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SARS-CoV-2 RNA Isolation Method from Sewage Sludge, Application in Field Samples and Comparison with Bacteriophage Loads

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Abstract

Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) is mainly transmitted through the respiratory tract. It can also be found in faeces leading to its detection in wastewater and potentially in sewage sludge. This one can be used in agriculture as a soil amendment. In France, the spreading of sludge is controlled in order to limit the dissemination of pathogenic microorganisms including SARS-CoV-2 since the pandemic. However, the control only concerns the analysis of bacteriophages. The present study was carried out to assess the presence of the virus in sewage sludge and compare with bacteriophages results. It describes the validation of a method for the isolation of SARS-CoV-2 RNA for detection by RT-PCR, using a surrogate virus. Two virus concentration methods and three nucleic acid extraction methods were compared. After validation, the most efficient method was applied to field samples (n=34) from Normand sewage treatment plants during the pandemic. Then, the results were compared with bacteriophage loads. According to our results, PEG precipitation followed by a nucleic acid extraction based on cleared lysate with phenol:chloroform:isoamyl alcohol, then concentrated and purified on anion-exchange column was selected. This process resulted in a yield of 39.6±37.3%. The field study confirmed the presence of SARS-CoV-2 in both primary and hygienized sludges. The comparative analysis suggested that the study of the effectiveness of sanitation on bacteriophages does not appear representative of that on SARS-CoV-2. In addition to the bacteriophages test, a direct search for the SARS-CoV-2 is recommended to evaluate the sanitation of sludge.

Keywords: SARS-CoV-2, Sewage sludge, Viral concentration, RNA isolation

1 Introduction

Since its emergence in Wuhan (China) at the end of 2019, the Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) is responsible for the worldwild Coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV-2 is classified as a β-coronavirus and belongs to the Coronaviridae family under the Cornidovirineae sub-order, the Nidovirales order and the Orthornavirae kingdom [1]. It is an enveloped, positive-sense, single-stranded RNA virus, which has an affinity for epithelial cell and respiratory system [2]. COVID-19 is a highly contagious respiratory disease spread through nasal secretion but several studies have highlighted the presence of the virus in various human samples [3], including stool samples [4] [5] [6]. Because of their excretion in faeces, enteric viruses and non-enteric viruses such as SARS-CoV-2 are present in wastewater [7], and consequently found in sewage treatment plant residues [8] [9]. The treatment of wastewater involves several steps. First, thickening and dehydration to reduce their volumes and, in a second time, hygienization to decrease the concentration of microorganisms. The treatment leads to the formation of sludge, which is the main product of wastewater plants. The resulting sludge can be incinerated, but it also can be used in agriculture. Its abundance of organic matters and fertilising elements confer it agricultural benefits [10]. Their release into the environment is a serious concern and represents a potential health risk due to the presence of pathogenic microorganisms [11]. In France, the spreading of sludge from processed wastewater is highly regulated [12] [13]. There are standardised methods for testing the viability of enteric viruses (enterovirus) [14], germs (*Escherichia coli*) [15] or parasites (worms) [16] in sludge. But due to the SARS-CoV-2 pandemic, authorities must tighten controls before their release into the environment. To date, the only mandatory test to assess the sanitary compliance of sludge prior to land application with respect to the presence of viruses is a bacteriophage search. But no analysis for SARS-CoV-2 is required to assess its presence. As reported in several studies, SARS-CoV-2 is detected in sewage water [17] [18] [19]. Protocols for viral isolation and detection of SARS-CoV-2 by reverse transcriptase-polymerase chain reaction (RT-PCR) from sewage are available [18] [20]. But, at the time of the study, no detection method and few data concerning the presence of SARS-CoV-2 in sewage sludge were published, to our knowledge.

In the current study, two elution processes and three RNA extraction kits were compared in order to detect the virus by RT-PCR. The most efficient system was validated and used to test the presence of SARS-CoV-2 in field samples from

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Normand sewage treatment plants during the COVID-19 pandemic. The results were compared to the detection of bacteriophages from the same sludges.

2 Materials and methods

2.1 Development of a method to detect SARS-CoV-2 in sewage sludge

2.1.1 Spiking solution

In order to avoid any health risks, the method was developed using a surrogate virus: the Bovine Coronavirus (BCoV). Like SARS-CoV-2, BCoV is an enveloped, positive-sense, single-stranded RNA virus of Coronaviridae family [21]. Its genome is approximatively 31 kb [22] against near 30 kb for SARS-CoV-2 (NCBI Reference Sequence: NC_045512.2). A vaccine strain of BCoV (Scourvax® – Pfizer) was grown on human colon cells (HRT18 - ATCC® CRL-11663™) as reported in Hogue et al. [23]. The viral strain was titrated with the Spearman-Karber method [24] at $10^{3.1}$TCID50 (Median Tissue Culture Infectious Dose)/25 µl. Strain was aliquoted into single-use volumes and stored at -80°C.

2.1.2 Sewage sludge

From August to November 2020, sludges were collected from different treatment plants in Normandy (France). For each, pH and dry matter rate were calculated with conventional methods. To work with different types of sludge (alkaline/basic and dry/wet), three of them were selected. The absence of BCoV in these sludges was confirmed by specific RT-PCR (described below) and each was artificially inoculated with the spiking solution, directly into the mass.

2.1.3 Virus elution and concentration

For the elution, 100 mL of a beef extract (Gibco™) with glycine solution (beef extract 3% w/v, 50 mM glycine) were added to 10 g of artificially inoculated sludge. The mixture was homogenized under magnetic stirring after adjustment to pH 9.5 for a minimum of 15 min or until completely dissolved, at room temperature. From this solution, two concentration methods were performed: organic flocculation in acid medium and precipitation with polyethylene glycol 8000 (PEG8000) with NaCl.

Method 1 (Concentration by organic flocculation in acid medium): The virus elution mixture was centrifuged at 3,000 x g for 15 min at room temperature. The supernatant was collected, acidified to pH 4.0 using a hydrochloric acid solution (1N HCl) and mixed by magnetic stirring for 45 min at room temperature. The viral particles were pelleted at 3,500 x g for 45 min at +4±2°C. The supernatant was discarded and the pellet was re-suspended in 2 mL of sterile purified water. The pH was adjusted to pH 7.0 using a sodium hydroxide solution (1N NaOH).

Method 2 (Concentration by precipitation with PEG8000 and NaCl): The virus elution solution was centrifuged at 4,500 x g for 30 min at +4°C2°C and the supernatant was mixed with 10% (w/v) PEG8000 and 2.25% (w/v) NaCl. The mixture was agitated manually up to disappearance of the flakes and subsequently centrifuged at 12,000 x g for 2 h at +4 ±2°C. The supernatant was discarded without disturbing the pellet and a second centrifugation at 12,000 x g was performed for 5 min at +4 ±2°C. The supernatant was carefully removed and the pellet was resuspended in 2 mL of sterile purified water.

2.1.4 Acid nucleic extraction

After each concentration method, the viral RNA contained in the pellets was extracted using three kits: the silica column (SC) isolation and purification kit (QIAamp® Viral RNA Mini Kit – QIAGEN), the magnetic bead-based viral nucleic acid (MBB) isolation kit (NucleoMag™ Pathogen kit – MACHEREY NAGEL™) or the RNA isolation kit from soil and difficult environmental samples (RNA IS) (RNeasy® PowerSoil® Total RNA Kit – QIAGEN). All extractions were performed following the manufacturer's recommendations.

To assess the presence of PCR inhibitors and to define the most optimal process, all the extracts were analysed under three conditions: pure, diluted in nuclease free water (1:10) and purified form. The PCR inhibitor clean up kit (OneStep™ PCR Inhibitor Removal Kit ZYMO RESEARCH - OZYME) was used for purification following the manufacturer's instructions.

2.1.5 Amplification

The RNA extracts were amplified by TaqMan® real time RT-PCR. The primers and probe (developed and routinely used by the diagnostic virology department of LABÉO) target a gene encoding a membrane protein of the Bovine Coronavirus (GenBank ID BBM61442.1). The following oligos were used: forward primer BOCO-1 5'GGTGGAGTTTCAACCCAGAA3', reverse primer BOCO-2 5'CGCTTATACGTGAGCAGGTG3' and TaqMan® probe 5'TCTTTGTCAGATTTGCCAGC3' labelled with the 6-FAM fluorescent tag. The gene amplification was performed in a total volume of 25 µL composed with 5 µL of the 5x QuantiTect® Virus master mix (QIAGEN), 0.2 µL of each primers (20µM), 0.5 µL of probe (5 µM), 0.25 µL of 100x QuantiTect® Virus RT mix (QIAGEN), 13.85 µL of RNase-free water (QIAGEN) and 5 µL of RNA extract. Thermal cycling conditions, using a QuantStudio™ 12K Flex Real-Time PCR System (Thermofisher Scientific), were as follows: reverse transcription in 1 cycle of 50°C for 20 min followed by real-time PCR consisting of 1 cycle of 95°C for 5 min following by 45 cycles of 95°C for 15 s and 60°C 45 s. Positive (dilution of the strain extract) and negative (RNase-free water) controls were systematically included at each run. Data were exploited on QuantStudio™ 12K Flex System Software.

2.2 Validation of the selected method

2.2.1 Detection limit

The detection limit was determined using one neutral and one alkaline sludge. Each was artificially inoculated with 150 µL of the BCoV strain at different dilutions: 1:100, 1:500, 1:1000 and 1:5000, i.e. 76, 15, 8 and 2 TCID50 per 150 µL respectively. Each viral load level was tested in triplicate. After isolation and purification of the viral RNA a RT-PCR was performed on extracts using the system described above. The detection limit of the method was assessed at 100%, i.e. the last dilution where all triplicates gave positive PCR signals.

2.2.2 Repeatability

A RT-PCR was performed on extracts from four replicates of a sludge inoculated with 150 µL of a BCoV strain dilution corresponding to the detection limit. Repeatability was assessed by the coefficient of variation (CV) of the cycle threshold (Ct) values obtained.

2.2.3 Performance

The amount of detected virus was calculated from a standard curve generated from a tenfold serial dilution of an extract of the pure spiking solution. For each test the yield was estimated by the ratio of the amount of the virus detected to the amount initially inoculated.

2.3 Application in field samples

SARS-CoV-2 was tested using the validated method on field samples. It was applied to 34 sludges received at the laboratory during and after different pandemic peaks, from June 2021 to April 2022. Sludges were collected from several wastewater treatment plants and had different hygienization statuses: primary sludge or hygienized sludge (dried or limed). They were treated within 48 hours of receipt using the current method. The detection of SARS-CoV-2 was performed with the ARGENE® SARS-COV-2 R-GENE® kit (BIOMERIEUX) targeting the N and RdRp genes of SARS-CoV-2.

The analyses were validated using the BCoV strain spiking solution as a process control allowing a yield to be to calculated per analyse. Samples were loaded with a 1:10 dilution of the strain. BCoV specific real time PCR described above, was performed on extracts from each test to determine the method performance by calculating the ratio between the amount of virus detected and the amount of virus inoculated. Concurrently, the field samples were analysed for the presence of bacteriophages. Their detection was realized according to the ministerial decree of April 20, 2021 [12] and the norm NF EN ISO 10705-2 [25] describing the method of detection and quantification of somatic coliphages in sludge.

2.4 Statistical analysis

Results of SARS-CoV-2 tests were compared to results of bacteriophage enumerations. The sensibility (formula a), the specificity (formula b), and the accuracy (formula c) were calculated to check the adequation of positive tests, negative tests and overall respectively, between the two methods. The kappa index was selected to study the agreement of the two methods. The interpretation of kappa values is based on the classification of Landis and Koch [26].

N = number of samples. pos: positive. neg: negative.

Formula a (sensitivity):

$$Se = \frac{N\ pos\ bacteriophages\ and\ SARS - CoV - 2}{N\ pos\ bacteriophages}\ x\ 100$$

Formula b (specificity):

$$Sp = \frac{N\ neg\ bacteriophages\ and\ SARS - CoV - 2}{N\ neg\ bacteriophages} x100$$

Formula c (accuracy):

$$Ac = \frac{(N\ pos\ bacteriophages\ and\ SARS - CoV - 2 + N\ neg\ bacteriophages\ and\ SARS - CoV - 2)}{N\ total} x100$$

3 Results

3.1 Development

3.1.1 Virus concentration

The first assay of the development was carried out on an alkaline sludge (pH 12.20) with a dry matter content of 30.70% while the second and third assays were carried out on a single neutral sludge (pH 7.40) with a dry matter content of 3.67%. The two methods, precipitation by acidification (method 1) and precipitation in presence of PEG8000 (method 2), showed distinct results in the majority of cases. Differences in PCR detection between both methods were observed. Independently of the extraction kit, detection with method 2 was earlier than with method 1 in all three trials (Table 1). The Ct values obtained with the precipitation method were always lower with ΔCt value ranging from 0.66 to 4.63, compared to those obtained with the acidification method. Moreover, method 1 did not allow the detection of the RNA in the second assay with the MBB kit.

Table 1: Ct values of diluted extracts as a function of concentration and extraction method. The Ct values are inversely proportional to the amount of detected virus. MBB: magnetic bead-based viral nucleic acid isolation kit. SC: silica column isolation and purification kit. RNA IS: RNA isolation kit from soil and difficult environmental samples. n/a: data not available

	Concentration	MBB	SC	RNA IS
1st assay	method 1	35.59	35.63	31.83
	method 2	30.96	32.87	31.17
2nd assay	method 1	Not detected	42.43	27.85
	method 2	44.19	38.90	26.55
3rd assay	method 1	n/a	n/a	35.38
	method 2	n/a	n/a	34.00

3.1.2 Acid nucleic extraction

Alkaline sludge concentrated by acidification (first assay, method 1) showed the earliest PCR signal detection with the RNA IS kit with a ΔCt value of 3.76 and 3.80 compared to the two other kits. After concentration by precipitation (first assay, method 2), the SC kit showed the latest detection, with a ΔCt value of 1.91 and 1.70 with the others. The RNA IS and the MBB kits gave similar results with less than 0.30 Ct difference.

In the second assay, PCR on the MBB kit extract showed no PCR signal with acidification concentration (method 1) and a late Ct (44.19) with precipitation concentration (method 2). For both concentration methods, only an extract with the SC kit allowed the detection of the RNA virus with a Ct of less than 40. In contrast, the RNA IS kit gave early Ct values: 27.85 for method 1 and 26.55 for method 2. Ct values between pure, diluted, and purified extracts were compared (Table2). All diluted extracts had lower Ct values or similar (less than 1Ct) than the pure extracts. 66% of the diluted extracts showed better PCR signals than the purified extracts. While purification of the extract gave a low Ct value for three samples, it did not allow the detection of the virus for three other samples, though their respective diluted extracts were positive.

Table 2. Ct values of pure, diluted and purified extracts for all tests of the development. MBB: magnetic bead-based viral nucleic acid isolation kit. SC: silica column isolation and purification kit. RNA IS: RNA isolation kit from soil and difficult environmental samples. n/a: data not available. For each method and extraction kit, the lowest Ct values among pure, diluted and purified extracts are highlighted (bold)

Concentration method	Extraction kit	Pure	Diluted	Purified
method 1	RNA IS	31.13	31.83	**30.63**
		33.97	**27.85**	32.81
		42.66	**35.38**	n/a
	SC	Not detected	**35.63**	Not detected
		Not detected	**42.43**	Not detected
	MBB	35.26	35.59	**33.13**
		Not detected	Not detected	n/a
method 2	RNA IS	29.85	**26.55**	29.42
		38.45	**31.17**	n/a
		34.98	**34.00**	n/a
	SC	Not detected	**32.87**	38.26
		Not detected	**38.90**	Not detected
	MBB	33.30	30.96	**29.32**
		Not detected	**44.19**	n/a

For the further validation, the system using PEG precipitation (method 2) followed by the extraction with the RNA IS kit was chosen because of its better recovery of viral RNA from the eluate.

3.2 Validation of the selective method

3.2.1 Method detection limit

The validation of the method was carried out using two sludges: a neutral sludge (pH 7.40) and a limed sludge (pH 12.20) with dries matters of 3.70% and 30.75%, respectively. For both sludges, only extracts obtained with 2TCID50/150 µL spiking solution gave negative signals. Triplicates at lower dilutions were all positive (Table 3).

Table 3: Ct values of diluted extracts from triplicates of sludges artificially inoculated with different virus loads. CV of triplicates. Ct: Cycle threshold. CV: coefficient of variation

Load	Neutral sludge		Limed sludge	
	Ct value	CV	Ct value	CV
76 TCID50/150 µL	34.22 34.42 34.11	0.46	35.36 34.59 34.53	1.10
15 TCID50/150 µL	35.15 35.06 35.59	0.80	36.33 36.67 35.98	0.95
8 TCID50/150 µL	36.52 36.20 35.82	0.97	36.30 37.56 36.38	0.90
2 TCID50/150 µL	41.26 Not detected 40.16		Not detected Not detected Not detected	

3.2.2 Repeatability

At the detection limit (8 TCID/150 µL), diluted extracts of the four replicates had Ct values between 34.33 and 36.81 (Table 4). SD and CV were 1.02 and 2.86, respectively. Repeatability was acceptable and this was strengthened by the Ct values obtained during the estimation of the detection limit (Table 3). Each triplicate had a CV between 0.46 and 1.10 which confirmed the reliability of the method.

Table 4. Ct values of extracts from four replicates of a sludge inoculated with 8TCID/150 µL spiking solution. SD and CV calculated with values of the four replicates. SD: standard deviation. CV: coefficient of variation

Replicate	Ct value
1	35.67
2	35.85
3	34.33
4	36.81
SD	1.02
CV	2.86

3.2.3 Performance method

Yield was calculated on each development test, carried out with the different sludges and at different virus load levels. It ranged from 2.3% to 83.5%. The average values were 52.7±42.7% for neutral sludge and 19.9±12.2% for alkaline sludge.

3.3 Application

A total of 34 sludge samples (Table 5) were analysed from June 2021 to April 2022. The 10 first sludges (samples 1 to 10) were collected from distinct treatment plants in June 2021 which corresponded to a period following a peak of pandemic in Normandy. Those presented a great variability of dry matter content, ranging from 0.59% to 84.33%. Yields for these samples varied from less than 1% to 30.5%. 4 samples out of 10 showed a positive signal for SARS-CoV-2 real time PCR test. Ct values ranged from 35.9 to 38.5. Generally, only one of the two genes (N gene) targeted by the PCR was amplified in positive extracts. In a second time, nine unhygienized sludges (samples 11 to 19) were collected from a single water treatment plant between August and October 2021, at the rate of one sample a week. Dry matter contents were relatively similar, around 20.14±0.56%. As the precedent samples, a sludge showed a low yield, less than 1%. Others ranged from 5.5% to 25.2%. Four out of nine sludges obtained a positive signal for SARS-CoV-2. As in the first campaign, only the N gene was detected in positive extracts with a Ct higher than 36.9.

From October 2021 to November 2022, four primary sludges were collected (samples 20 to 23) in a single treatment plant. The same sludges were collected again after hygienization by drying over two to four months (samples 24 to 27). For primary sludges, dry matter contents were relatively equivalent for three out of the four sludges (18.03% to 19.10%), the last sludge was more liquid with 1.59% dry matter. SARS-CoV-2 RNA was detected in only one sample with an early Ct (32.8) and yields ranged from 1.6% to 23.6%. For dried sludges, dry matter contents ranged from 78.84% to 83.97%, two sludges showed a positive PCR signal for SARS-CoV-2 but yields were lower (less than 1% to 3.4%). To complete the data with sludge that undergone another treatment, seven limed sludges were analysed (samples 28 to 34). The dry matter content of these sludges ranged from 4.49% to 28.21%. Yields ranged from less than 1% to 15.8%. Two were positive for SARS-CoV-2 test with a late Ct and only the fragment on the N gene was amplified.

In parallel of the search for SARS-CoV-2, sludges were analysed for bacteriophage detection and enumeration which is currently an alternative way of checking the effectiveness of hygienization against viruses. The primary sludges, with the exception of sample 10, were all positive for bacteriophages at widely varying levels, ranging from 1 794 PFU/g MS to almost 189 000 PFU/g MS. In contrast, only six out of these 14 sludges (samples 10, 11, 12, 14, 17 and 20) were positives for SARS-CoV-2. The hygienized sludges showed lower bacteriophage loads, with eight sludges (samples 25 to28 and 30 to 33) containing less than 10 PFU/g MS. The remaining three sludges (samples 24, 29 and 34) containing 634 PFU/g MS at most. SARS-CoV-2 RNA was detected in four sludges (samples 24, 27, 32 and 34). The four sludges with the highest bacteriophage loads (samples 1,2,14 and 20) were also positive for SARS-CoV-2. By contrast, four other SARS-CoV-2 positive sludges (samples 9, 10, 27 and 32) were nearly free of bacteriophages. The bacteriophage kill rate between samples 20 and 24 (same sludge before and after drying) was 2.47 log. With regard to SARS-CoV-2, the difference in Ct value between the same samples showed a decrease of less than 1log (Ct of 32.8 for sample 20 and 35.2 for sample 24).

3.4 Statistical analysis

Positive samples for both bacteriophages and SARS-CoV-2 tests represented 37% of the samples (Table 6). The simultaneous absence of both microorganisms accounted for 60% of the samples. Overall, only 44% of sludges were concordant for the concomitant presence or absence of both microorganisms, representing 19 samples out of 34 that did not match. The kappa index was evaluated below 0, reflecting two discordant methods.

Table 5: Type of sludge and results of analysis of dry matter, SARS-CoV-2 detection and bacteriophages enumeration of field sludge samples. Yield of process determined on BCoV virus recovery. PFU/g MS: plaque-forming unit per gram of dry matter. n/a: data not available. PS: primary sludge. DS: dried sludge. LM: limed sludge

Sample	Hygienisation	Dry matter	SARS-CoV-2 PCR result (Ct)	Yield	Bacteriophages (PFU/g MS)
1	n/a	1.32%	POSITIVE (35.9)	17.8%	29 635
2	n/a	0.59%	POSITIVE (38.5)	1.0%	26 895
3	n/a	0.88%	negative	4.7%	23 810
4	n/a	15.47%	negative	30.5%	16 156
5	n/a	2.00%	negative	7.5%	15 496
6	n/a	5.72%	negative	3.9%	5 419
7	n/a	4.57%	negative	23.7%	1 532
8	n/a	1.33%	negative	2.1%	3 752
9	n/a	84.33%	POSITIVE (37.7)	<1%	<10
10	PS	1.86%	POSITIVE (38.3)	3.1%	<10
11	PS	20.17%	POSITIVE (36.9)	6.6%	15 371
12	PS	19.91%	POSITIVE (38.1)	25.2%	7 533
13	PS	19.80%	negative	15.1%	12 625
14	PS	19.62%	POSITIVE (38.1)	12.6%	48 921
15	PS	19.39%	negative	6.8%	10 313
16	PS	20.06%	negative	<1%	6 981
17	PS	20.32%	POSITIVE (37.2)	5.5%	7 383
18	PS	20.78%	negative	6.2%	4 811
19	PS	21.18%	negative	21.2%	1 794
20	PS	1.59%	POSITIVE (32.8)	23.6%	189 119
21	PS	19.10%	negative	12.9%	7 329
22	PS	18.77%	negative	21.4%	5 062
23	PS	18.03%	negative	1.6%	17 197
24	DS	78.84%	POSITIVE (35.2)	3.4%	634
25	DS	80.20%	negative	<1%	<10
26	DS	83.97%	negative	1.7%	<10
27	DS	80.30%	POSITIVE (39.7)	1.1%	<10
28	LS	28.21%	negative	<1%	<10
29	LS	5.38%	negative	4.4%	370
30	LS	26.94%	negative	<1%	<10
31	LS	4.49%	negative	5.1%	<10
32	LS	22.38%	POSITIVE (39.5)	2.0%	<10
33	LS	15.38%	negative	15.8%	<10
34	LS	25.57%	POSITIVE (38.5)	3.1%	235

Table 6: number of positive and negative sample for bacteriophages and SARS-CoV-2. *Se*: Sensitivity. S*p*: Specificity. *Ac*: Accuracy

	Bacteriophage positive >10 PFU/g MS	Bacteriophage negative <10 PFU/g MS
SARS-CoV-2 positive	9	4
SARS-CoV-2 negative	15	6
	Se = 37%	Sp = 60%
	Ac = 44%	

4 Discussion

The development of an effective method for detecting SARS-CoV-2 in sludge was the first step to investigate the presence of the virus in sewage sludge. This enabled field samples to be monitored to compare the results with the current analysis used to verify the sludge sanitation. PEG precipitation, which is commonly use to concentrate viruses from water [9] [27] [28] [29] and has even been published for SARS-CoV-2 [20] appeared to be a good approach for the concentration of SARS-CoV-2 from sludge. During development, PEG precipitation was able to consistently recover more virus than the acidification method. For extraction, the RNA IS kit was the only one allowing the detection of viral RNA in the pure, diluted and purified forms of the extract in all cases. In addition, more viral RNA was detected with this kit than with the other two kits. Sludge components, such as metals, detergents or chemicals [11], can make detection by amplification enzymes difficult due to the presence of many inhibitory substances [30]. The comparison of Ct values between pure and diluted extracts confirmed the presence of inhibition of amplification reaction. Analysis of the purified extracts showed that a 1:10 dilution in nuclease free water is more effective in reducing inhibition than purification of the extract with the kit used in the current study.

The technique allowed detection of the virus up to 8 TCID50 in 10 g of sludge, for both neutral and limed sludge with an acceptable repeatability. Unfortunately, no quantified genetic material of the BCoV strain was available at the laboratory to determine a detection limit in terms of viral genome copy number. Whatever the physical and chemical composition of the sludge, the average yield was 39.6%±37.3% during development and varied from <1% to 30.5% for field samples. These results were relatively consistent with Barril et al [20] who recovered from 0 to 26.4% of SARS-CoV-2 from wastewater. Kocamemi et al [31] tested the concentration of avian Coronavirus by PEG adsorption from wastewater and observed a virus loss of 1 to 1.5log equivalent to a yield of approximately 3 to 10%. Studies performed with the PEG precipitation method have reported variable recoveries. D'Aoust et al [32] recovered 8.4±3.6% (post-grit solids) and 9.3±4.9% (primary clarified sludge) of a surrogate virus in spiking samples while Balboa et al [33] were able to recover up to 32.1±15.8% of the bacteriophage MS2.

As previously mentioned, SARS-CoV-2 is found in wastewater but at the time of development, little data on its presence in sewage sludge were available. In order to confirm the presence of SARS-CoV-2 in sewage sludge from Normandy, the developed method was applied to field samples. Previous works did not detect the virus in digested sludge [33] [34] but as Serra-Comte et al [35] the current study showed that SARS-CoV-2 was present in primary and also in hygienized sludge. The quantities detected were very low, close to the detection limit. This could explain why, in most of the cases, only one of the two genes targeted by the RT-PCR gave positive signal. This observation has already been described and may be a consequence of the lower detection limit inherent in multiplex PCR [33]. The bacteriophage test is an alternative method as indicators of the effectiveness of sludge sanitation against viruses including SARS-CoV-2 [12]. The two methods of hygienization, drying and liming, significantly reduced the bacteriophage load. Bacteriophages and SARS-CoV-2 were not systematically detected simultaneously. The search for the two parameters, SARS-CoV-2 and bacteriophages, did not show similar results on the presence and absence of both microorganisms. The sensibility and the specificity between both parameters were low with respectively 37% and 60%. Moreover, the statistical measure of kappa showed a clear discordance between the two methods. This, raise the question of the interest to search bacteriophages in order to judge the effectiveness of sludges sanitation against SARS-CoV-2.

Based on our results, the presence of bacteriophages and their quantification did not appear to be representative of the presence of the SARS-CoV-2 in sludge. The results, with reference to SARS-CoV-2, did not corroborate previous work on the link between the presence of bacteriophages and pathogenic viruses [36] [37]. The kill rates of both microorganisms before and after sanitation were different between SARS-CoV-2 and bacteriophages. For example, the analyse of one sludge showed a higher decrease (two to three times greater) of bacteriophages than SARS-CoV-2 between the primary and hygienized sludge. Another sludge highlighted also a significant decrease of the bacteriophage load. However, SARS-CoV-2 RNA was not detected in the first sample, whereas it was detected in the second. The negative result for SARS-CoV-2 test in the first sample might be due to the small amount of virus present but also to the small quantity of sludge tested compared to the large volume of sewage sludge. However, it should be noted that the virus was still present after desinfection. The sample 10, a primary sludge that contained a very low quantity of bacteriophages, was however positive for SARS-CoV-2. A second check of this sludge after treatment did not allow a decrease in the bacteriophage level to be

observed, given their low initial concentration. The effectiveness of its hygienization cannot therefore be verified and SARS-CoV-2 could therefore persist in this sludge. Unfortunately, we were not able to recover the sludge after hygienization to check for the presence of SARS-CoV-2.

5 Conclusions

Our results showed that the described method allowed the detection of SARS-CoV-2 in sludge and overcomes difficulties due to physical and chemical matrix constituents. The most appropriate method for concentrating coronavirus after an elution with a beef extract solution and glycine consisted of a concentration by centrifugation in presence of PEG8000. The nucleic acid extraction based on cleared lysate with phenol:chloroform:isoamyl alcohol, concentrated and purified on an anion-exchange column was most appropriate for isolating and purifying viral RNA from the concentrate. However, a 1:10 dilution of the RNA extract was required to obtain an efficient detection. According to field samples results and previous study SARS-CoV-2 persists in treated sludge and further analysis should be carried out to confirm the relevance of the bacteriophage testing as an indicator of the effectiveness of sanitation of sludge against SARS-CoV-2. Knowing that the concentration method was based on the physical characteristics of the virus, the method allows the recovering of only the enveloped virus. In spite of all, PCR detection based on DNA amplification does not allow the viability of the virus to be determined. In order to conclude on a potential risk to humans from the handling or spreading the sludge, it might be interesting to test the infectivity of the SARS-CoV-2 virus isolated from it.

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Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

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Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing. YQ and ST contributed to data collection and analyses. YQ wrote the manuscript. ST, HB and MH reviewed the manuscript. MH supervised the study.

References

1 https://ictv.global/taxonomy. https://ictv.global/taxonomy.
2 Machhi J, Herskovitz J, Senan AM, Dutta D, Nath B, Oleynikov MD, Blomberg WR, Meigs DD, Hasan M, Patel M, Kline P, Chang RCC, Chang L, Gendelman HE, Kevadiya BD. The Natural History, Pathobiology, and Clinical Manifestations of SARS-CoV-2 Infections. Journal of Neuroimmune Pharmacology. 2020. https://doi.org/10.1007/s11481-020-09944-5
3 Bora I, Gogoi S, Venkatasubramanian V, Mathew R, Mohindra R. Persistence of sars-cov-2 in body fluids: A bystander or whistle blower. Iranian Journal of Microbiology. 2020. DOI: 10.18502/ijm.v12i5.459
4 Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA - Journal of the American Medical Association. 2020. DOI: 10.1001/jama.2020.3786
5 Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for Gastrointestinal Infection of SARS-CoV-2. Gastroenterology. 2020. DOI: 10.1053/j.gastro.2020.02.055
6 Zhang Y, Chen C, Zhu S, Shu C, Wang D, Song J, Song Y, Zhen W, Feng Z, Wu G, Xu J, Xu W. Isolation of 2019-nCoV from a Stool Specimen of a Laboratory-Confirmed Case of the Coronavirus Disease 2019 (COVID-19). China CDC Weekly. 2020. DOI: 10.46234/ccdcw2020.033
7 McCall C, Wu H, Miyani B, Xagoraraki I. Identification of multiple potential viral diseases in a large urban center using wastewater surveillance. Water Research. 2020. https://doi.org/10.1016/j.watres.2020.116160
8 Bibby K, Peccia J. Identification of viral pathogen diversity in sewage sludge by metagenome analysis. Environmental Science and Technology. 2013. DOI: 10.1021/es305181x
9 Gholipour S, Ghalhari MR, Nikaeen M, Rabbani D, Pakzad P, Miranzadeh MB. Occurrence of viruses in sewage sludge: A systematic review. Science of the Total Environment. 2022. DOI: 10.1016/j.scitotenv.2022.153886
10 Suhadolc M, Schroll R, Hagn A, Dörfler U, Schloter M, Lobnik F. Single application of sewage sludge - Impact on the quality of an alluvial agricultural soil. Chemosphere. 2010. https://doi.org/10.1016/j.chemosphere.2010.08.024
11 Fijalkowski K, Rorat A, Grobelak A, Kacprzak MJ. The presence of contaminations in sewage sludge – The current situation. Journal of Environmental Management. 2017. DOI : 10.1016/j.jenvman.2017.05.068
12 Journal Officiel République Française. JORF n°0121 du 27 mai 2021. 2021. https://www.legifrance.gouv.fr/download/pdf?id=tnDkLomDEXzUnDetotFJNWgPlRv7gOmIegJaPV6kUU0=
13 Journal Officiel République Française. JORF n°23 du 31 janvier 1998. 1998. https://www.legifrance.gouv.fr/jorf/id/JORFTEXT000000570287
14 AFNOR. NF T 90-451 Essais des eaux - Recherche des entérovirus - Méthode par concentration sur laine de verre et détection par RT-qPCR, et/ou par culture cellulaire. 2020. https://www.boutique.afnor.org/fr-fr/norme/nf-t90451/essais-des-eaux-recherche-des-enterovirus-methode-par-concentration-sur-lai/fa156831/260786
15 AFNOR. FD CEN/TR 15214-2 Caractérisation des boues - Détection et dénombrement des Escherichia coli dans les boues, les sols, les amendements du sol, les supports de culture et les biodéchets - Partie 2 : méthode miniaturisée (nombre le plus probable) par ensemencement en milieu liquide. 2006. https://www.boutique.afnor.org/fr-fr/norme/fd-cen-tr-152142/caracterisation-des-boues-detection-et-denombrement-des-escherichia-coli-da/fa137923/27377
16 AFNOR. FD X33-040 Caractérisation des boues - Dénombrement et viabilité des oeufs d'helminthes parasites - Méthodes de dénombrement. 2013. https://www.boutique.afnor.org/fr-fr/norme/fd-x33040/caracterisation-des-boues-denombrement-et-viabilite-des-oeufs-dhelminthes-p/fa177182/41359
17 Foladori P, Cutrupi F, Segata N, Manara S, Pinto F, Malpei F, Bruni L, la Rosa G. SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review. Science of the Total

Environment. 2020. https://doi.org/10.1016/j.scitotenv.2020.140444
18 Sharma D, Nalavade U, Kalgutkar K, Gupta N, Deshpande J. SARS-CoV-2 detection in sewage samples: Standardization of method & preliminary observations. Indian Journal of Medical Research. 2021. DOI: 10.4103/ijmr.IJMR_3541_20
19 Orive G, Lertxundi U, Barcelo D. Early SARS-CoV-2 outbreak detection by sewage-based epidemiology. Science of The Total Environment. 2020. DOI: 10.1016/j.scitotenv.2020.139298
20 Barril PA, Pianciola LA, Mazzeo M, Ousset MJ, Jaureguiberry MV, Alessandrello M, Sánchez G, Oteiza JM. Evaluation of viral concentration methods for SARS-CoV-2 recovery from wastewaters. Science of the Total Environment. 2021. https://doi.org/10.1016/j.scitotenv.2020.144105
21 Clark MA. Bovine coronavirus. British Veterinary Journal. 1993. DOI: 10.1016/S0007-1935(05)80210-6
22 Suzuki T, Otake Y, Uchimoto S, Hasebe A, Goto Y. Genomic characterization and phylogenetic classification of bovine coronaviruses through whole genome sequence analysis. Viruses. 2020. https://doi.org/10.3390/v12020183
23 Hogue BG, King B, Brian DA. Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59. Journal of Virology. 1984. https://doi.org/10.1128/jvi.51.2.384-388.1984
24 Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World Journal of Virology. 2016. https://doi.org/10.5501/wjv.v5.i2.85
25 AFNOR. NF EN ISO 10705-2 Qualité de l'eau - Détection et dénombrement des bactériophages - Partie 2 : dénombrement des coliphages somatiques. 2001. https://www.boutique.afnor.org/fr-fr/norme/nf-en-iso-107052/qualite-de-leau-detection-et-denombrement-des-bacteriophages-partie-2-denom/fa117463/19113?pk_source=google-adwords&pk_medium=cpc&gclid=EAIaIQobChMI-5zWjIOo-QIVD7h3Ch2sBQXHEAAYASAAEgLiXfD_BwE.
26 Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977. https://doi.org/10.2307/2529310
27 Amdiouni H, Faouzi A, Fariat N, Hassar M, Soukri A, Nourlil J. Detection and molecular identification of human adenoviruses and enteroviruses in wastewater from Morocco. Letters in Applied Microbiololy. 2012. DOI: 10.1111/j.1472-765X.2012.03220.x
28 Masclaux FG, Hotz P, Friedli D, Savova-Bianchi D, Oppliger A. High occurrence of hepatitis E virus in samples from wastewater treatment plants in Switzerland and comparison with other enteric viruses. Water Research. 2013. https://doi.org/10.1016/j.watres.2013.05.050
29 Strubbia S, Schaeffer J, Oude Munnink BB, Besnard A, Phan MVT, Nieuwenhuijse DF, De Graaf M, Schapendonk CME, Wacrenier C, Cotton M, Koopmans MPG, Le Guyader FS. Metavirome sequencing to evaluate norovirus diversity in sewage and related bioaccumulated oysters. Frontiers in Microbiology. 2019. https://doi.org/10.3389/fmicb.2019.02394
30 Prado T, Bonet Guilayn W de CP, Coimbra Gaspar AM, Pereira Miagostovich M. The efficiency of concentration methods used to detect enteric viruses in anaerobically digested sludge. Memorias do Instituto Oswaldo Cruz. 2013. DOI: 10.1590/S0074-02762013000100013
31 Kocamemi BA, Kurt H, Hacıoglu S, Yaralı C, Saatci AM, Pakdemirli B. First Data-Set on SARS-CoV-2 Detection for Istanbul Wastewaters in Turkey. medRxiv. 2020. DOI: 10.1101/2020.05.03.20089417
32 D'Aoust PM, Mercier E, Montpetit D, Jia JJ, Alexandrov I, Neault N, Baig AT, Mayne J, Zhang X, Alain T, Langlois MA, Servos MR, MacKenzie M, Figeys D, MacKenzie AE, Graber TE, Delatolla R. Quantitative analysis of SARS-CoV-2 RNA from wastewater solids in communities with low COVID-19 incidence and prevalence. Water Research. 2021. https://doi.org/10.1016/j.watres.2020.116560
33 Balboa S, Mauricio-Iglesias M, Rodriguez S, Martínez-Lamas L, Vasallo FJ, Regueiro B, Lema JM. The fate of SARS-COV-2 in WWTPS points out the sludge line as a suitable spot for detection of COVID-19. Science of the Total Environment. 2021. https://doi.org/10.1016/j.scitotenv.2021.145268
34 Pourakbar M, Abdolahnejad A, Raeghi S, Ghayourdoost F, Yousefi R, Behnami A. Comprehensive investigation of SARS-CoV-2 fate in wastewater and finding the virus transfer and destruction route through conventional activated sludge and sequencing batch reactor. Science of the Total Environment. 2022. https://doi.org/10.1016/j.scitotenv.2021.151391
35 Serra-Compte A, González S, Arnaldos M, Berlendis S, Courtois S, Loret JF, Schlosser O, Yàñes AM, Sorai-Soria E, Fittipaldi M,Saucedo G, Pinar-Méndez A, Paraira M, Galofré B, Lema JM, Balboa S, Mauricio-Iglesias M, Bosch A, Pintó Rosa, Bertrand I, Gantzer C, Montero C, Litrico X. Elimination of SARS-CoV-2 along wastewater and sludge treatment processes. Water Research. 2021. DOI: 10.1016/j.watres.2021.117435
36 Martín-Díaz J, Lucena F, Blanch AR, Jofre J. Review: Indicator bacteriophages in sludge, biosolids, sediments and soils. Environmental Research. 2020. https://doi.org/10.1016/j.envres.2020.109133
37 Dias E, Ebdon J, Taylor H. The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. Water Research. 2018. DOI: 10.1016/j.watres.2017.11.022