CORONASTEP Report 01
SARS-CoV-2 sewage surveillance in Luxembourg

Introduction – Context – Objectives

In case of pandemics, a clear image of the prevalence in the population is essential to manage the containment, particularly with regard to its release. Tracking mild or asymptomatic cases that do not require care or testing in the general population is costly, logistically difficult and presents a delay in implementation at the onset of a pandemic of a new nature. However, as soon as the pathogen is excreted in significant amounts in faeces, a monitoring of the wastewater is proving to be an effective way to obtain detailed dynamics of the viral prevalence. This strategy is particularly recommended by WHO in the context of the global poliovirus eradication. Recent reports show that SARS-CoV-2 has been detected in stool of COVID-19 cases worldwide [1-6] as well as in sewage [7-12]. **The collection of information on the occurrence and fate of this new virus in sewage is important to determine the extent to which sewage surveillance can be used to monitor the circulation of SARS-CoV-2 in population to complement current clinical surveillance and ultimately serve as early warning of (re)-emergence of COVID-19 at the national level**.

The Environmental Microbiology Group of the LIST has long been involved in monitoring of viruses in wastewater [13-16]. As soon as the SARS-CoV-2 outbreak spread outside of China, discussions were held between the country's major microbiology research institutes, highlighting great interest in a nationwide molecular epidemiology study. Through coordination with the staff of the wastewater treatment plants (WWTPs), LIST began to set SARS-CoV-2 monitoring in wastewater on March 31st. A composite (24-hour) influent sample has since been collected weekly from several WWTPs.

Materials and Methods

Sewage samples

From March 31st to May 11th 2020, six WWTP were sampled at the inlet of the plant according to the planning presented in Table 1. The operators of the WWTP sampled a 24-h composite sample of 96 samples of 125 mL every 15 min. Composite sample was stored at 4°C until sample processing.

Table 1- Sewage samples collected before and during the CORONASTEP study.

		Before First Covid-19 case							After First Covid-19 case								
WWTP	Inhabitants	08-Oct-20	20-Oct-20	12-Nov-20	17-Dec-20	14-Jan-20	12-Feb-20	24-Feb-20	12-Mar-20	30-Mar-20	05-Apr-20	16-Apr-20	22-Apr-20	28-Apr-20	04-May-20	10-May-20	**Total**
Beggen	139,731									x	x	x	x	x	x	x	7
Bettembourg	53,606															x	1
Schifflange	68,143	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	15
Mersch	30,473													x	x	x	3
Pétange	59,481	x	x	x	x	x	x	x	x					x	x	x	11
Hesperange	15,479													x	x	x	3
Total	366913																**40**

Sample processing

The samples were transported to the laboratory at 4°C and viral RNA was isolated on the day of sampling. Larger particles (debris, bacteria) were removed from the samples by pelleting using centrifugation at 2,400 x g for 20 min at 4°C. A volume of 120 mL of supernatant was filtered through Amicon® Plus-15 centrifugal ultrafilter

with a cut-off of 10 kDa (Millipore) by centrifugation at 3,220 x g for 35 min at 4°C. The resulting concentrate was collected and 140 µL of each concentrate was then processed to extract viral RNA using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's protocol. Elution of RNA was done in 60 µl of elution buffer.

Real-time One-Step RT-PCR

Samples are screened for the presence of *Sarbecovirus* (*Coronaviridae, Betacoronaviruses*) and/or SARS-CoV-2 virus RNA by two distinct real-time one-step RT-PCR, one on the E gene (Envelope small membrane protein) and the second on the N gene (nucleoprotein). The E gene real-time RT-PCR can detect *Sarbecoviruses*, i.e. SARS-CoV, SARS-CoV-2 and closely related bat viruses. In the context of the COVID19 pandemic, it can be assumed that only SARS-CoV-2 strains will be detected by this assay given that SARS-CoV virus has been eradicated and other bat viruses do not commonly circulate in the human population. The E gene assay is adapted from Corman et al. [17]. The N1 gene real-time RT-PCR assay specifically detects SARS-CoV-2 virus. It is adapted from the CDC protocol[1]. The two primers/probe sets were presented in Table 2. The RT-qPCR protocols and reagents were all provided by the LIH.

Table 2 – RT-qPCR primer-probe sets

Target	Primer name	Primer sequence (5' to 3')	References
E gene	E_Sarbeco_F1	5-ACAGGTACGTTAATAGTTAATAGCGT-3	Corman et al., 2020
	E_Sarbeco_R2	5-ATATTGCAGCAGTACGCACACA-3	
	E_Sarbeco_P1	5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	
N gene	2019-nCoV_N1_Fw	5'-GAC CCC AAA ATC AGC GAA AT-3'	CDC
	2019-nCoV_N1_Rv	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	
	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	

Each reaction contained 5 µl of RNA template, 5 µl of TaqPath 1-step RT-qPCR MasterMix (A15299, Life Technologies), 0.5 µL of each primer (20 µM) and probe (5 µM) and the reaction volume was adjusted to a final volume of 20 µl with molecular biology grade water. Thermal cycling reactions were carried out at 50 °C for 15 min, followed by 95 °C for 2 min and 45 cycles of 95 °C for 3 sec and 58°C (E gene) or 53°C (N gene) for 30 sec using a Viia7 Real-Time PCR Detection System (Life Technologies). Reactions were considered positive (limit of detection – LOD) if the cycle threshold was below 40 cycles.

Controls

A non-target RNA fragment commercially available (VetMAX™ Xeno™ IPC and VetMAX™ Xeno™ IPC Assay, ThermoFischer Scientific) was added to the viral RNA extract from sewage concentrates as an internal positive control (IPC). This IPC-RNA is used to control the performance of the RT-qPCR (E gene) and to detect the presence of RT-qPCR inhibitors.

Viral RNA copies quantification of both targeting genes in wastewater samples was performed using a RT-qPCR standard curves generated using EDX SARS-CoV-2 Standard (Biorad). This standard is manufactured with synthetic RNA transcripts containing 5 gene targets (E, N, S, ORF1a, and RdRP genes of SARS-CoV-2, 200,000 copies/mL each).Using such a standard, the limits of quantification (LOQ) of both RT-qPCR assays were estimated to 1 RNA copy per reaction (Figure 1).

[1] https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf

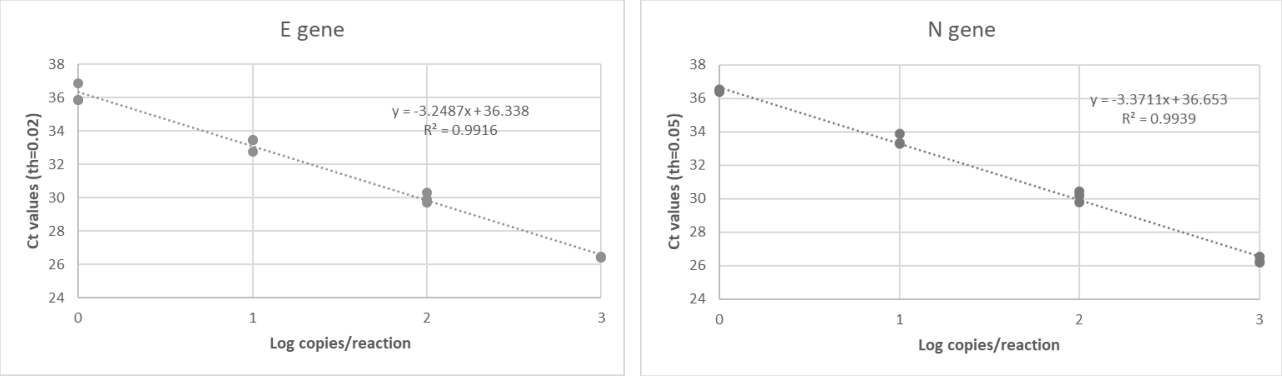

Figure 1 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

Results

Broadly speaking, a wastewater sample is declared positive if both RT-qPCR present a positive signal. If only one RT-qPCR assay give a positive signal, the sample is suspected as positive. In this case, a supplementary analysis could be carried out to confirm the result.

Qualitative results – The Table 3 presents an overview of all results gathered to date. All samples collected prior to the first confirmed Covid-19 case and tested so far are scored negative for the detection of the SARS-CoV-2, with the exception of the sample collected on February 25th. This particular sample was suspected to be positive for the SARS-CoV-2 because it showed a positive signal for the E gene only, with a Ct value above the LOD but below the LOQ. All samples collected after the first confirmed case of Covid-19, and between March 11 and May 4, are declared positive for the presence of SARS-CoV-2 RNA. Since May 10th, the viral RNA is no longer detectable in wastewater at most of the treatment plants tested, with the exception of Beggen.

Table 3 – Results of the screening of SARS-CoV-2 in 24h composite samples of incoming wastewater at different WWTP in Luxembourg approx. 4.5 months before and 3 months after the first COVID-19 case. ND: sampled but not tested yet.

		Before First Covid-19 case							After First Covid-19 case										
WWTP	Inhabitants	08-Oct-20	20-Oct-20	12-Nov-20	17-Dec-20	14-Jan-20	12-Feb-20	25-Feb-20	11-Mar-20	30-Mar-20	05-Apr-20	16-Apr-20	22-Apr-20	28-Apr-20	04-May-20	10-May-20	Tested samples	Positive samples	Positive rate (%)
Beggen	139731									+	+	+	+	+	+	+	7	7	100
Bettembourg	53606															-	1	0	0
Schifflange	68143	-	-	-	-	-	-	+/-	+	+	+	+	+	+	+	-	15	7	47
Mersch	30473													+	+	-	3	2	67
Pétange	59481	ND	ND	ND	ND	ND	ND	ND	ND					+	+	-	3	2	67
Hesperange	15479													+/-	+	+/-	3	3	100
Total	366913																**32**	**21**	**66**

Quantitative Results – Quantitative results are shown in Figure 2 and 3 for the WWTP of Schifflange and Beggen, respectively; both treatment plants were sampled weekly starting in mid-March.

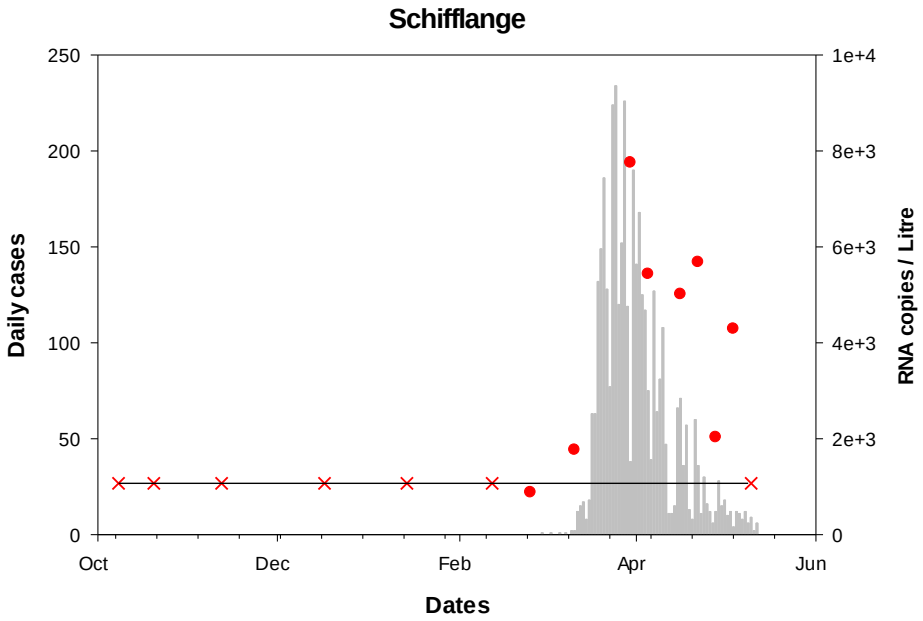

Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from Schifflange wastewater treatment plant (from Oct 8th to May 11th). Grey squares: daily confirmed cases[2], red dots: positive samples, red cross: tested but negative samples, black line: limit of quantification (LOQ).

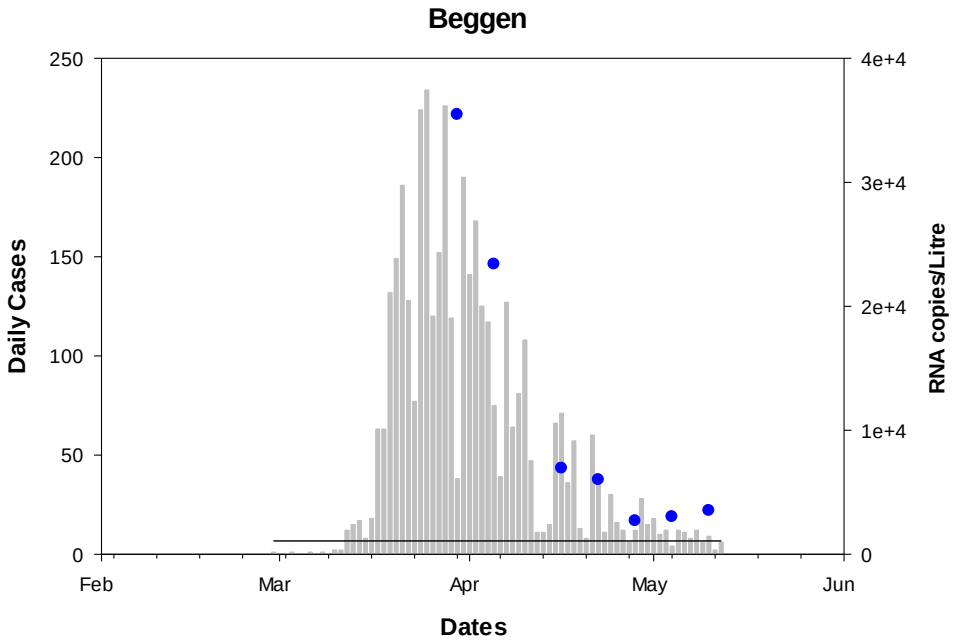

Figure 3 - RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from Beggen wastewater treatment plant (from March 31st to May 11th). Grey squares: daily confirmed cases[1], blue dots: positive samples, blue cross: tested but negative samples, black line: limit of quantification (LOQ).

[2] *https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6*

We compared the average level of SARS-CoV-2 genome in wastewater samples over time with the number of confirmed Covid-19 cases in Luxembourg. As assumed, we confirmed that the increase and the subsequent decrease of the viral RNA copies in wastewater influents accurately followed the increase and the decrease of the Covid-19 cases observed at the national level (Figures 2 and 3). This is all the more true and all the more visible for the Beggen WWTP, which is the largest in the country.

In conclusion, this preliminary work has demonstrated that quantitative monitoring of SARS CoV-2 RNA in wastewater influents can provide additional relevant information for a better monitoring and surveillance of the circulation of SARS CoV-2 at the national level. Indeed, our results showed that sewage contamination and viral genome detection can take place before the start of the exponential growth of the epidemic. These interesting results argue, in particular, in favor of long-term wastewater monitoring, which would make it possible to alert the authorities to the occurrence of a possible epidemic and the emergence of new viruses circulating in the population.

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