, determining whether an individual is infected or not, but as cited by the UK Scientific Advisory Group for Emergencies (2021), significant barriers exist in prison environments for these to be solely relied upon. Due to local issues of staff resources, prisoner compliance, identifying symptoms or exposure and time to testing, and issues with the sensitivity and specificity of the tests themselves, the use of clinical testing in prisons is 'limited and variable' (O'Moore 2020: 2). Amidst these unique and challenging conditions, prisons frequently experience fast and enduring outbreaks of infectious disease, as observed in our analysis. Therefore, the most effective public health approach to managing infectious diseases in enclosed settings is to identify infections and respond as early as possible.

As wastewater monitoring is a low-staff resource and a relatively unbiased and accurate population-level surveillance method that does not require compliance – after all, sampling and analysis are anonymous and prisoners cannot choose where they defaecate – it complements other surveillance methods, informing public health practitioners whether infections are present and whether the trend is increasing or not. In summary, there is no public health surveillance panacea to prevent outbreaks in prison settings, but wastewater monitoring provides an important contribution to identifying and responding to infectious disease outbreaks.

The novel contribution of wastewater-based surveillance in prisons has subsequently been acknowledged by the UK Government. Results from this pilot informed the development of an ongoing national wastewater-based surveillance programme in prisons (The Telegraph 2021) and national guidance for the management of COVID-19 outbreaks in prisons (UKHSA 2022). Reflecting on the findings of our analysis, public health practitioners were regularly informed of wastewater-based signals of SARS-CoV-2 presence and trend and guided on how to respond with clinical testing. Wastewater-based surveillance informed the identification of and response to outbreaks and supported closures by indicating whether shedding of infection was present or not, providing public health information in a unique setting that may otherwise have not been possible.

CONCLUSIONS AND IMPLICATIONS

This study supports the use of routine WBE to monitor SARS-CoV-2 levels in prisons, providing a potential method to monitor the overall health of prison populations. This study focused on COVID-19 and SARS-CoV-2, but methods should be equally valuable for other pathogens and targets of concern. Samples were collected as overnight 14-h composites, 4 days per week for the duration of the study; however, data indicate that shorter sampling windows may be appropriate. Data

also suggest that for routine monitoring, two samples a week would provide comparable overall trends. However, we recommend that for the use of WBE as an early warning system, particularly in prisons where infectious diseases can spread quickly, more frequent sampling is required. Comparisons between the detection of SARS-CoV-2 using the *N1* versus *E* gene both provide a reasonable estimate of virus levels. We recommend both be used in monitoring because this will reduce reporting of false negatives from wastewater samples. Population normalisation was assessed for *N1* versus *E* gene signals for comparison with case data, but normalisation by population size and ammonia levels both reduced the r^2 of correlations with case data compared with non-normalised data. We suggest that further work on virus normalisation is needed where larger case datasets are available. Biomarkers, such as PMMoV and crAssphage, should be investigated for use in prison WBE.

SARS-CoV-2 concentrations quantified in wastewater samples positively correlated with available positive COVID-19 case data. This is consistent with observations made in other SARS-CoV-2 WBE studies at near-source settings such as nursing homes, schools, and university accommodation (Betancourt *et al.* 2021; Davó *et al.* 2021; Gibas *et al.* 2021; Castro-Gutierrez *et al.* 2022; Fielding-Miller *et al.* 2023). However, unlike these settings where resources and compliance are less of an issue, WBE provides an important 'safety net' that has been used to identify COVID-19 cases that were not detected by clinical monitoring programmes, flagging asymptomatic cases when clinical testing may not be possible. Near-source WBE can, therefore, complement public health surveillance approaches, mitigating local biases and challenges.

At the time of this study, only symptomatic testing was being performed at the six prisons investigated. In addition to this testing policy, which may have missed asymptomatic and pre-symptomatic cases, it has been speculated that incarcerated individuals may hide symptoms of infectious disease to avoid further isolation, which in prisons is already a form of punishment (Crick *et al.* 2014; Besney *et al.* 2017). Therefore, although clinical testing is the 'gold standard' for the detection of SARS-CoV-2, in near-source settings such as prisons, where testing may be logistically difficult and mass testing unavailable, wastewater surveillance is a strong alternative. WBE provides a passive sampling method, which can identify the overall infection trends of a community and will include all asymptomatic, symptomatic, and pre-symptomatic cases if faecal shedding is occurring. WBE shows great promise for monitoring and health protection, and it is hoped that the results herein can guide the use of WBE in other near-source settings as it continues to do so in prisons across England and Wales at the time of writing.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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