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Rapid RT-PCR identification of SARS-CoV-2 in screening donors of fecal microbiota transplantation

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ABSTRACT

Since its first appearance in late 2019 in Wuhan, China, severe acute respiratory syndrome caused by Coronavirus 2 (SARS-CoV-2) has had a major impact on healthcare facilities around the world. Although in the past year, mass vaccination and the development of monoclonal antibody treatments have reduced the number of deaths and severe cases, the circulation of SARS-CoV-2 remains high. Over the past two years, diagnostics have played a crucial role in virus containment both in health care facilities and at the community level. For SARS-CoV-2 detection, the commonly used specimen type is the nasopharyngeal swab, although the virus can be identified in other matrices such as feces. Since fecal microbiota transplantation (FMT) assumes significant importance in the treatment of chronic gut infections and that feces may be a potential vehicle for transmission of SARS-CoV-2, in this study we have evaluated the performance of the rapid cartridge-based RT-PCR test STANDARD™ M10 SARS-CoV-2 (SD Biosensor Inc., Suwon, South Korea) using fecal samples. The results obtained indicates that STANDARD™ M10 SARS-CoV-2 can detect SARS-CoV-2 in stool samples even at low concentration. For this reason, STANDARD™ M10 SARS-CoV-2 could be used as reliable methods for the detection of SARS-CoV-2 in fecal samples and for the screening of FMT donors.

1. Introduction

Since its emergence in late 2019, Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) has led to considerable pressure on hospital wards and, more generally, on the world population [1–3]. Today (April 2023), circa three years after the start of the pandemic, has been registered 762.201.169 cases and 6.893.190 deaths globally due to SARS-CoV-2 infection [4]. Since the beginning of the pandemic, the most used sample for detecting SARS-CoV-2 has been the nasopharyngeal swab (NPS), although the virus can also be detected in other matrices such as saliva, bronchoalveolar lavage and feces [5–9]. Therefore, most diagnostic tests on the market today are only validated on NPS, which is consider the "gold standard" sample type [10]. In parallel with the analysis of nasopharyngeal swabs since the beginning of the pandemic circulation of SARS-CoV-2, the presence of the virus has been observed in feces and its possible implications [11]. Nowadays, at one stage we have moved from virus containment to coexistence with the virus, and SARS-CoV-2 diagnoses are requested on different matrices, in particular for specific

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applications in the hospital environment [6,12,13]. One important emerging practice is fecal microbiota transplantation (FMT), required to treat patients with recurrent intestinal infections due to presence of *C. difficile* [14,15]. When FMT takes place, due to the persistent virus circulation, it appears appropriate to exclude the presence of SARS-CoV-2 in donor feces [12,16,17]; (Di Pilato et al., 2022). In particular, the request for a diagnostic test that can detect SARS-CoV-2 in faecal samples to be used for FMT has been advocated by Khanna and colleagues since the early stages of the pandemic [18]. This concept is taken up in Ref. [19]; also citing an alert from the FDA itself [19]. Although some assays designed for the use of nasopharyngeal swabs have already been evaluated on other sample types such feces [19]; (Di Pilato et al., 2022), the number of tests currently commercially available to assess the presence of SARS-CoV-2 in stool samples is extremely scarce. For this reason, in this study we analyze the performance of STANDARD™ M10 SARS-CoV-2 (SD Biosensor Inc, Suwon, South Korea) using fecal samples. STANDARD™ M10 is a cartridge-based RT-PCR test processed exclusively on the modular STANDARD™ M10 instrument. The latter is a walk-away and random access all-in-one system processing the sample from extraction to results. Recent publications show that the performance of the STANDARD™ M10 SARS-CoV-2 with nasopharyngeal swabs is comparable to that of the main commercially available systems [20–22], but there isn't yet no study about its use with stool samples. Given the on-demand nature of the test, its ease of set-up and its speed of execution (60 min), evaluating the performance of STANDARD™ M10 SARS-CoV-2 on fecal samples can greatly improve the detection of SARS-CoV-2 in donor material destined for FMT.

2. Results

2.1. Negative assessment for SARS-CoV-2 in the selected samples

Before investigating the 10 stool samples, we evaluated whether the selected samples were negative for the presence of SARS-CoV-2. The 10 stools samples have been diluted in UTM in order to obtain a fecal suspension characterized by a final concentration of 6 mg/mL, this control has been named Control 1. Although sterility is guaranteed by the manufacturer, we also decided to test the diluent used i.e., the UTM buffer transport medium (named Control 2) mainly to demonstrates that cross-contamination did not occur as a result of handling SARS-CoV-2-positive samples during the performance of the experiments.

The results of these tests are summarized in Table 1: both fecal suspension and UTM for each samples were negative after processing with STANDARD™ M10 SARS-CoV-2. This result shows that the 10 stool samples selected for experiments are not characterized by inherent positivity to the virus, a condition that would have rendered the tests invalid due to the presence of the analyte in the blank. Verification of SARS-CoV-2 negativity of the fecal samples used for the validation tests was necessary because the clinical history of the patients from whom these samples came was not investigated before the experiments were performed. Consequently, given the high prevalence of COVID-19 during the months in which the experiments were conducted, the presence of viral particles in the stool samples due to ongoing or past COVID-19 positivity of the patients could have been likely.

In addition, the absence of invalid or inconclusive results when the STANDARD™ M10 SARS-CoV-2 assay was used to process the 10 fecal suspensions eluted in UTM at the final concentration of 6 mg/mL attests to the correct execution of the rRT-PCR and, consequently, the absence of amplification reaction inhibitory agents in the samples under investigation. This was confirmed for each fecal suspension by the positivity of the internal control (IC) in conjunction with the lack of amplification of target E and ORF1ab (Table 1). The absence of inhibitory agents is also confirmed by examining the cycle threshold (C_T) ranges of the IC present in each test, which as can be seen in Table 1 do not differ in the two conditions. For these reasons, the 6 mg/mL concentration of feces was therefore used in all subsequent experiments in this work.

2.2. Evaluation of STANDARD™ M10 SARS-CoV-2 performance using stool samples spiked with known SARS-CoV-2 positive samples

The presence of SARS-CoV-2 was assessed in the samples added with dilutions containing the virus using SD Biosensor STANDARD™ M10 SARS-CoV-2 assay. Table 2 shows the C_T values of the E and ORF1ab targets genes and the results of the STANDARD™ M10 SARS-CoV-2 assay performed on the 60 samples added with 1:2, 1:1000 and 1:1,000,000 dilutions of SARS-CoV-2, 30 of which consisted of Fecal suspension + SARS-CoV-2 positive UTM (referred to from here on as FS + SARS-CoV-2 samples) and 30 consisted of positive SARS-CoV-2 UTM (referred to from here on as UTM + SARS-CoV-2 samples, Table 2).

In FS + SARS-CoV-2 samples the STANDARD™ M10 SARS-CoV-2 assay was positive in 100% of samples both at 1:2 dilution and at 1:1000 dilution; the positivity decreased to 60% of samples at 1:1,000,000 dilution of virus (Tables 2 and 3).

In the UTM + SARS-CoV-2 samples the STANDARD™ M10 SARS-CoV-2 assay resulted positive in 100% of the samples with 1:2

Table 1
Presence assessment of SARS-CoV-2 in the fecal suspension and diluent (UTM) used in this work.

	Control 1	Control 2
	Fecal suspension	UTM
STANDARD™ M10 Result – SARS-CoV-2 Negative	10/10 (100%)	10/10 (100%)
E target negative	10/10 (100%)	10/10 (100%)
ORF1ab target negative	10/10 (100%)	10/10 (100%)
Internal control (IC) positive	10/10 (100%)	10/10 (100%)
Internal control (IC) C_T range	22,93–26,23	23,68–26,09

Table 2
Cycle threshold (C_T) for each sample in both fecal suspension and UTM. Res = Result obtained by STANDARD™ M10 instrument, P = Positive, PP = Presumptive Positive, N = Negative.

	Fecal suspension + SARS-CoV-2 positive UTM (FS + SARS-CoV-2)									Positive SARS-CoV-2 UTM (UTM + SARS-CoV-2)								
	Dilution 1:2			Dilution 1:1000			Dilution 1:1,000,000			Dilution 1:2			Dilution 1:1000			Dilution 1:1,000,000		
Sample N	**E gene**	**ORF1ab gene**	**Res**	**E gene**	**ORF1ab gene**	**Res**	**E gene**	**ORF1ab gene**	**Res**	**E gene**	**ORF1ab gene**	**Res**	**E gene**	**Orf1AB gene**	**Res**	**E gene**	**Orf1AB gene**	**Res**
1	15,51	16,31	P	23,92	23,43	P	33,46	0	PP	12,15	13,55	P	24,02	25,59	P	29,74	31,01	P
2	15,79	17,13	P	24,88	26,25	P	32,48	33,03	P	15,75	16,41	P	22,56	24,02	P	0	32,3	P
3	17,94	18,97	P	25,4	25,78	P	35,23	0	PP	15,23	16,23	P	24,25	25,39	P	32,94	33,73	P
4	19,25	19,93	P	28,78	29,28	P	0	0	N	20,38	21,10	P	26,51	27,25	P	34,33	0	PP
5	15,88	17,35	P	26,37	24,52	P	34,58	33,57	P	16,51	17,94	P	24,8	26,47	P	33,25	34,49	P
6	15,93	16,77	P	24,57	24,44	P	34,73	0	PP	15,69	16,09	P	24,5	25,09	P	30,82	31,68	P
7	20,3	21,38	P	29,87	30,38	P	0	0	N	18,74	19,43	P	26,17	26,71	P	36,27	0	PP
8	23,22	23,74	P	31,19	31,17	P	0	0	N	21,08	21,58	P	26,95	27,3	P	0	0	N
9	19,4	19,9	P	27,23	27,34	P	35,71	0	PP	19,4	19,9	P	26,13	26,75	P	35,02	33,88	P
10	17,67	18,06	P	25,49	25,2	P	0	0	N	17,63	18,07	P	25,15	25,86	P	35,79	35,26	P

dilution, 100% of the samples with 1:1000 dilution, and 90% of the samples with 1:1,000,000 dilution of the virus (Tables 2 and 3). In all positive replicates of 1:2 and 1:1000 dilutions, using as diluent both fecal suspension or UTM the result was determined by amplification of both targets (Tables 2 and 3).

Regarding the FS + SARS-CoV-2 samples diluted 1:1,000,000 in 3 out of 10 cases we observed amplification of the E gene alone; this phenomenon also occurs in 2 out of 10 cases of the UTM + SARS-CoV-2 matches diluted 1:1,000,000. Accordingly with the manufacturer this result was recorded as presumptively positive (PP). Given the local epidemiological context at the time of this study, the observation of a presumptive positive result was considered to be highly indicative of the presence of SARS-CoV-2 and subsequently a positive result.

Analyzing in detail the FS + SARS-CoV-2 samples at the 1:1,000,000 dilution, it is observed that the STANDARD™ M10 SARS-CoV-2 test identifies 6 out of 10 samples as positive (Tables 2 and 3). Concerning the 4 samples provided as negatives, it is worth noting that 3 of them, namely samples 4,7,10 diluted in UTM (UTM + SARS-CoV-2 samples) provided results characterized by very elevate C_T, suggesting a low viral load. The 1:1,000,000 dilution is the higher used in this work and it is possible that some diluted positive samples are no longer identified as such by the test. This is because the dilution results in a final copy number in the sample of SARS-CoV-2 that is below the limit of detection (LoD) of the STANDARD™ M10 SARS-CoV-2 test. In particular for sample 4, 7 and 10, the C_T were higher than 34, 36 and 35 respectively thus indicating a low viral load due to dilution operated (Table 2). In addition, sample 8 gave at dilution 1:1,000,000 a negative result even when diluted in UTM (UTM + SARS-CoV-2 samples). Notably this sample, among the 10 selected, showed the highest C_T, even at the lowest dilution factor both in UTM and in fecal suspension, thus suggesting a low viral load within the starting sample (Table 2).

The differences in C_T values between the two experimental conditions (fecal suspension + SARS-CoV-2 positive sample/UTM + SARS-CoV-2 positive sample) were not found to be statistically significant for either target E (p-value 0.8015 calculated with Mann-Whitney test because the numeric variable does not have a normal distribution - Fig. 1A) or target ORF1ab (p-value 0.0797 – calculated with t Student test because the numerical variable has a normal distribution - Fig. 1B).

3. Materials and methods

3.1. Specimen collection

This validation study was performed using 10 fresh stool samples from residual specimens from as many patients whose health status is not known. These samples came from the routine laboratory and were totally anonymized.

In order to spiked the 10 fresh stool sample described above we selected 10 positive nasopharyngeal samples collected with flocked swab and stored in Universal Transport Medium™ (UTM®, Copan Diagnostic, Italy). Nasopharyngeal samples were analyzed for the presence of SARS-CoV-2 using the Xpert® Xpress SARS-CoV-2 (Cepheid, Sunnyvale, USA) rRT-PCR assay. We selected samples characterized by high viral load; in particular the C_T of the target genes ranged between 14 and 17 (data not shown).

3.2. Preparation of fecal suspension spiked with SARS-CoV-2

For each of the 10 experiments, a stool sample was diluted in 3 mL of sterile UTM to a final concentration of 6 mg/mL (average value) and eight aliquots were then prepared. Of these, the first was spiked with an equal volume of SARS-CoV-2 positive UTM, while the other six were spiked with a 10-fold serial dilution of the same SARS-CoV-2 positive UTM. Thus, from the first aliquot it was obtained the 1:2 dilution of SARS-CoV-2 positive UTM in the fecal suspension and from the subsequent six were obtained 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions respectively. The last aliquot was not artificially infected and was used as negative control to exclude the SARS-CoV-2 positivity of the stool sample (Fig. 2A).

In parallel eight aliquots of sterile UTM were prepared with the same samples and the same scheme described above and the experiment was reproduced in order to compare the data obtained between the two different experimental conditions (Fig. 2B).

For both fecal suspension and UTM the aliquot tested are the dilutions 1:2, 1:1000 (10^{-3}), 1:1,000,000 (10^{-6}) and the controls, for this reason in Fig. 2 their color appear clear and not opaque. Fecal suspensions with spiked virus always have a concentration very close

Table 3
Overview of STANDARD M10 SARS-CoV-2 test results of samples consisting of fecal suspension with added dilutions of SARS-CoV-2 and UTM with added dilutions of SARS-CoV-2 in the ten validation samples.

	Fecal suspension + SARS-CoV-2 positive UTM (FS + SARS-CoV-2)			Positive SARS-CoV-2 UTM (UTM + SARS-CoV-2)		
	Dilution 1:2	**Dilution 1:1000**	**Dilution 1:1,000,000**	**Dilution 1:2**	**Dilution 1:1000**	**Dilution 1:1,000,000**
STANDARD™ M10 result – SARS-CoV-2 Positive[a]	10/10/ (100%)	10/10 (100%)	6/10 (60%)	10/10 (100%)	10/10 (100%)	9/10 (90%)
E gene target positive	10/10/ (100%)	10/10 (100%)	6/10 (60%)	10/10 (100%)	10/10 (100%)	8/10 (80%)
ORF1ab gene target positive	10/10/ (100%)	10/10 (100%)	2/10 (20%)	10/10 (100%)	10/10 (100%)	7/10 (70%)

[a] In positive result are included also the presumptive positive ones.

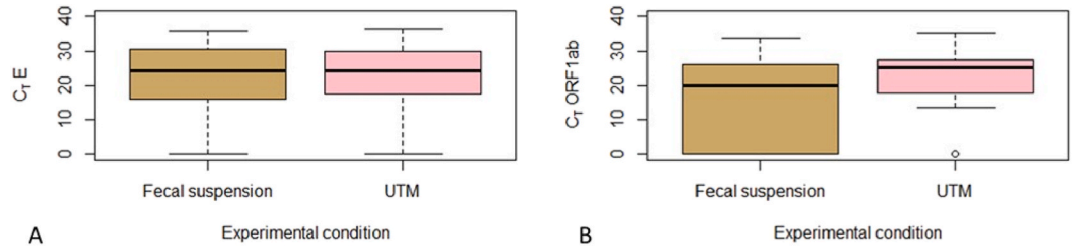

Fig. 1. Distribution of C_T values of target E and ORF1ab in known SARS-CoV-2 samples at various concentration in the presence and absence of fecal material. The two horizontal lines at the ends of the graph represent the highest and the lowest C_T value. The 'colored box' is bounded by the first quartile and the third quartile. Within this range is the dark line identifying the median: the central value of the data distribution. **1A** C_T distribution of E gene target The differences in these values between the two experimental conditions are not statistically significant (p-value 0.8015). **1B** C_T distribution of ORF1ab target gene. The differences in these values between the two experimental conditions were not statistically significant (p-value 0.0767).

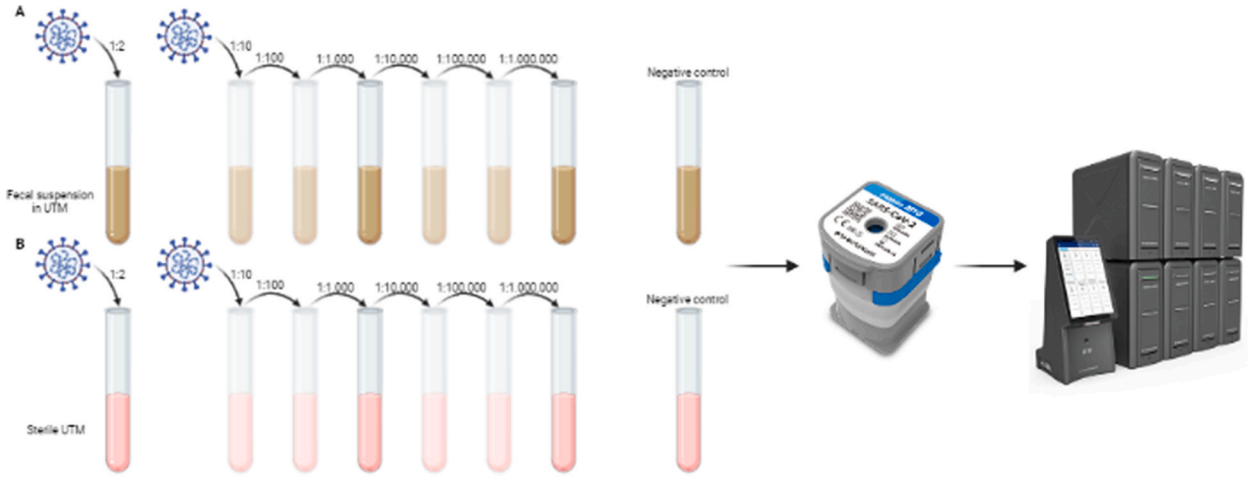

Fig. 2. Schematic representation of the experiment conducted (A) in presence and (B) in absence of stool.

to 6 mg/mL, except for the 1:2 dilution; only in this specific case the fecal suspension has a concentration of 3 mg/mL.

3.3. Performing rRT-PCR for SARS-CoV-2 detection

Eight assays were performed for each of the 10 samples, specifically the 1:2, 1:1000 (10^{-3}), 1:1,000,000 (10^{-6}) dilutions and the negative control sample were tested for each experimental condition (Fig. 2). 600 ml of each dilution or control have been introduced through a pasteur pipette in the STANDARD™ M10 SARS-CoV-2 (SD Biosensor Inc, Suwon, South Korea) device (Fig. 2). STANDARD™ M10 SARS-CoV-2 is an "all-in-one" cartridge-based test; the cartridge contains extraction reagents, PCR buffers and lyophilized real-time RT-PCR reagents. The user only needs to insert 600 μl of sample directly into the cartridge and load it into the STANDARD™ M10 instrument, a modular system which allows continuous loading and random access of the samples (Fig. 2). In 60 min the instrument performs the whole analysis and provides the result automatically. The target sequences of the test are envelope (E), and ORF1ab genes. A positive result occurs only if both targets are detected, or if only ORF1ab is detected. If only detection of E occurs the instrument will provide a presumptive positive result. Each STANDARD™ M10 SARS-CoV-2 test contains synthetic RNA as internal control (IC) to evaluate the presence of inhibitors of extraction and PCR reaction. STANDARD™ M10 SARS-CoV-2 provides C_T values for both target E and ORF1ab and for IC.

3.4. Statistical analysis

Quantitative comparison between C_T values obtained by testing samples with and without human fecal material was analyzed by Mann-Whitney test for target E and by Student's t-test for ORF1ab. The comparison between the total number of positive results and negative results in the two experimental conditions was carried out using Fisher's exact test. P-values <0.05 were considered statistically significant. Statistical analysis was conducted using R Studio software (version March 1, 1093).

3.5. Ethics considerations

The "RETRAMI Registry study" has been designed to collect data related to Fecal Microbiota Transplants performed at SS Antonio e Cesare Arrigo Hospital. This study was approved by the Institutional Ethics Committee (Comitato Etico Interaziendale Alessandria, protocol number ASO. Gastro.21.01). All study procedures complied with the 1946 Declaration of Helsinki, the Good Clinical Practices guidelines and relative updating. Informed consent was obtained from all participants.

4. Discussion

In this study, a technical evaluation of the diagnostic performance of STANDARD™ M10 SARS-CoV-2 test for SARS-CoV-2 RNA detection in human fecal material was presented in order to screen fecal donors for the practice of human fecal microbiota transplantation.

Currently, an optimal protocol to be used for validating commercial tests for SARS-CoV-2 in human feces has not been described yet. In the few studies already available in the literature, the authors mainly exploit two different approaches. The first is to collect stool samples or anal swabs from a more or less large number of SARS-CoV-2 positive and negative patients tested on respiratory secretions. After that, as there is evidence showing the possible presence of the virus in the feces of COVID-19-infected individuals, the concordance of results between respiratory and fecal samples is checked using the commercial test undergoing validation [23,24]. The second method, on the other hand, involves artificially infected human fecal material with SARS-CoV-2 particles and using the resulting samples to evaluate the performance of the commercial test in detecting the presence of viral RNA in fecal samples [25]; Di Pilato et al., 2022).

For the present study, it was decided to use the second approach since in our experimental setting it is less prone to bias.

The results obtained show that STANDARD™ M10 SARS-CoV-2 assay, even if validated to process test samples from nasopharyngeal swab, can effectively detect SARS-CoV-2 target sequences in human feces as well (Tables 2 and 3 in result section). In addition, the data in Table 1 of result section show that the STANDARD™ M10 SARS-CoV-2 test is not adversely affected by the nature of the sample itself, as processing feces or UTM does not affect the amplification of the exogenous control in the cartridge and also the specific genes C_T values are not significantly different. This underscores the excellent performance because although rRT-PCR test is one of the most powerful tools in molecular biology, its potential can be limited by substances called "PCR inhibitors" of which feces are particularly rich [23]; Di Pilato et al., 2022). These inhibitory substances varies from sample to sample [26] and the best known are: bile salts [27,28], bilirubin degradation products [27], calcium, lipids, glycolipids, uric acid, urea, heparin, chlorophyll derived from residues of plant substances introduced into the body through the diet [29] and blood, either visible or occult [30].

Even if the presence of PCR-inhibiting substances in the feces was suspected in our work, it was preferred not to apply protocols for removing or reducing their effect, which are generally recommended when there is a need to apply PCR directly to complex biological samples [31]. This choice, dictated by the need to avoid preanalytical treatments that are often laborious, costly, and time-consuming, was proved successful because the results obtained show that the performances of STANDARD™ M10 SARS-CoV-2, especially at dilution 1:2 and 1:1000 (Tables 2 and 3), are comparable in the presence and absence of human feces within the test sample. Consequently, the PCR inhibiting substances likely present in the fecal samples not only did not prevent the test from performing correctly, but also did not result in significant deviations in the results compared with the control samples without fecal material (Fig. 1).

The choice of not applying sample preparation protocols was also successful in this case because it was shown that the results of the STANDARD™ M10 SARS-CoV-2 test were not affected even by the presence of visibly floating particles in the test samples consisting of fecal suspension.

Moreover, the main advantage of avoiding fecal material preparation operations in this study was to shorten the duration of the preanalytical phase, which fulfil the need to make the performance of the STANDARD™ M10 SARS-CoV-2 test on the fecal sample as quick and easy to perform for seamless integration of the test within the laboratory screening protocol on biological donor samples of intestinal microbiota.

5. Conclusions

In this study, the performance of the STANDARD™ M10 SARS-CoV-2 test was evaluated by utilizing fecal samples. The functionality of the STANDARD™ M10 SARS-CoV-2 test do not appear to be affected by the nature of the fecal sample itself as no invalid results were obtained.

The performance of the STANDARD™ M10 SARS-CoV-2 test on fecal samples supplemented with a positive SARS-CoV-2 sample does not appear to be inferior to that obtained on UTM with the exception of three samples at the lowest dilution tested i.e. 1:1,000,000.

In this study, it was decided to set up a protocol that involved no fecal processing other than a dilution to achieve the final concentration of 6 mg/mL. This choice depended on avoiding overly laborious and time consuming preanalytical procedures. The STANDARD™ M10 SARS-CoV-2 is a rapid RT-PCR test with a duration of 60 min and is characterized by the complete automation of the sample extraction and amplification steps that require no operator intervention. Therefore, due to both the characteristics of the preanalytical protocol developed and performance observed, the STANDARD M10 SARS-CoV-2 test can be used as a valuable aid to assess the presence of SARS-CoV-2 in fecal samples. Continued efforts in evaluation and validating molecular assay for detection of SARS-CoV-2 in stool are needed to support adoption of FMT donor screening and banking programs to the era of COVID-19 pandemic.

Author contribution statement

Sara Scaglione; Andrea Rocchetti: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Franca Gotta; Daria Vay; Christian Leli: Analyzed and interpreted the data.
Annalisa Roveta; Antonio Maconi: Contributed reagents, materials, analysis tools or data.

Data availability statement

The data that has been used is confidential.

Declaration of competing interest

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

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