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Comparison of Automated and Manual Nucleic Acid Extraction Methods for Detection of Enterovirus RNA

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Automated nucleic acid extraction is an attractive alternative to labor-intensive manual methods. We compared two automated methods, the BioRobot M48 instrument (Qiagen, Inc.) and MagNA Pure (Roche Applied Sciences) methods, to two manual methods, the QIAamp Viral RNA Mini kit (Qiagen) and TRIzol (Invitrogen), for the extraction of enterovirus RNA. Analytical sensitivity was assessed by dilution analysis of poliovirus type 2 Sabin in cerebrospinal fluid. The sensitivity of PCR was equivalent after RNA extraction with QIAamp, BioRobot M48, and MagNA Pure. All 18 replicates of 100 PFU/ml were detected after extraction by the four methods. Fewer replicates of each successive dilution were detected after extraction by each method. At 10^{-1} PFU/ml, 17 of 18 replicates were positive by QIAamp, 15 of 18 replicates were positive by BioRobot M48, and 12 of 18 replicates were positive by MagNA Pure; at 10^{-2} PFU/ml, 4 of 17 replicates were positive by QIAamp, 2 of 18 replicates were positive by BioRobot M48, and 0 of 18 replicates were positive by MagNA Pure. At 10^{-3} PFU/ml, no replicates were detected. Evaluation of TRIzol was discontinued after nine replicates due to a trend of lower sensitivity (at 10^{-3} PFU/ml eight of nine replicates were positive at 100 PFU/ml, four of nine replicates were positive at 10^{-1} PFU/ml, and zero of nine replicates were positive at 10^{-2} PFU/ml). Concordant results were obtained in 24 of 28 clinical specimens after extraction by all methods. No evidence of contamination was observed after extraction by automated instruments. The data indicate that the sensitivity of enterovirus PCR is largely similar after extraction by QIAamp, BioRobot M48, and MagNA Pure; a trend of decreased sensitivity was observed after TRIzol extraction. However, the results of enterovirus PCR were largely concordant in patient samples, indicating that the four extraction methods are suitable for detection of enteroviruses in clinical specimens.

Enteroviruses are the primary cause of aseptic meningitis in adults and can cause devastating disseminated disease in neonates. Enterovirus infections have conventionally been identified by virus isolation. PCR assays have improved the diagnosis of enterovirus infections since they are more sensitive than culture; they allow detection of enterovirus types, such as coxsackie A virus, that are not readily isolated (3); and results are obtained more quickly.

The detection of enteroviruses in cerebrospinal fluid (CSF) and blood is sufficient to diagnose an active, disease-producing infection. RNA must be extracted prior to PCR since specimens contain heme or other unidentified constituents that inhibit the activity of thermostable polymerases (1, 4). Enterovirus genomic RNA can be purified from blood and CSF by manual methods that employ organic solvents, magnetic beads (13), or chaotropic salts with or without silica gel columns (2, 18). There are numerous disadvantages to these methods, including labor-intensiveness, the need for specially trained staff to perform extraction, limited throughput, and technician-dependent variability in the efficacy of extraction. The advent of automated instruments to extract nucleic acids from clinical specimens may represent an improvement in molecular tests since they have the potential to improve some of the problems posed by manual extraction.

A growing number of automated extraction platforms are available, and there are multiple reports of their use in assays for pathogen detection (6–8, 10–12, 16, 17, 19, 20). However, there are few studies that compare the performance of these instruments. In the setting of molecular diagnostics for the detection of herpes simplex virus in dermal and genital specimens, manual (IsoQuick) and automated extraction methods (MagNA Pure [Roche Applied Sciences] and BioRobot 9600 [Qiagen]) performed comparably (5). In blood product screening for hepatitis C virus by Roche COBAS Amplicor, the greatest sensitivity was obtained with the BioMerieux NucliSens extractor compared to the BioRobot 9600 and a manual guanidinium thiocyanate method (9). A comparative study of the extraction of RNA targets by automated and manual extraction methods has not been described outside the blood product screening setting.

The goal of our study was to compare the performance of an enterovirus PCR assay with RNA extracted by two manual methods, TRIzol (Invitrogen) and QIAamp (Qiagen), and with two automated instruments, BioRobot M48 (Qiagen) and MagNA Pure LC (Roche Applied Sciences). The analysis included analytical sensitivity, a cross-contamination study, performance with clinical specimens, and comparison of extraction times and reagent costs. The BioRobot M48 and MagNA Pure platforms were chosen for investigation because they process 48 and 32 specimens, respectively. These instruments are therefore suitable for use by laboratories with intermediate testing volumes. The comparative performance of the BioRobot M48 in molecular assays for infectious diseases has not been previously reported.

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MATERIALS AND METHODS

Virus stock. Poliovirus type 2 Sabin (PV2S; Division of Biological Standards at the National Institutes of Health, Bethesda, Md.) was used as a representative enterovirus. A virus stock was grown and quantitated in A549 lung carcinoma cells. Virus was diluted in viral transport media (VTM; minimal essential medium with 0.125% gentamicin, 5% fetal calf serum, and 0.1% amphotericin B) when adjustment of sample input volume was required.

RNA extraction methods. RNA was extracted manually by two methods: the use of TRIzol solution (Invitrogen; 100-μl sample input, 50-μl output) and the use of a QIAamp Viral RNA kit (Qiagen; 140-μl sample input, 70-μl output). The performance of two automated nucleic acid extraction instruments, MagNA Pure LC (Total Nucleic Acid Isolation kit [Roche Applied Sciences]; 200-μl sample input, 100-μl sample output) and BioRobot M48 (MagAttract Viral RNA M48 kit [Qiagen], formerly Geno M48 instrument and GenoPrep Viral RNA kit [Genovision]; 300-μl sample input, 150-μl output) was tested. Input and output volumes were chosen based on the manufacturer's recommendations.

Analytical sensitivity and reproducibility experiments. Panels of PV2S were created by serial dilution (10^0 to 10^{-3} PFU/ml; three replicates of each dilution per panel) in pooled CSF, which was found to have no detectable enterovirus RNA by reverse transcription-PCR. The CSF pool was tested for enterovirus 10 times to exclude the possibility that the pool contained a low level of enteroviral RNA that would be variably detected by PCR. Virus dilutions were extracted by each method and enterovirus RNA was detected by reverse transcription-PCR as described below. Except for TRIzol, six panels were tested per extraction method, for a total of 18 datum points per dilution per method. Evaluation of TRIzol was terminated after three panels were tested (nine replicates) due to the relative insensitivity of enterovirus PCR after RNA extraction with this method.

Contamination studies. The occurrence of cross contamination during the operation of the MagNA Pure and BioRobot M48 instruments was assessed by extraction of PV2S and a negative control (VTM only) in various alternating patterns, including sample-by-sample, row-by-row, column-by-column, and plate-by-plate patterns. Alternating column experiments were not performed on the BioRobot M48 instrument because of its configuration (specimens are extracted in blocks containing offset rows rather than columns). The concentration of PV2S approximated the highest amount of virus (10^5 PFU/ml) that might be found in a clinical sample (14).

Patient samples. Extraction and amplification of diverse enterovirus serotypes from a variety of specimen types were assessed by testing clinical specimens, previously tested as positive or negative for enterovirus, that had been stored at −70°C. With the exception of specimens 29 and 30, results of enterovirus testing for clinical samples were based on TRIzol extraction, followed by PCR. Specimens 29 and 30 were identified as containing enterovirus by tissue culture isolation. For comparison of different extraction methods, the TRIzol extraction was repeated. A panel of 30 specimens comprised of stool (n = 11), CSF (n = 7), nasopharyngeal aspirates (n = 6), rectal swabs (n = 4), and sera (n = 2) was tested. Enteroviruses were isolated from six of the patient specimens described above, and serotypes were determined by neutralization with Lem Benyesch Melnick pools (World Health Organization). Four different enterovirus types were identified (poliovirus type 1, poliovirus type 3, echovirus 17, and coxsackie B2 virus). Attempts to determine the type of enterovirus in the remaining positive specimens (n = 24) by PCR and cycle sequencing of the VP-1 region (15) were not successful.

Enterovirus PCR. Reverse transcription-PCR was performed as described previously. Briefly, forward (E1, 3′-ACCTAACCGGTAGGCCAC-5′) and reverse (E2, 5′-TCCGGCCCCTGAATG-3′) primers amplified a 196-bp fragment of the 5′-nontranslated region (3). Reactions contained 300 μM concentrations of deoxynucleoside triphosphates, 0.5 μM concentrations of each primer, 3.5 mM manganese acetate, 1× EZ buffer (Applied Biosystems, Foster City, Calif.), 5 U r*Tth* DNA polymerase (Applied Biosystems), and 10 μl of extracted sample in a total volume of 50 μl (J. Ticehurst, M. Forman, W. Merz, and P. Charache, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996, abstr. C–130, p. 24, 1996). The positive control on each PCR assay consisted of a sample containing 10^0 PFU of PV2S/ml that was extracted alongside test samples on each run. Thermocycling was performed by using a GeneAmp PCR system 9600 instrument (Applied Biosystems) programmed with the following parameters: 1 cycle of 60°C for 30 min; 1 cycle of 94°C for 1 min; 40 cycles of 94°C for 15 s and 60°C for 30 s; and finally 60°C for 7 min, with a hold temperature of 4°C (Ticehurst et al, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996). PCR products were detected by Southern blot analysis after electrophoresis through 4% agarose (NuSieve 3:1; BioWhittaker Molecular Applications, Rockland, Maine). Amplicons were transferred onto nylon membrane (GeneScreen Plus; Perkin-Elmer Life Sciences, Boston, Mass.) and hybridized for 1 h at 42°C with a ^{32}P-end-labeled oligonucleotide probe (E3 probe, 5′-ACACGGACACCCAAAGTAG-3′) in hybridization solution (3× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; pH 7.7], 5× Denhardt solution, 0.5% sodium dodecyl sulfate, 250 μg of yeast tRNA/ml). Blots were washed in 6× SSPE (twice at room temperature and twice at 60°C). Amplicons were detected by autoradiography after an overnight exposure at −70°C.

Time analysis. The amount of time elapsed during extraction of a comparable number of specimens by QIAamp, BioRobot M48, and MagNA Pure was measured by using a stopwatch. A single run was timed.

TABLE 1. Analytical sensitivity of four extraction methods[a]

Extraction method	No. positive/total no. tested at a viral input (PFU/ml) of:			
	10^0	10^{-1}	10^{-2}	10^{-3}
TRIzol	8/9	4/9	0/9	0/9
QIAamp	17/18	17/18	4/17*	0/17*
BioRobot M48	18/18	15/18	2/18	0/18
MagNA Pure	18/18	12/18	0/17*	0/17*

[a] Evaluation of TRIzol was discontinued after nine replicates. *, A single replicate was excluded from analysis because bands on autoradiographs were smeared and not interpretable.

RESULTS

Analytical sensitivity of enterovirus PCR in CSF samples after extraction of RNA by four different methods. The sensitivity of PV2S detection by PCR after extraction by two manual methods and two automated methods was compared (Table 1). After manual extraction with TRIzol, the enterovirus PCR assay detected eight of nine replicates at 10^0 PFU/ml, four of nine replicates at 10^{-1} PFU/ml, and zero of nine replicates at 10^{-2} PV2S. In contrast, all replicates at 10^0 PFU/ml were detected by PCR after manual QIAamp, BioRobot M48, and MagNA Pure extraction (18 replicates per method). PV2S RNA was also detected by PCR in most replicates of 10^{-1} PFU/ml after extraction by these three methods. Enterovirus PCR detected PV2S RNA in a smaller number of replicates of the 10^{-2} PFU/ml dilution after extraction of CSF by QIAamp and BioRobot M48. No replicates of this concentration were detected by MagNA Pure. No replicates of PV2S at 10^{-3} PFU/ml were detected by PCR after extraction by all four methods.

Analytical specificity of enterovirus PCR after automated extraction. One potential disadvantage of extraction by automated instrumentation is the generation of false-positive results due to contamination. This was investigated in a series of experiments in which positive (VTM spiked with 10^5 PFU of PV2S/ml) and negative (VTM only) specimens were alternated by position (checkerboard), column (MagNA Pure only), row, and plate (Table 2). The samples in the MagNA Pure instrument are set up in a four-by-eight plate (n = 32). The BioRobot M48 is configured in two offset rows of 24 (n = 48). No evidence of contamination was observed after extraction by MagNA Pure. Two false-positive results were observed in a checkerboard experiment on the BioRobot M48. The instrument was reprogrammed with a new protocol after this result was obtained, and no evidence of contamination was observed in subsequent checkerboard, alternating-row, and alternating-plate experiments.

Enterovirus detection in clinical specimens after extraction by four different methods. In order to compare the efficacies of

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TABLE 2. Cross-contamination studies on automated extraction instruments

Extraction instrument	True-positive/false-positive results[a] (*n*)			
	Checkerboard pattern	Column-by-column pattern	Row-by-row pattern	Plate-by-plate pattern
MagNA Pure	16/0 (32)	16/0 (32)	16/0 (32)	32/0 (64)
BioRobot M48 v.1	24/2 (48)	ND*	ND	ND
BioRobot M48 v.2	24/0 (48)	ND*	24/0 (48)	48/0 (96)

[a] Positive and negative specimens were alternated by position (checkerboard), column, row, and plate. After two false-positive results were found with checkerboard analysis by BioRobot M48 (BioRobot M48, v.1), the instrument software was rewritten. The new software (BioRobot M48, v.2) was retested with the four specimen configurations. ND, not done. *, The BioRobot M48 extraction platform is not configured in columns; therefore, this was not tested.

enterovirus RNA extraction from different specimen types and the efficacies of extraction of different enterovirus serotypes, clinical specimens tested previously for enterovirus were extracted and enterovirus PCR was performed (Table 3 [positive specimens, $n = 19$; enterovirus not detected, $n = 11$]). CSF, nasopharyngeal aspirate, stool, and rectal swab specimens were extracted by each method. Sera were extracted by QIAamp, BioRobot M48, and MagNA Pure. The results of PCR were concordant in 24 of 28 specimens that were extracted by all four methods (13 of 17 positive for enterovirus RNA; 11 of 11 enterovirus RNA were not detected). Enterovirus RNA was detected in the two serum specimens that were extracted by automated instruments. Discordant results were obtained from stool, rectal swab, nasopharyngeal aspirate, and CSF specimens (one each) in which enteroviruses were previously detected. In 2 of 4 specimens with discordant results, enterovirus RNA was detected after extraction by two methods (one after extraction with QIAamp and BioRobot M48, one after extraction with MagNA Pure and BioRobot M48). The remaining two discordant results were obtained from specimens that were positive after extraction by one of the four methods (one QIAamp and one TRIzol).

TABLE 3. Enterovirus detection in clinical specimens after extraction by four different methods

Specimen	Source	Enterovirus type[a]	Detection by:			
			TRIzol	QIAamp	BioRobot M48	MagNA Pure
6	CSF	unknown	+	+	+	+
10	CSF	unknown	−	+	+	−
19	CSF	unknown	+	+	+	+
20	CSF	unknown	−	−	−	−
21	CSF	unknown	−	−	−	−
22	CSF	unknown	−	−	−	−
23	CSF	unknown	−	−	−	−
1	Stool	PV1	+	+	+	+
4	Stool	PV1	+	+	+	+
5	Stool	PV1	+	+	+	+
7	Stool	PV3	+	+	+	+
8	Stool	unknown	+	+	+	+
9	Stool	unknown	+	+	+	+
11	Stool	unknown	+	+	+	+
12	Stool	unknown	+	+	+	+
13	Stool	unknown	−	−	−	−
14	Stool	unknown	−	−	+	+
15	Stool	unknown	+	−	−	−
17	Stool	unknown	+	+	+	+
18	Stool	unknown	+	+	+	+
27	Stool	unknown	−	−	−	−
28	Stool	unknown	−	−	−	−
16	NP	unknown	−	−	−	−
2	NP	CB2	+	+	+	+
3	NP	ECHO17	−	+	−	−
24	NP	unknown	−	−	−	−
25	NP	unknown	−	−	−	−
26	NP	unknown	−	−	−	−
29	Serum	unknown	ND[b]	+	+	+
30	Serum	unknown	ND	+	+	+

[a] PV1, polio virus type 1; PV2, polio virus type 2; CB2, coxsackie B2 virus; ECHO17, echovirus 17.
[b] ND, not done.

The enterovirus type was known for 6 of 19 positive specimens (Table 3) and consisted of four different viruses. Three of the four virus types (poliovirus type 1, poliovirus type 3, and coxsackie B2 virus) were detected after extraction by all four techniques. The nasopharyngeal specimen containing echovirus 17 was detected only after QIAamp extraction.

Time and cost analysis. The amount of time required for specimen processing by QIAamp, BioRobot M48, and MagNA Pure was determined (Table 4). The total instrument time for runs with a comparable number of specimens was greater for BioRobot M48 than for MagNA Pure. The disparity in the length of time for a full run on each instrument was even greater. The times required for extraction of a comparable number of specimens by the manual QIAamp and MagNA Pure methods were equivalent. The list prices of the processing kits (i.e., the cost per specimen) for the manual Qiagen and BioRobot M48 methods were comparable. MagNA Pure reagents for RNA extraction were slightly more expensive.

DISCUSSION

Our data demonstrate that the analytical sensitivity of enterovirus detection by PCR is similar after RNA extraction by the two automated methods and by the QIAamp manual method. Studies with serially diluted PV2S demonstrated a

TABLE 4. Time and cost comparison for manual spin column and two automated extraction instruments

Extraction method	No. of extracted specimens	Time (min)/run	Instrument time (min)/specimen	Cost (U.S. dollars)/specimen
QIAamp	30	100	NA[a]	2.64
BioRobot M48	36	130 (173[b])	3.6	2.71[c], 2.46[d]
MagNA Pure	32	92	2.9	3.58

[a] NA, not applicable.
[b] Calculated time required for a run of 48 specimens.
[c] List price for kits with reagents for 96 extractions.
[d] List price for kits with reagents for 480 extractions (the cost of plastics is not included).

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trend of higher sensitivity after extraction by manual QIAamp and automated BioRobot M48 methods. However, the results of enterovirus PCR after extraction of clinical specimens were largely concordant, and all methods effectively extracted the low concentration of enterovirus (10^0 PFU/ml) that serves as the positive control for the clinical assay (Table 1).

Currently, there at least 64 different enterovirus types. In the present study, a limited number of types were known. Three different types were detected after extraction by all four methods. It is likely that the ability to detect numerous different enteroviruses within a single PCR method is determined more by primer/probe sequences than by extraction method. Accordingly, our results suggest that the genomic RNA from diverse enteroviruses can be effectively extracted with all methods assessed.

Discordant results of enterovirus PCR were observed from four clinical specimens that were previously demonstrated to contain enterovirus by culture or PCR. The source of this discordance is unclear. Specimen storage is a potential explanation, since these specimens had been maintained at −70°C for ca. 15 years and had been frozen and thawed a number of times. The lack of concordance does not appear to correlate with the effectiveness of the extraction technique (as defined by sensitivity studies above) since PCR was positive by one or two of each of the techniques. It is also unlikely that specimens were initially erroneously classified as positive since one discrepant result was obtained from a specimen from which enterovirus was isolated and the serotype was determined, whereas the remaining three specimens were determined to be positive by PCR by using a template that had been extracted at least twice by the same or different techniques.

A major concern in the implementation of automated instrumentation to extract nucleic acid for use in amplification assays is the potential for cross-contamination of negative specimens as a consequence of aerosolization, faulty robotics, or robotic error. We did not observe any specimen cross-contamination after extraction by MagNA Pure. Initial results with the BioRobot M48 were not as promising; however, no evidence of contamination was observed once the instrument was programmed to change tips after the platform was checked. This software upgrade is available to all users. These data suggest that specimen contamination during automated RNA extraction by both methods remains a theoretical rather than a real concern. We did not test specimen contamination during the manual methods, but it is a potential concern given the technician-dependent nature of manual extraction efficacy.

Practical considerations such as volume, throughput, flexibility, and cost are important when instrumentation is being selected. The BioRobot M48 can extract a greater number of specimens per run than MagNA Pure (48 versus 32 specimens); however, extraction on the BioRobot M48 is significantly slower. In addition, extractions on the BioRobot M48 platform must be performed on batches of six specimens. In contrast, the MagNA Pure instrument has a more flexible extraction format (any number of specimens [up to 32] can be extracted per run). List prices for MagNA Pure reagents are greater than for the BioRobot M48 and manual QIAamp extractions.

The goal of our studies was to compare the RNA extraction performance of two mid-sized automated platforms to manual methods. Our results show that the BioRobot M48 and MagNA Pure instruments perform comparably to one another and to manual extraction methods. These platforms are realistic options for laboratories with test volumes for enteroviruses and other pathogens that are not great enough to justify the acquisition of high-throughput extractors.

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