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Development of a Collection Kit for Efficient Isolation of SARS-CoV-2 RNA from Urine and Stool Samples

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background Information: Consider adding a brief sentence about the significance of detecting SARS-CoV-2 in urine and stool samples. For example, mention how these samples can serve as alternative sources for diagnosis, potentially leading to better patient outcomes.
Objective Statement: This study aimed to develop a collection kit for the effective extraction of SARS-CoV-2 RNA from urine and stool samples. The study presents a significant advancement in the field of viral diagnostics by introducing a novel collection kit specifically designed for the extraction of SARS-CoV-2 RNA from urine and stool samples. The demonstrated efficacy of the kit in isolating the virus with high success rates from alternative biological fluids, as well as the superior RNA.

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Methodology: The kit contains a swab of medical polyester and three tubes, two of them used to inactivate the virus, one contains 3% phenol and the other 1% phenol, and the third tube contains 2 ml of (PBS) Phosphate Buffer Saline added to it 4% of skim milk. Urine or stool samples from Covid-19 patients who reported positive by nasopharyngeal swab during RT-PCR was adde consequently in these three tubes then assessed through RT-PCR testing.
Results: The kit succeeded in isolating coronavirus from the urine and stool of patients who reported positive by nasopharyngeal samples. From 20 positive nasopharyngeal samples, there was 18 (90%) of them were positive by using stool sample using this kit, and 15 (75%) of them were give positive result when urine samples were used by this kit. The concentration of RNA of coronavirus which was isolated from urine and feces was higher than in the nasopharyngeal swab during RT-PCR. Mean Ct value of the 20 samples was showed that the mean Ct of nasopharyngeal was 31.5, while urine and stool samples Ct means less concentration of virus. were 25.75 and 24.32 respectively. High The kit was able to serve RNA that was isolated from urine and feces for about 5,10, and 30 days in a percentage of 100%, 94.1%, and 94.1% respectively.
Conclusion and Implications: The kit is produce to confirm that the patient is infected with by COVID-19 and to preserve the virus in stool or urine samples of the infected person for a long period until laboratory tests through RT-PCR are conducted on the samples. The kit showed great ability to test SARS-CoV-2, even if it was at low concentrations of virus compared to the examination of nasopharyngeal swabs.

Keywords: SARS-COV-2; RNA preservation; viral detection; molecular detection.

1. INTRODUCTION

The emerging coronavirus, SARS-CoV2 (Severe Acute Respiratory Syndrome), is one of the developed viral types of the coronavirus family, which causes acute respiratory infection, from which its name is derived (Anand et al., 2020). At the end of 2019, a disease resembling the common flu appeared and developed into bronchitis, causing death. The disease appeared in the Chinese city of Wuhan and then was recorded in many countries of the world until the World Health Organization declared it a global epidemic on the 3rd of November 2020 (World Health Organization, 2020).

The health protocols in force from the World Health Organization mentioned its recommendation for taking samples from the pharynx or/and nasopharynx of infected people (World Health Organization, 2020). Many studies showed that isolating the virus from sputum samples was more effective than isolating it from the nasal area (Han & Ivanovski, 2020), and the virus was also isolated from saliva by using the drool saliva collection method (Azzi et al., 2020). The most common method used to diagnose COVID-19 is the detection of SARS-CoV-2 in upper and lower respiratory tract specimens, including nasopharyngeal swabs, pharyngeal swabs, sputum, lower respiratory tract aspiration, and bronchoalveolar lavage. Genetic testing methods, such as real-time reverse transcription polymerase chain reaction (RT-PCR), are the standard laboratory testing methods for COVID-19 currently used in most countries (To et al., 2020).

A study reported that the virus can be detected in body fluids such as serum, urine, and feces, along with respiratory samples (Kim et al., 2020). A survey of 39 studies from 12 different countries was done on a total of 533 patients who were tested for coronavirus during their stay in hospitals and up to 52 days after the onset of symptoms. The results confirmed the presence of the virus in urine samples in 20% of the samples studied in China, Korea and Japan (Kim et al., 2020; Peng et al., 2020). Viruses were found in urine samples at different times, from the first day to 52 days, and in varying proportions that depended mainly on the disease state of the infected person (Thiel et al., 2020).

One of the experiments conducted by Sun et al., (2020). showed that the virus isolated from the urine remains active and capable of infecting Vero E5 cells *in vitro* and causing a devastating effect on the cells.

In addition to the discovery of the virus in the urine by numerous researchers, the virus was also found in the feces of patients in about 32-67% of samples during and after 21 days of infection by using Cepheid Xpert Xpress SARS-CoV-2 and Hologic Panther Fusion real-time RT-PCR assays (Szymczak et al., 2020). Various solutions were used to preserve stool and urine

samples to test for the presence of viruses, or to preserve DNA samples isolated from stool and urine. Amies media was used, which consisted of sodium, potassium, calcium and magnesium chloride salts, in addition to potassium and disodium phosphate with charcoal (Amies, 1967; Isenberg, 2004). Whereas, since the late sixties, the charcoal medium has been used to transmit viruses, which contains potassium chloride, sodium chloride, dipotassium phosphate (Leibovitz, 1969). Genefec solution with EDTA was also used to preserve DNA pieces while isolating them from the urine (Carozzi & Sani, 2013). Also, Cary and Blair's media is still used to isolate viruses from urine and excretion, and it is a medium that contains sodium thioglycolate, disodium phosphate, sodium chlorate and calcium (Cary & Blair, 1964) during the Corona pandemic. Normal saline (0.85%) was used to transport stool and urine samples to testing laboratories (Szymczak et al., 2020). In addition to using Viral Transport Media in transporting stool and urine samples, as well as using it in transporting nasopharyngeal, pharynx, and rectal swabs to testing laboratories (Xu et al., 2020).

This study presented a new method to isolate the virus from the urine and feces by using a kit that contains stabilizers and other neutralizing and preservative chemicals components so that the sample can be kept for a longer period before performing a RT-PCR test.

2. MATERIALS AND METHODS

This is a quasi-experiment research which used to compare between three types of samples for Covid-19 positive patients. Nasopharyngeal, urine and stool samples were used. The kit contains a polyester swab was used for stool sampling, consisting of polyester with a wooden stick and a tube of 15 ml with a cap (Fig. 1). The kit also contains a plastic dropper of 5 ml to take a urine sample and to transfer the sample from one tube to another.

- **The first tube:** contains 2ml of normal saline solution to which 0.5ml of Penicillin-Streptomycin antibiotic solution (110000U/ml) from (MENAMIRI, Italy) and 500µg of Amphotericin B from (BPRL, India) are added to prevent microbial growth in the sample.
- **The second tube:** contains 2 ml of a 1 ml phenol solution with a concentration of (10%) phenol with 4% sodium dodecyl sulfate (SDS) with 0.5M of sodium chlorate.
- **The third tube:** contains 1 ml of 0.5% phenol.
- **The fourth tube:** contains 1 ml of (10%) phosphate buffer saline solution, to which 4% skim milk was added.

2.1 Sampling

Because of the restriction due to COVID-19 crisis, only 20 positive patients were tested. A stool and urine sample were taken from 20 people infected with the Coronavirus who showed symptoms of diarrhea, and their results were positive for the virus by examining the nasal swab samples by RT-PCR. Stool samples were taken using polyester swabs and transferred to the kit.

The swab is placed in the first tube containing saline solution containing antibiotics with shaking to try to lower the sample into the tube. Leave the sample in the tube for 30 min, during which the sample is mixed well with the antibiotic solution in the tube using a plastic pipette. Then it was centrifuged at a speed of 4000 pm for 4 min at a temperature of 25°C.

Transfer 1 ml of the filtered solution in the first tube to the second tube containing phenol with sodium dodecyl sulfur with sodium chlorate to extract the genetic material of the virus. Leave the sample for 10 min, then treat it with a centrifuge for 4 min under the previous conditions. Then 1 ml of the sample filtrate is transferred from the second tube to the third tube containing phenol at a concentration of 0.5% to preserve the nucleic acid in the sample and left for 5 min after which centrifugation is used again under the same conditions and 1 ml of the filter is taken to the fourth tube containing phosphate saline with skim milk to stabilize and preserve the RNA of the virus. This sample can be kept for a long time reached to a month in a normal refrigerator at a temperature of 4°C until the examinations are conducted.

2.2 Efficiency of the Kit in Preserving Samples

Fecal and urine samples that showed positive results by RT-PCR were kept for different periods of 5, 10 and 30 days in the solution at a temperature of 4°C.

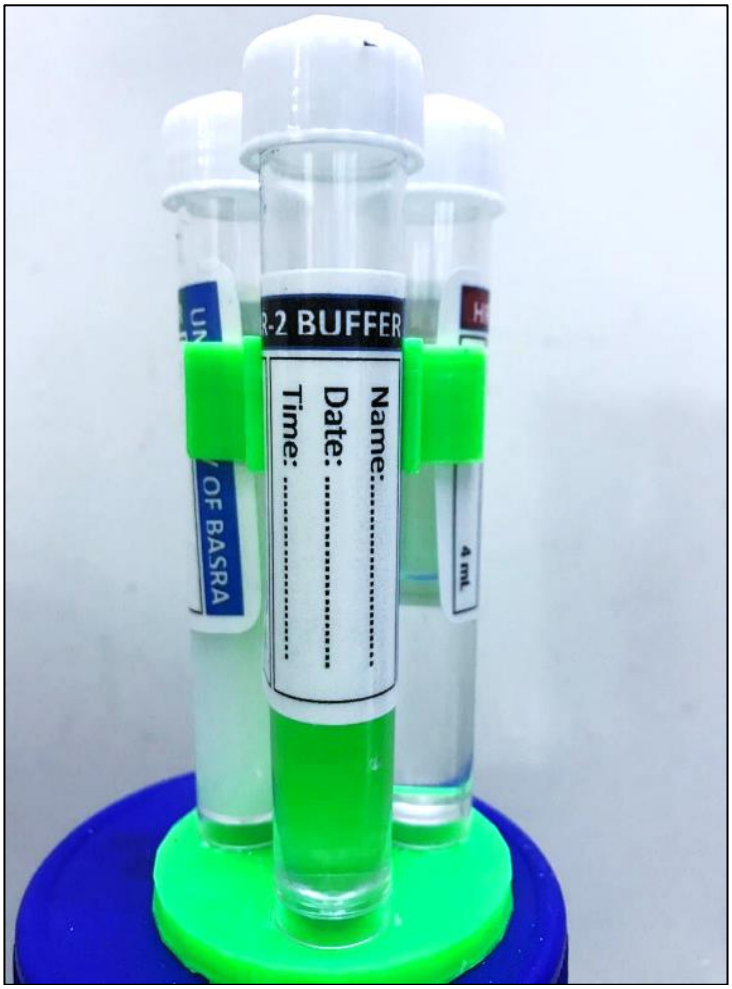

Fig. 1. Kit component, the fourth tube in a holder with the label of each one

2.3 Calculation of RNA Concentration

RNA extraction samples absorbance were measured at 260 nm and 280 nm, and the ratio 260/280 was performed to qualify the purity RNA concentrations by ng/ml according to Wilfinger et al. (1997). Calculating the gene cycle threshold value for the ORF1ab gene, which indicates the concentration of RNA in the sample. The value of ($Ct \geq 35$) was low, ($25 < Ct < 35$) medium, and ($Ct \leq 25$) high, according to the kit used.

2.4 Examination of Coronavirus by Real-Time PCR

The routine examination of the virus was carried out using the RT-PCR technique in the central laboratory of the Basra Health Department, Basra Health Directorate, Basra, Iraq.

3. RESULTS AND DISCUSSION

The kit is designed to isolate the Coronavirus from stool and urine, which may be considered a confirmatory test for infection with the emerging coronavirus. The kit contained four tubes: the first was used to remove microbial contamination using an antibiotic solution consisting of Penicillin-Streptomycin to get rid of bacteria and Amphotericin to get rid of fungi. These antigens were used in many types of virus-carrying media and solutions used for the same purpose, and they proved their efficiency in eliminating bacterial contamination that may cause interference in the results of examining samples using RT-PCR (Eagle et al., 1952; Bishai & Labzoffsky, 1974).

A 1ml of the solution in the first tube was transferred after being treated with antibiotics solution to the second tube containing phenol with sodium dodecyl sulfur with sodium chlorate,

which was used as an extraction solution for RNA, which is a modified step from the RNA extraction method used by Nwokeoji et al., (2005). where the cell analysis solution was used without phenol. The use of phenol has been tried to contain stool and urine samples on salts and food residues and phenol can stop its effectiveness against extraction. This method has also been used in other research works (Nwokeoji et al., 2016; Rio et al., 2010). In addition, SDS was used because of its efficiency in binding to proteins in the stool and urine samples of patients, and thus proteins precipitate with SDS by centrifugation (Chomczynski & Sacchi, 2006).

A phenol solution with a lower concentration was used in the third tube as a safe substance to preserve the RNA of the virus (Nwokeoji et al., 2016).

In the fourth stage, 1 ml of the sample is transferred in the third tube to the fourth tube containing a phosphate-saline solution that provides a suitable medium for the survival of the genetic material. Skimmed milk was added to the solution to provide a solution with high stability for RNA (Akinwole & Babarinde, 2019) and thus It can be saved for a longer time and its efficiency has been confirmed.

In infected persons for whom nasopharyngeal swabs showed positive results, urine and stool samples were taken from them, and the results were positive by 75% and 90%, respectively, as shown in Table 1.

The RNA extracts of the positive urine and stool samples were kept for 5, 10 and 30 days at a temperature of 4°C. The RNA samples extracted from the urine preserved during the mentioned periods showed results of RNA stability of 100%, 94.1%, and 94.1%. One sample appeared negative after storage for 10 days.

As for the RNA extracts that were isolated from the positive stool samples, they were stable in the storage periods by 100% during the 5, 10 and 30 days, as shown in Table 2.

Previous studies showed that some of the results of the examination of emerging COVID-19 patients are inconsistent between the examination of nasal and nasopharyngeal swab samples and stool or urine swabs in many research (Szymczak et al., 2020; Lau et al., 2005; Chen et al., 2020). This may be due to the medium in which the sample is taken. In all samples studied, VTM or Phosphate Buffer Saline was used to collect urine or stool samples without observing an appropriate sample preservation process or an extraction process in which the effect of enzymes that destroy the genetic material of the virus RNases is reduced (Sun et al., 2020; Zhang et al., 2020).

Table 1. Positive and negative samples of patients using nasal swabs in the usual way, urine and feces using the kit method

Number of Patients	Nasal Sample	Stool Sample	Urine Sample
1	+	+	-
2	+	+	+
3	+	+	+
4	+	+	-
5	+	+	+
6	+	+	+
7	+	-	-
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	-	-
14	+	+	-
15	+	+	+
16	+	+	+
17	+	+	+
18	+	+	+
19	+	+	+
20	+	+	+

Table 2. Results of keeping sample extracts for different periods

Sample Numbers	5 Days		10 Days		30 Days	
	Urine	Stool	Urine	Stool	Urine	Stool
1	+	+	-	+	-	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
11	+	+	+	+	+	+
12	+	+	+	+	+	+
13	+	+	+	+	+	+
14	+	+	+	+	+	+
15	+	+	+	+	+	+
16	+	+	+	+	+	+
17	+	+	+	+	+	+

Table 3. Cycle threshold (Ct) values in nasopharyngeal, excretory and diuresis swab samples

Sample Number	Ct Value of Urine Extraction	Ct Value of Stool Extraction	Ct Value of NS
1	22.6	18.9	31.21
2	26.71	22.82	30.98
3	22.78	25.76	32.44
4	30.81	19.82	31.04
5	27.62	21.22	34.22
6	40.00	28.31	28.51
7	39.89	27.73	27.10
8	23.01	22.2	25.25
9	30.66	28.31	34.21
10	39.9	18.91	33.61
11	30.11	24.25	30.01
12	28.00	23.32	28.8
13	40.01	30.3	34.71
14	31.12	28.90	39.2
15	39.88	26.42	36.61
16	30.09	22.2	33.42
17	27.65	24.56	28.71
18	23.41	22.1	25.77
19	30.72	24.7	31.4
20	30.02	25.6	33.3

It is clear from Table 3 and Fig. 2 that the use of the kit showed a higher concentration of viral RNA in the urine and feces than in the nasopharyngeal swab samples. The figure shows the RNA concentration, which represents the value of the cycle threshold (ct), where it is noted that the ct value decreases using the kit compared to nasal swabs that were taken normally without using the virus isolation kit. It should be noted that the ct value reflects the concentration of the nucleic acid of the virus, where And based on the health specifications approved by the health departments and adopted by the World Health Organization for the new Corona test kit with the RT-PCR device, the value (Ct≥35) means a small number of the virus's nucleic acid, i.e. the percentage of virus presence is low, (25<Ct<35) medium, (Ct ≤ 25) high, meaning that the concentration of DNA is large, which was observed using the kit for urine and stool samples to a greater extent compared to nasopharyngeal swab samples taken using the VTM carrier medium.

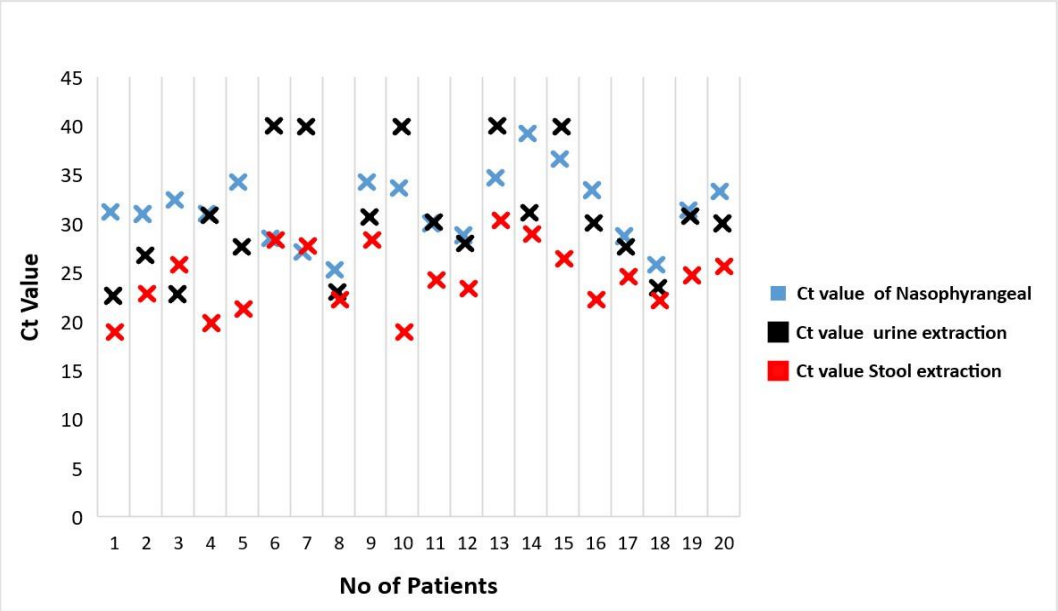

Fig. 2. Distribution of the Ct value of the nasopharyngeal, urine and fecal samples of the examined patients

4. CONCLUSION

Production of a kit to isolate the emerging coronavirus from urine and stool samples. The kit is used to isolate and extract the RNA of the Coronavirus for the purpose of RT-PCR examination and saves RNA viruses for a long period. It reduces contamination that may occur during the transfer of samples and during the examination through materials that inhibit the virus to stop its ability to infect workers.

5. RECOMMENDATION

The health authorities in the province approved taking stool and urine samples from only 20 people infected with the disease due to Covid-19 crisis and the restrictions from health directorate. Further research is needed to study a large size sample to confirm the comparative result.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

This research was approved by the Ethics Committee of the Al-Zahraa College of Medicine, University of Basrah under code number E/T 35.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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