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Multicenter Comparison of Nucleic Acid Extraction Methods for Detection of Severe Acute Respiratory Syndrome Coronavirus RNA in Stool Specimens

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The emergence of a novel coronavirus (CoV) as the cause of severe acute respiratory syndrome (SARS) catalyzed the development of rapid diagnostic tests. Stool samples have been shown to be appropriate for diagnostic testing for SARS CoV, although it has been recognized to be a heterogeneous and difficult sample that contains amplification inhibitors. Limited information on the efficiency of extraction methods for the purification and concentration of SARS CoV RNA from stool samples is available. Our study objectives were to determine the optimal extraction method for SARS CoV RNA detection and to examine the effect of increased specimen volume for the detection of SARS CoV RNA in stool specimens. We conducted a multicenter evaluation of four automated and four manual extraction methods using dilutions of viral lysate in replicate mock stool samples, followed by quantitation of SARS CoV RNA using real-time reverse transcriptase PCR. The sensitivities of the manual methods ranged from 50% to 100%, with the Cortex Biochem Magazorb method, a magnetic bead isolation method, allowing detection of all 12 positive samples. The sensitivities of the automated methods ranged from 75% to 100%. The bioMérieux NucliSens automated extractor and miniMag extraction methods each had a sensitivity of 100%. Examination of the copy numbers detected and the generation of 10-fold dilutions of the extracted material indicated that a number of extraction methods retained inhibitory substances that prevented optimal amplification. Increasing the volume of sample input did improve detection. This information could be useful for the extraction of other RNA viruses from stool samples and demonstrates the need to evaluate extraction methods for different specimen types.

The emergence of a novel coronavirus (CoV) as the cause of severe acute respiratory syndrome (SARS) and its spread throughout the world catalyzed the development of rapid diagnostic tests. SARS CoV has been shown to replicate in the gastrointestinal tract (4), and consistent with this, stool samples were shown to be appropriate for diagnostic testing for SARS CoV. Peiris et al. (7) found a positivity rate of 97% (65/67 samples) for detection of SARS CoV nucleic acid in stool samples at 14 days after the onset of symptoms. By contrast, Chan et al. (1) found a lower overall stool positivity rate of 26.2% (70/267), with a 42.9% (9/21) positivity rate within 1 week of the onset of symptoms, a 68.0% (17/25) positivity rate between 1 and 2 weeks of onset, and a 70.8% (34/48) positivity rate between 2 and 4 weeks of onset. Preliminary studies performed in our laboratories indicated that variations in RNA extraction methods could explain the differences seen in these studies. We anticipated that the optimization of extraction methods for stool samples could potentially increase the sensitivity of amplification detection of SARS CoV, especially early in infection. Stool samples have been recognized to be heterogeneous and difficult samples for use for molecular analysis. Bile salts, hemoglobin, and polysaccharides have been identified as factors that inhibit amplification assays (11, 6); and reverse transcriptase (RT) has been described to be particularly sensitive to inhibition (12). Although these amplification inhibitors are well known, limited information is available on the efficiency of various extraction methods for the purification and concentration of SARS CoV RNA from stool samples.

Our primary study objective was to determine the optimal extraction method for the detection of SARS CoV RNA in stool samples, and our secondary objective was to examine the effect of an increased volume of stool specimen on the ability to detect SARS CoV RNA. We conducted an international multicenter evaluation of automated and manual extraction methods using dilutions of inactivated viral lysate in replicate mock stool samples, followed by single-site quantitation of SARS CoV RNA by real-time RT-PCR.

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MATERIALS AND METHODS

Virus. A heat-inactivated cell culture lysate of SARS CoV strain Tor-2 was supplied by Martin Petric, British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada. The lysate, which contained 10^8 50% tissue culture infective doses per ml of SARS CoV, was used to spike the stool samples.

Generation of mock stool suspensions. Five stool samples submitted to the Regional Virology Laboratory in Hamilton, Ontario, Canada, for routine testing were pooled and diluted with RNase-free water to make a 10% (vol/vol) suspension. An aliquot was extracted and tested by RT-PCR (5) to ensure that it was negative for SARS CoV nucleic acid. SARS CoV lysate was serially diluted in the 10% stool suspension to generate 10-fold dilutions ranging from 10^{-2} to 10^{-7}. An approximate detection limit was determined by extracting a 140-μl aliquot of each dilution by use of a QIAamp viral RNA kit (QIAGEN Inc., Mississauga, Ontario, Canada) and performing a quantitative RT-PCR targeting the nucleocapsid gene with primers APNF and APNR and a TaqMan probe, as described previously (5), in replicates of three.

Panel composition. A panel of 20 samples was assembled and sent to seven laboratories. The panel contained 12 potentially positive samples and 8 negative samples. The four dilutions of the SARS CoV viral lysate in stool suspension contained in the panel comprised samples with 1 dilution lower than the endpoint dilution, the endpoint dilution, and 2 dilutions higher than the endpoint dilution (10^{-4} and 10^{-7}). It was anticipated that the 10^{-7} dilution would not be positive by all extraction methods. Three replicates for each SARS CoV dilution were included for a total of 12 possible positive results. The negative samples comprised two stool suspensions (10%; vol/vol) to which nothing was added and six stool suspensions spiked with other viral lysates (parainfluenza virus 3, influenza A virus, human coronaviruses 229E and OC43, respiratory syncytial virus, and adenovirus). The order of the samples was randomized by a computer-based random numbers sequence, and test panels were sent to two laboratories to ensure that the samples performed as expected prior to wider distribution to all seven laboratories. The participating laboratories included St. Joseph's Healthcare, Hamilton, Ontario, Canada (site 1); Mount Sinai Hospital, Toronto, Ontario, Canada (site 2); Hospital for Sick Children, Toronto, Ontario, Canada (site 3); British Columbia Center for Disease Control, Vancouver, British Columbia, Canada (site 4); Edmonton Public Health Laboratory, Edmonton, Alberta, Canada (site 5); Central Public Health Laboratory, Etobicoke, Ontario, Canada (site 6); the Centers for Disease Control and Prevention, Atlanta, Ga. (site 7); Sunnybrook and WHC HSC, Toronto, Ontario, Canada (site 8).

Nucleic acid extraction. The panels were extracted by eight different methods (seven commercial methods and one in-house method) at seven different laboratories. Two extraction methods were each performed at two different laboratories: QIAamp Viral RNA kit with 140 μl at two sites (sites 1 and 6) and the NucliSens automated extractor method (bioMérieux, St. Laurent, Quebec, Canada) with 100 μl and 200 μl at sites 4 and 7, respectively. Four methods were performed with two different sample input volumes: the QIAamp Viral RNA method with 140 μl and 560 μl, the miniMag method (bioMérieux) with 140 μl and 200 μl, the NucliSens automated extractor method (bioMérieux) with 100 μl and 200 μl, and the QIAamp UltraSens method (QIAGEN Inc.) with 140 μl and 500 μl. The participating laboratories were asked to elute RNA into a final volume of 50 μl, dispense the volume into two aliquots, and send the extracted nucleic acid back to a central site for testing by RT-PCR. Two laboratories (site 7 and site 4) did not extract into 50 μl, as they followed protocols that they had optimized in-house: the QIAamp MDx method (QIAGEN Inc.) with 80 μl and the bioMérieux NucliSens automated extractor method with 30 μl. All methods were performed according to the manufacturers' protocols. The extraction methods included four manual methods (QIAamp UltraSens, QIAamp Viral RNA, Magazorb [Cortex Biochem, San Leandro, Calif.], and in-house guanidinium thiocyanate methods), three automated methods (the MagNA Pure [Roche Diagnostics, Laval, Quebec, Canada], QIAamp MDx [QIAGEN Inc.], and bioMérieux NucliSens automated extractor methods), and one semiautomated method (the bioMérieux MiniMag method), with sample input volumes ranging from 100 μl to 560 μl for the different methods (Table 1). One manual in-house method used guanidinium thiocyanate buffer, followed by isopropanol precipitation, as described by Johnson et al. (3). The MagNA Pure LC TNA isolation kit, which uses a lysis buffer, followed by binding of nucleic acid to magnetic particles coated with silica, was used with the MagNA Pure method. The miniMag and Cortex Biochem methods also use magnetic beads covered with silica in a less automated procedure, while the automated NucliSens method uses standard silica. The QIAamp Viral RNA kit, QIAamp UltraSens method, and the MDx method from QIAGEN use a silica gel membrane to capture the nucleic acid in a manual or automated format.

TABLE 1. Description of extraction methods and volumes used at eight different sites to compare the recovery of SARS coronavirus nucleic acid from mock stool specimens

Site	Method	Extraction method	Starting vol (μl)	Elution vol (μl)	Mode of extraction
1	A	bioMeriéux miniMag	140	50	Semiautomated
1	D	bioMeriéux miniMag	200	50	Semiautomated
7	C	bioMeriéux NucliSens automated	200	50	Automated
4	H	bioMeriéux NucliSens automated	100	30	Automated
5	B	Cortex Biochem Magazorb	200	50	Manual
2	E	Roche MagNA Pure	200	50	Automated
3	F	Guanidinium Thiocyanate	100	50	Manual
1	G	QIAamp Viral RNA	140	50	Manual
8	I	QIAamp Viral RNA	140	50	Manual
1	M	QIAamp Viral RNA	560	50	Manual
6	J	QIAamp UltraSens	140	50	Manual
6	L	QIAamp UltraSens	500	50	Manual
7	K	QIAamp MDX	265	80	Automated

Quantitation by RT-PCR. The extracted material was sent to a central site for quantitation by a commercially available real-time RT-PCR assay (RealArt HPA Coronavirus kit; artus GmbH, Hamburg, Germany) for detection of SARS CoV nucleic acid. Five microliters of purified sample was tested by the RealArt SARS coronavirus HPA RT-PCR (artus) assay undiluted and at a 1:10 dilution in RNase-free water to detect the presence of inhibitors. A LightCycler 1.2 or 2.0 instrument (Roche Diagnostics) was used for amplification. It was previously determined by an evaluation of standards and test samples that the results obtained with the two LightCycler instruments were interchangeable (data not shown). The control samples used to generate the standard curve were provided by the manufacturer. An attempt was made to test the same sample extracted by different methods in the same run. This was not always possible, so crossing points for the standards were collected for all runs and analyzed. Significant differences between runs were not detected, suggesting that there was little interrun variability (data not shown).

Data analysis. For qualitative test results, the results of the extraction techniques were compared by the Cochran Q test for three or more repeated samples, followed by pairwise comparisons by the McNemar test (SPSS for Windows 11.5). For quantitative test results, robust multilevel-modeling linear regression models were constructed to determine the impacts of the extraction method, the concentration, and the 1:10 dilution on the copy number (the dependent variable) detected. The model fit was assessed. Concentrations and copy numbers were log transformed, and 0.001 was added to the results with a copy number of zero. Robust regression was performed with S-Plus for Windows, version 7.0 (Insightful Corporation, Seattle, Wash.). A P value <0.05 was taken as statistically significant.

RESULTS

Panels consisting of 20 mock-infected stool samples were extracted by eight methods, including four manual methods, three automated methods, and one semiautomated method, in seven different laboratories (Table 1). The extracted material was sent to a central site for quantitation by using a commercially available real-time RT-PCR assay (RealArt) for the detection of SARS CoV RNA. The samples were tested undiluted and at a 1:10 dilution to detect the presence of inhibitors.

All unspiked samples or those spiked with other viral lysates (parainfluenza virus 3, influenza A virus, human coronaviruses 229E and OC43, respiratory syncytial virus, and adenovirus) tested negative by the real-time PCR, indicating a specificity of 100% (8/8) for the RT-PCR with all extraction methods, for a total of 64/64 negative results (8 sets of 8 samples).

Manual extraction methods. For the stool specimens that were analyzed undiluted, the sensitivities of the manual meth-

ods ranged from 50% (6/12) for the QIAamp UltraSens method (140 μl; site 8) to 91.7% (11/12) for the QIAamp Viral RNA method (140 μl) at site 1 and 66.7% (8/12) at site 3 and from 83.3% (10/12) for the in-house guanidinium thiocyanate method at site 4 to 100% (12/12) for the Cortex Biochem Magazorb method at site 5 (Table 2). The QIAamp Viral RNA method (with 140 μl of sample), which was tested at two different laboratories, had a sensitivity of 91.7% (11/12) at one site and a sensitivity of 66.7% (8/12) at the other, although this difference was not statistically significant (McNemar test, $P = 0.25$). There did not appear to be a difference between the extraction of 140 μl and the extraction of 560 μl with the QIAamp Viral RNA kit, as the overall numbers of positive results was similar: 91.7% (11/12) and 83.3% (10/12), respectively ($P = 1.0$). The QIAamp UltraSens method with a starting volume of 140 μl used undiluted detected 6/12 (50.0%) positive samples, whereas 10/12 (83.3%) positive samples were detected at a 1:10 dilution. A similar pattern was seen when the QIAamp UltraSens kit was used with a 500-μl sample volume (undiluted, 8/12; 1:10 dilution, 10/12). Therefore, there was a slight increase in positivity rates when larger volumes of stool specimen were used. The additional dilution of 1:10 appeared to enhance the positivity rates by dilution of anticipated inhibitors for both volumes, although the increase was not statistically significant.

Automated and semiautomated extraction methods. The sensitivities obtained by the automated methods (Table 3) ranged from 100% (12/12) for both the bioMérieux NucliSens automated extractor method (200 μl; site 7) and the bioMérieux miniMag method (140 μl; site 1) to 75.0% (9/12) for the QIAamp MDx, bioMérieux miniMag (200 μl; site 1), and the bioMérieux NucliSens automated extractor (200 μl; site 4) methods with the undiluted specimen. The results from the two sites performing the bioMérieux NucliSens automated extractions showed markedly different sensitivities, with site 4 having only 9/12 positive results with the undiluted specimen and 6/12 positive results with the specimen diluted 1:10, whereas site 7 had 12/12 positive results with the undiluted and the diluted specimens. Site 4 used half the volume of starting material (100 μl, whereas site 7 used 200 μl) and eluted their samples into 30 μl. It was identified following investigation that the samples at site 2 had been extracted following an additional freeze-thaw step, which may have caused some of the RNA present to degrade, decreasing the template available for amplification.

Comparison of numbers of copies of nucleic acid. Recovery of SARS CoV RNA was also evaluated by comparing the numbers of copies of the specific target detected. While the copy numbers determined by quantitative PCR were not adjusted for sample input volume, they demonstrate the absolute numbers detected when the manufacturers' recommendations for extraction were followed or when an increase in the sample volume was attempted to improve sensitivity. Under these conditions, the semiautomated bioMérieux miniMag method with a starting volume of 140 μl (Table 3, method A, site 1) and the Cortex Biochem Magazorb 200 μl (Table 2, method B, site 5) had the highest rate of recovery of SARS CoV target RNA, with approximate stepwise 10-fold decreases between dilutions. The in-house guanidinium thiocyanate method with a 1:10 dilution showed a similar rate of recovery (Table 2, site 3). To visualize the extraction efficiency over 4 log units of viral

TABLE 2. Number of SARS CoV RNA copies recovered by four manual extraction methods performed at five sites[a]

Viral lysate dilution	No. of copies/5 μl															
	Method I (site 8, QIAamp Viral RNA, 140 μl)		Method G (site 1, QIAamp Viral RNA, 140 μl)		Method M (site 1, QIAamp Viral RNA, 560 μl)		Method J (site 6, QIAamp UltraSens, 140 μl)		Method L (site 6, QIAamp UltraSens, 500 μl)		Method F (site 3, guanidinium thiocyanate, 100 μl)		Method B (site 5, Cortex Biochem Magazorb, 200 μl)	
	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10
10^{-4}	142	465	161	692	140	250	137	634	115	293	312	1,020	12,100	1,270		
10^{-4}	130	338	165	664	128	176	89.2	578	97.2	261	489	1,290	15,300	1,280		
10^{-4}	152	384	204	809	79.5	183	120	600	104	330	604	1,500	14,300	2,120		
10^{-5}	46.2	76	44.2	86.2	43.5	57.0	41.4	122	39.8	79.4	122	285	1,090	192		
10^{-5}	35.1	65.4	54.5	131	36.0	45.5	40.9	83.8	33.6	82.4	65.1	157	976	209		
10^{-5}	30.5	70.2	55.5	105	46.1	55.0	51.8	95.5	35.2	66.5	79.0	161	1,150	184		
10^{-6}	17.5	47.1	29.7	47.7	23.3	37.1	17.9	41.4	13.1	36.7	62.2	45.9	201	54.5		
10^{-6}	17.5	38.3	33.6	48.9	19.9	36.6	13.8	39.7	0	37.8	26.0	44.2	197	61.4		
10^{-6}	0	37.4	24.7	44.3	20.1	40.3	0	48.2	0	41.1	36.1	57.0	137	46.2		
10^{-7}	0	27.2	7.9	27.7	10.4	0	0	0	12.2	0	0	0	424	28.7		
10^{-7}	0	0	8.9	28.1	0	0	0	27.2	0	0	7.1	0	425	27.9		
10^{-7}	0	29.4	0	0	0	27.7	0	0	0	28.7	0	0	520	35.4		
Total no. positive/total no. tested (%)	8/12 (66.7)	11/12 (91.7)	11/12 (91.7)	11/12 (91.7)	10/12 (83.3)	10/12 (83.3)	6/12 (50.0)	10/12 (83.3)	8/12 (66.7)	10/12 (83.3)	10/12 (83.3)	9/12 (75.0)	12/12 (100)	12/12 (100)		

[a] SARS CoV RNA copy numbers were determined by using the RealArt HPA kit (artus). In each case the copy number indicated represents the numbers of SARS CoV RNA molecules in 5 μl of extracted specimen for the specimen dilution (undiluted or 1:10) and the elution volume shown in Table 1.

TABLE 3. Number of SARS CoV RNA copies recovered by four automated or semiautomated extraction methods performed at four sites[a]

Viral lysate dilution	No. of copies/5 μl											
	Method A (site 1, bioMeriéux miniMag, 140 μl)		Method C (site 7, bioMeriéux NucliSens, 200 μl)		Method D (site 1, bioMeriéux miniMag, 200 μl)		Method E (site 2, Roche MagNA Pure, 200 μl)		Method H (site 4, bioMeriéux NucliSens, 200 μl)		Method K (site 7, QIAamp MDx, 265 μl)	
	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10	Undiluted	1:10
10^{-4}	19,500	1,330	6,670	890	2,720	406	962	710	204	46.4	84.1	15.7
10^{-4}	13,800	1,530	10,900	1,270	1,650	314	1,020	750	161	50.6	62.5	13.0
10^{-4}	15,900	1,520	5,330	829	2,850	555	1,150	762	209	55.5	81.1	16.1
10^{-5}	1,800	162	644	157	619	65.3	203	130	105	36.9	7.6	1.8
10^{-5}	1,750	214	708	101	422	31.6	183	142	52.9	32.5	8.1	1.7
10^{-5}	2,130	215	976	203	337	26.2	219	137	54.6	32.5	7.3	1.6
10^{-6}	151	12.3	100	31.0	68.5	4.3	67.3	54.5	0	0	5.7	0
10^{-6}	173	20.9	105	37.0	12.8	0	38.5	36.6	0	0	2.0	0
10^{-6}	158	10.1	139	26.8	3.1	9.2	68.1	48.0	18.2	0	0	0
10^{-7}	19.1	0	30.8	18.4	0	0	0	33.9	0	0	0	0
10^{-7}	5.0	1.0	9.4	22.0	0	0	0	33.9	15.4	0	3.3	0
10^{-7}	12.1	0	16.7	23.6	0	0	24.3	38.5	21.6	0	0	0
Total no. positive/total no. tested (%)	12/12 (100)	10/12 (83.3)	12/12 (100)	12/12 (100)	9/12 (75.0)	8/12 (66.7)	10/12 (83.3)	12/12 (100)	9/12 (75.0)	6/12 (50.0)	9/12 (75.0)	6/12 (50.0)

[a] SARS CoV RNA copy numbers were determined by using the RealArt HPA kit (artus). In each case the copy number indicated represents the numbers of SARS CoV RNA molecules in 5 μl of extracted specimen for the specimen dilution (undiluted or 1:10) and the elution volume shown in Table 1.

RNA, the results were graphed according to the log dilution of the copy numbers obtained for amplification of 5 μl of extracted material and extraction method (Fig. 1A and B). The bioMérieux miniMag method had the highest copy number overall for all dilutions, followed by the Cortex Biochem Magazorb method. This was consistent with all concentrations of template. More variability was seen between the methods at the lower concentrations, presumably due to the effect of sampling. Dilution of the purified RNA prior to testing appeared to improve the RNA recovery for a number of methods. These sites used the Roche MagNA Pure method, the in-house guanidinium thiocyanate method, the QIAamp Viral RNA method (140 μl), and the QIAamp MDx method. Following dilution, however, not all of these methods were able to detect the lowest concentration of spiked specimen (e.g., the QIAamp MDx and guanidinium thiocyanate methods).

Concentrations calculated with no dilution and 1:10 dilution. All samples were tested at a 1:10 dilution to look for the presence of inhibitors. Actual data are shown; hence, if no inhibitors were present, a 10-fold drop in the calculated numbers should have been seen between the undiluted and the diluted samples. A number of the extraction methods, including the Roche MagNAPure and the QIAamp UltraSens methods, showed increased copy numbers and/or more positive results in tests with the diluted samples. This suggests that inhibitors present in the undiluted sample decreased the level of amplification and that this effect could be diluted out. A 10-fold drop in the calculated concentrations was seen with the Cortex Biochem Magazorb, bioMérieux NucliSens automated extractor, and bioMérieux miniMag methods (Tables 2 and 3). To illustrate the presence of inhibitors based on the copy numbers obtained from real-time amplification, analysis by robust regression (to account for outliers and correlation among samples) of the log dilution versus the log-transformed mean concentration of undiluted and diluted samples was performed. Figure 2A and B was derived from quantitative counts obtained from material extracted by the bioMérieux miniMag method (140 μl) at site 1 and the QIAamp Viral RNA method (140 μl) at site 8, respectively. In Fig. 2A and B, the robust linear regressions for the undiluted and diluted samples are graphed as solid and dashed lines, respectively. In the regression model for method A at site 1, each log concentration was associated with an increased copy number of 0.98 ± 0.02 (versus an expected increase of 1.0), and the samples diluted 1:10 had an estimated concentration of -0.95 ± 0.04 (versus an expected log concentration of -1.0). This is demonstrated in Fig. 2A as two parallel lines, with the log concentration of the undiluted samples being approximately 1 log greater than that of the diluted samples. Hence, no inhibitors were detectable with material extracted by the bioMérieux miniMag method. By contrast, for the QIAamp Viral RNA method (140 μl) extraction in Fig. 2B, the regressions found a weaker association with the log concentration of 0.45 ± 0.8 (versus an expected log concentration of 1.0), and the samples diluted 1:10 were associated with a 0.40 ± 0.16 greater concentration (versus an expected log concentration of -1.0; $P = 0.02$). This is demonstrated in Fig. 2B, in which the lines representing the undiluted and the diluted samples are reversed from those in Fig. 2A. The diluted specimens were associated with a higher copy number, indicating partial amplification inhibition for

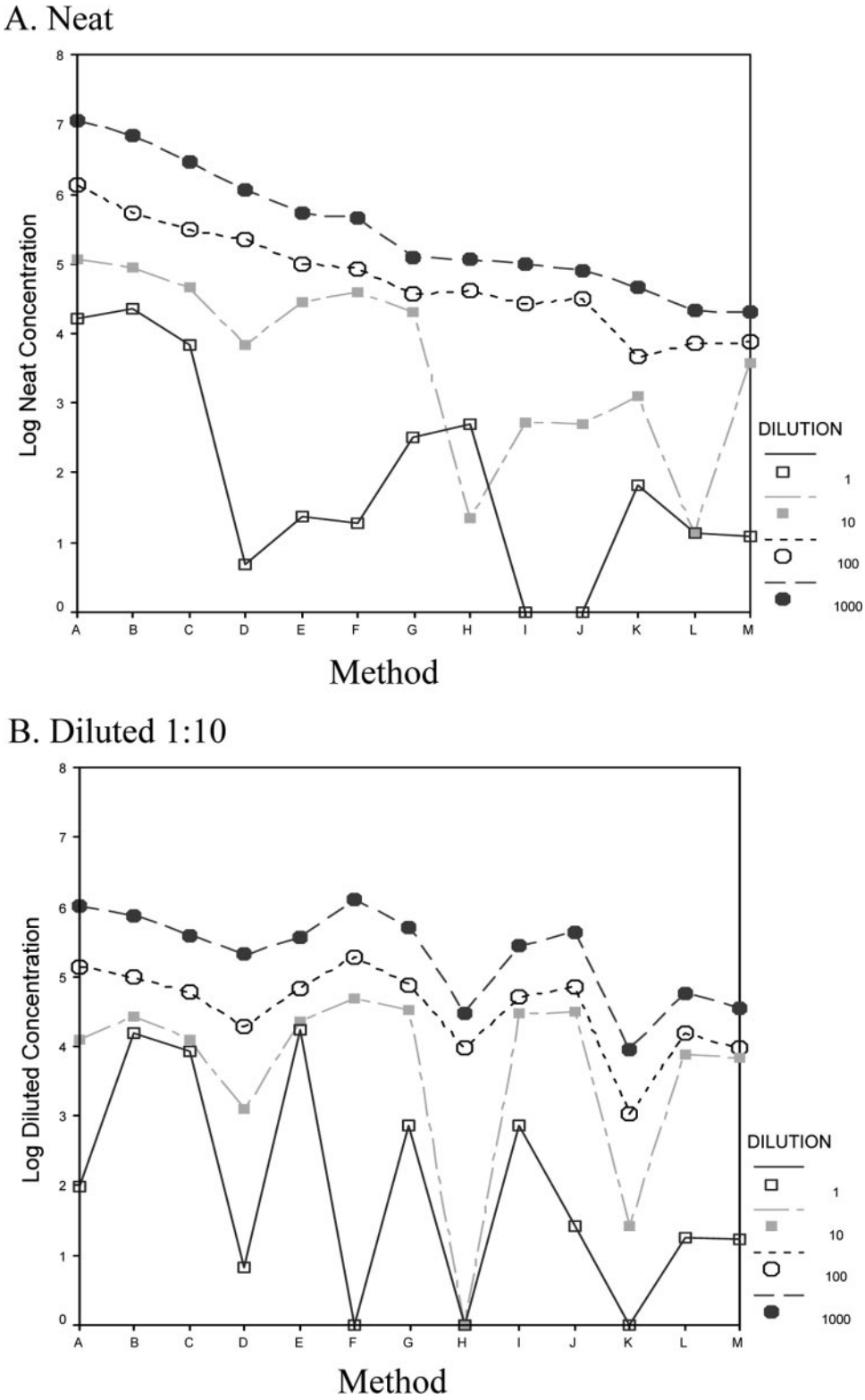

FIG. 1. Comparison of log undiluted concentration (number of copies of SARS CoV in 5 μl) obtained by quantitative real-time RT-PCR following nucleic acid purification by 13 different extraction methods (methods A to M) performed at seven different laboratories. Both undiluted (A) and diluted (B) purified material was tested by each extraction method. The methods are arranged in order from the most sensitive to the least sensitive, as determined by the mean quantities of the most concentrated samples (10^{-4}). The methods are as follows: A, bioMérieux miniMag method (140 μl), site 1; B, Cortex Biochem Magazorb method, site 5; C, bioMérieux NucliSens automated extractor method, site 7; D, bioMérieux miniMag method (200 μl), site 1; E, Roche MagNA Pure method, site 2; F, guanidinium thiocyanate method, site 3; G, QIAamp Viral RNA method (140 μl), site 1; H, bioMérieux NucliSens automated extractor method, site 4; I, QIAamp Viral RNA method (140 μl), site 8; J, QIAamp UltraSens method (140 μl), site 6; K, QIAamp MDx method, site 7; L, QIAamp UltraSens method (500 μl), site 6; M, QIAamp Viral RNA method (560 μl), site 1. For the purposes of performing the statistical methods, dilutions of 1,000, 100, 10, and 1 in the figure correspond to spiked dilutions of viral lysate of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, respectively, used to generate the mock-infected samples.

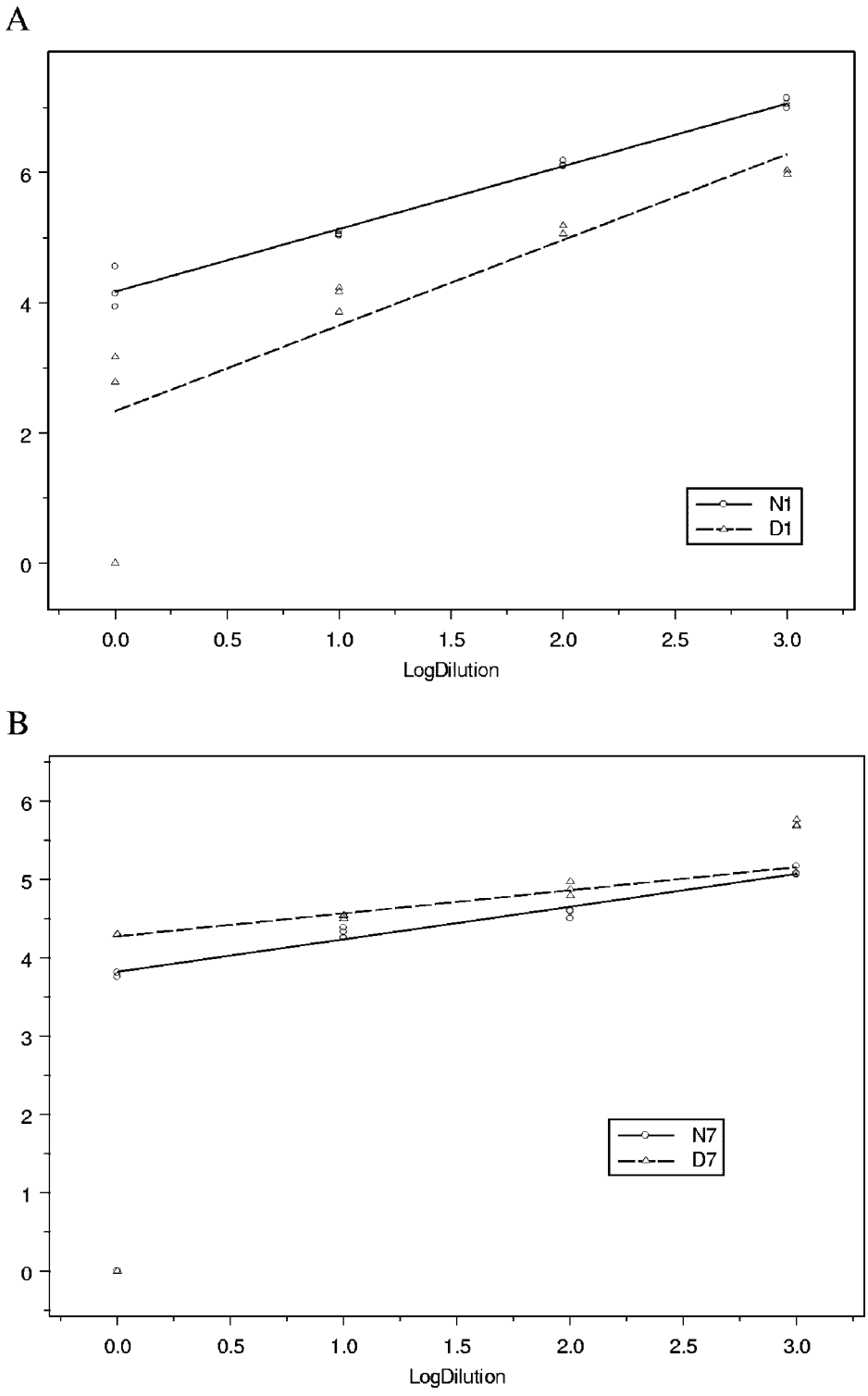

FIG. 2. Robust multilevel-modeling linear regression model (S-Plus 7.0) of log dilution versus log-transformed mean concentration of undiluted (N1) and diluted (D1) samples extracted by the bioMérieux miniMag method (140 μl) at site 1 (A) and by the QIAamp Viral RNA method (140 μl) at site 8.

undiluted specimens. The internal control from the RT-PCR kit was amplified by all of the assays, even though the presence of discrepant results between the undiluted and the diluted samples demonstrated the existence of inhibitory substances.

Effect of increasing the sample volume. To assess whether increasing the input sample volume would decrease the detection limit, two sample volumes were extracted by three methods: the QIAamp Viral RNA method (140 μl and 560 μl), the

QIAamp UltraSens method (140 μl and 560 μl), and the bioMérieux miniMag method (140 μl and 200 μl). Comparison of the quantitative numbers of the lower and higher sample input volumes showed that increasing the sample volume did not increase the numbers of target copies detected by real-time PCR. In fact, with the miniMag extraction method, the number of RNA copies was lower when a larger volume of specimen was extracted (140 μl versus 200 μl).

DISCUSSION

The optimal method for the extraction of RNA for PCR detection would ideally provide pure nucleic acid free of amplification inhibitors and at the same time recover RNA quantitatively across a range of concentrations. We evaluated different extraction methods for the purification of RNA from stool specimens for the optimal detection of SARS CoV RNA using a commercially available quantitative real-time RT-PCR. Four 10-fold dilutions of SARS CoV cell lysates were spiked into negative stool samples so that the lowest concentration would be at or close to the assay endpoint. Not all extraction methods were expected to be able to recover detectable levels of RNA. The two methods that gave the highest overall numbers of copies per 5 μl for all samples were the miniMag (bioMérieux) and the Magazorb (Cortex Biochem) methods. Both methods use magnetic beads coated with silica in their methodologies. The Roche MagNA Pure method also uses magnetic beads; however, it did not perform as well, giving lower copy numbers and lower percentages of positivity, often in the presence of amplification inhibitors. Without knowledge of the proprietary ingredients of the kits, the reason for the improved performance of the miniMag and Cortex Biochem Magazorb methods compared with those of the other methods can only be surmised but presumably is due to the composition of the magnetic beads or the surface area available for the binding of nucleic acid. The nature of the wash procedures, including the buffer volume and composition, may also affect the purity and yield of the nucleic acid. The internal control of the RealArt assay was detected in tests with all undiluted and diluted samples; however, the crossing point (the cycle threshold number) was shifted later in some cases by as much as three cycles, which represents a 1-log quantity of RNA. With such a high internal control signal, weakly inhibited samples may still have a positive internal control signal but the inhibition would be enough to make a low-level-positive sample negative. This implies that users should view the internal control signal cautiously and should understand that the concentration of RNA in the internal control may be higher than that found in some weakly positive samples. Users may wish to examine crossing points for internal controls to see if they have shifted and/or consider running a dilution of the sample if they believe that inhibition is possible.

The inclusion of replicates of dilutions at or near the detection limit in the panel, as previously described by our group for *Chlamydia pneumoniae* detection (2, 9), were meant to aggressively challenge the extraction methods. All methods have positive results for the samples at the higher RNA concentrations; however, as the concentration drops, differences between the methods can clearly be determined. The use of replicates minimizes the differences obtained over those obtained by the interpretation of results based on a single sample point, especially with samples that contain a low concentration of template and that are therefore susceptible to sampling bias. This strategy allowed us to demonstrate that there are differences in the ability of extraction methods to purify and recover RNA from stool samples that could be clinically significant. Automated extractors may not perform the best with this specimen type, and the advantages of high throughput may sacrifice performance. In our hands, a manual method and a semiautomated method performed the best for the quantitative recovery of RNA, suggesting that laboratories with limited resources and/or lower sample volumes can find manual extraction methods that do not require expensive instrumentation. For life-threatening infections (SARS and avian influenza), performance cannot be jeopardized, particularly when specimens that are taken early in the course of the infection and that may have low viral RNA copy numbers are tested.

In this study, five individual stool samples were pooled and a 10% suspension was generated; thus, a single sample matrix spiked with different concentrations of SARS CoV was used for all comparisons. One limitation of this approach was that it may not represent the specimen heterogeneity found in this sample type. Fortuitously, this particular sample matrix contained PCR amplification inhibitors, making it an excellent matrix for comparison of the abilities of various extraction methods to extract RNA and remove inhibitors. Performing quantitation with samples that were undiluted and diluted 1:10 allowed us to look for partial or complete inhibition. Only three of the extraction methods (the miniMag, Magazorb, and NucliSens automated extractor methods) clearly showed a 10-fold drop in counts from undiluted to diluted material, demonstrating a lack of inhibitors in the purified preparation. Some of the methods actually had higher numbers for the diluted sample than for the undiluted sample, suggesting the presence of a low level of amplification inhibitors. It is clear from this evaluation that ideal results, including assay sensitivity and quantitation, require an optimal extraction method. Not only were there differences in the number of positive samples detected following different extraction methods, but there were also considerable variations in the number of copies of SARS CoV template recovered by each extraction method. In this study the MiniMag extraction method (site 1) with the 10^{-4} dilution of viral lysate had a copy number of approximately 17,000, while the QIAamp MDx method demonstrated only ~80 copies/5 ml. The assay was positive by both extraction methods with this concentration of target in the sample, demonstrating that reliable quantitative results can be achieved only when a good extraction method is married to a good amplification assay and linear results are obtained over a range. Care must be taken to ensure that a linear result is achieved over a large dynamic range of template concentration by using optimal extraction and amplification methods.

Poon et al. (8) were able to increase the sensitivity of the early detection of SARS CoV from nasopharyngeal aspirates from 22% to 80% by increasing the sample volume extracted (140 μl to 540 μl) and by incorporating a quantitative real-time RT-PCR for amplification. Wang et al. (10) were able to achieve a detection rate of 80% with 116 plasma samples from 44 SARS patients by increasing the amount of viral RNA input at three steps during the assay: (i) by increasing the sample

input for extraction, (ii) by increasing the volume of purified nucleic acid in the RT reaction, and (iii) by using 100% of the cDNA from the RT step in the subsequent PCR. We could not verify a similar increase in sensitivity when we increased the sample input using three extraction methods. In contrast, the sensitivity remained the same or was decreased. This may be due to the difference in the type of sample tested and the load of cells and/or bacteria present in the sample. Too much nucleic acid in a sample may saturate the binding material used for extraction and exclude the desired target. Increasing the amount of starting material for some sample types will also increase the amount of inhibitors present in the purified eluate. High concentrations of nucleic acid are also known to interfere with amplification and prevent primers from finding the desired target nucleic acid.

This study identified the optimal methods for the extraction of SARS CoV RNA in stool samples. The results may be generalizable to other viruses present in stool samples; however, the physical properties of the virus (e.g., enveloped or nonenveloped) and the nature of the disease caused by the virus, which may result in variations in the sample matrix or differences in the virus copy numbers present, must be considered. Ideally, an evaluation of extraction methods should be undertaken for each virus and sample type being examined. Similar studies are warranted for optimization of the recovery of viral RNA and DNA in other specimens, in particular, respiratory specimens, for the detection of viruses which carry a high mortality rate, such as SARS CoV and avian influenza virus.

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