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Impact of nucleic acid extraction platforms on hepatitis virus genome detection

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Abstract

Detection and quantification of viral nucleic acids are important for diagnosing current viral infections and monitoring response to antiviral therapy. Automated nucleic acid extraction and purification platforms are routinely used during the first step in these processes in clinical and research laboratories. Here, we compare the extraction efficiencies of four MagNA Pure magnetic bead-based nucleic acid extraction platforms and associated kits using samples positive for nucleic acids from HAV, HBV, HCV, HDV, and HEV. These five hepatitis viruses are diverse in their virion structures and type of nucleic acid that compose their genomes. We found that the most efficient nucleic acid extraction platform and corresponding kit, when averaged across all tested viruses, was the MagNA Pure 96, which yielded twice as much detectable nucleic acid as the other platforms. However, the relative efficiencies of the different platforms varied by virus type, suggesting that an extraction platform that is more efficient for one virus type will not necessarily function better with a different virus type. Our results show that the choice of a nucleic acid extraction platform influences the sensitivity of the methodology and has the potential to generate false-negative results especially in samples with low levels of viral nucleic acids.

Keywords

Nucleic acid testing; Viral hepatitis

1. Introduction

It is estimated that hundreds of millions of individuals are infected with hepatitis viruses worldwide (Ly and Klevens, 2015; Schweitzer et al., 2015; Thrift et al., 2017; Hakim et al., 2017). Acute and chronic infection by these viruses can cause severe morbidity and are a contributing factor to over one million deaths annually (Stanaway et al., 2016). The major public health burden from hepatitis viruses necessitates the development of sensitive serological and molecular detection methods. Serological methods for antigen and antibody detection are commercially available for routine diagnosis of hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D (delta) virus (HDV), and

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Declaration of Competing Interest

None.

hepatitis E virus (HEV) infections (Nainan et al., 2006; Villar et al., 2015; Noureddin and Gish, 2014; Mirazo et al., 2014). However, nucleic acid testing (NAT) is currently the only method for diagnosing current HCV infection and is crucial for monitoring response to antiviral therapy in HBV, HCV, HDV and HEV-infected patients (Villar et al., 2015; Easterbrook et al., 2017; Kodani et al., 2013; Germer et al., 2017). Furthermore, detection of viral nucleic acids is important for characterization of circulating viral isolates, genotyping, outbreak investigations and transplant related transmission studies (Collier et al., 2014; Bixler et al., 2019). A first step of any NAT-based methodology is the extraction of viral nucleic acids from clinical samples and for this purpose both manual methods and various automated platforms are widely used (Berensmeier, 2006). The automated nucleic acid extraction platforms, which require little hands-on time, have recently gained popularity in both clinical and research laboratories.

All five hepatitis viruses replicate in hepatocytes and cause an illness with similar signs and symptoms that are clinically indistinguishable. The hepatitis viruses are diverse in their modes of transmission, genome and virion structure, and disease progression. HAV and HEV are non-enveloped positive-sense RNA viruses shed in feces and transmitted via the fecal-oral route (Cao and Meng, 2012; Lemon et al., 2018). HBV is an enveloped virus with a small partially double-stranded DNA genome that is transmitted parenterally (Seeger and Mason, 2015). HDV is a satellite virus that can only replicate in HBV infected individuals (Negro, 2014). Hepatitis D virions are enveloped, using the same surface protein as HBV (HBsAg), and contain a small circular single-stranded RNA genome (Sureau and Negro, 2016). HCV, a parenterally transmitted enveloped virus, possesses a positive-sense RNA genome (Dubuisson and Cosset, 2014). All five hepatitis viruses can be detected in the serum or plasma of infected patients using various NAT methodologies. Viremia is short-lived for hepatitis A and hepatitis E, while chronic infection with HBV, HCV, and HDV can persist for decades. Sensitive NAT is necessary for accurate monitoring of disease progression and treatment outcomes.

To determine whether there are differences in the efficiency of viral nucleic acid extraction that could influence the sensitivity of various PCR methodologies for HAV, HBV, HCV, HDV or HEV detection, we evaluated four different automated nucleic acid extraction kits based on magnetic-bead technology from Roche Life Sciences: the MagNA Pure Compact Nucleic Acid Isolation Kit I, the MagNA Pure LC 2.0 Total Nucleic Acid Isolation Kit, the MagNA Pure LC 2.0 Total Nucleic Acid Kit - High Performance, and the MagNA Pure 96 DNA and Viral NA Small Volume Kit.

2. Materials and methods

2.1. Samples

We used 32 well-characterized samples positive for HAV RNA (n = 6), HBV DNA (n = 6), HCV RNA (n = 7), HDV RNA (n = 6) and HEV RNA (n = 7) to compare four nucleic acid extraction methods (Table 1). Twenty-seven of these samples were de-identified, anonymized human plasma or serum samples obtained from the viral hepatitis specimen repository. Three were de-identified 10% stool suspensions in phosphate buffered saline (PBS) (HAV (n = 1), HEV (N = 2)) from the viral hepatitis specimen repository. Two were

cleared cell culture supernatants (HAV (n = 1), HEV (n = 1)). These samples spanned a range of viral titers for each virus. Viral titers were determined independently of the nucleic acid extractions described in our results using the FDA-approved COBAS Ampliprep/ TaqMan assays for HBV and HCV (Roche Applied Science, Indianapolis, IN), and laboratory developed tests (LDT) for quantitative PCR (described below) with standards containing known amounts of viral nucleic acids for HAV, HDV, and HEV (Kodani et al., 2013; Costafreda et al., 2006; Jothikumar et al., 2006; Kodani et al., 2014). Five of the 32 samples were determined to be hepatitis virus positive by qualitative means and were not quantitatively titered. Due to volume limitations, some plasma samples were diluted in negative human serum (Seracare, Milford, MA) and stool was diluted in PBS prior to nucleic acid extraction (Table 1). Eight additional de-identified human plasma specimens with HCV titers below 1000 IU/mL (ranging from 41 to 822 IU/mL) were used for comparing detection of low-titer samples. This analysis was considered research involving non-identifiable human samples and thus exempt from review by the CDC IRB.

2.2. Nucleic acid extraction

Total nucleic acids were extracted from each positive sample using four different extraction methods on Roche MagNA Pure platforms (Roche Applied Science) using recommended manufacturer's protocols (Table 2). The Nucleic Acid Isolation Kit I (Roche Applied Science) was used on the MagNA Pure Compact platform. Both the Total Nucleic Acid Isolation Kit (Roche Applied Science) and the Total Nucleic Acid Kit - High Performance kit (Roche Applied Science) with the maximum sensitivity and recovery protocol (HS) were used on the MagNA Pure LC 2.0 platform. The MagNA Pure 96 platform was used with the DNA and Viral NA Small Volume Kit (Roche Applied Science). Each extraction processed 200 μL of sample and eluted nucleic acid in a final volume of 50 μL. To test the effect of input volume on nucleic acid detection, low-titer HCV samples (< 1000 IU/mL HCV RNA) were also processed with 400 μL or 500 μL input volumes and 50 μL elution volumes using the MagNA Pure Compact or the LC 2.0 High performance kit, respectively. All four extractions of a sample were performed on the same day. Extracted nucleic acids were stored at −80 °C and thawed only once prior to nucleic acid detection.

2.3. Nucleic acid detection

Quantitative PCR was used to compare the efficiencies of the four nucleic acid extraction methods. Primer and probe sequences and concentrations are listed in Table 3. All probes were modified with 5′ 6-carboxyfluorescein, 3′ Iowa BFQ, and an internal ZEN quencher nine bases from the 5′ end (Integrated DNA Technologies, Coralville, IA). HAV RNA was measured using SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) on a LightCycler 480 II (Roche Applied Science with the following cycling parameters; 50 °C for 30 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and 58 °C for 1 min (Costafreda et al., 2006). HBV DNA was measured using Express qPCR Supermix (Invitrogen) on a LightCycler 480 II with the following cycling parameters; 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min (Mixson-Hayden et al., 2014). HCV RNA was measured using SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen) on a LightCycler 480 II with the following cycling parameters; 50 °C for 30 min, 95 °C for 5 min, and 55 cycles

of 95 °C for 15 s and 58 °C for 1 min (Mixson-Hayden et al., 2014). HDV RNA was measured using AgPath-ID One-Step RT-PCR (Applied Biosystems, Foster City, CA) on a 7500 Real-Time PCR System (Applied Biosystems) as previously described (Kodani et al., 2013). HEV RNA was reverse transcribed using SuperScript VILO (Invitrogen) with random hexamers followed by qPCR using PerfeCTa SYBR Green SuperMix (Quantabio, Beverly, MA) on a LightCycler 480 II with the following cycling parameters; 95 °C for 5 min, and 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s (Jothikumar et al., 2006; Kodani et al., 2014). The cycle threshold (Ct) value was determined for each reaction using the second derivative maximum method. Three technical replicates were included on each PCR plate for all nucleic acid extraction samples. All nucleic acid extractions from all samples for a particular virus type were run on the same qPCR reaction plate to allow for direct comparison of Ct values.

2.4. Statistical analysis

Relative nucleic acid yields from the four extraction methods for each sample were calculated by subtracting the Ct value obtained for one method from the average Ct value of the four methods. These relative yields were compared for individual viruses and for aggregated data using a one-way ANOVA with the Tukey correction for multiple comparisons in R (version 3.5.0).

3. Results

The nucleic acid extraction efficiencies of four kits from Roche Life Science were compared using samples containing hepatitis viruses A–E. Each kit uses similar reagents, protocols, and magnetic-bead technology to purify nucleic acids from lysed samples. These kits differ in their cost per sample, run length, number of samples that can be processed in a single run, and the automated extraction platform with which they are used (Table 2). The MagNA Pure Compact has the fastest extraction procedure, but it is also the most expensive and the lowest throughput, allowing only eight samples per run. The two intermediate throughput kits used with the MagNA Pure LC 2.0 are the least expensive per sample, but also take the most time. The MagNA Pure 96 kit can process the most samples in one run and has an intermediate cost per sample and run time compared to the other kits. Each of these kits uses a similar method for nucleic acid extraction and purification. Samples are lysed using chaotropic salts and proteinase K treatment. Nucleic acids (both DNA and RNA) are bound to magnetic glass particles and washed several times to remove proteins and other contaminants. Nucleic acids are then eluted from the magnetic glass particles and ready to use for subsequent applications.

Nucleic acid extractions using each of the four kits were performed on samples containing diverse source materials (plasma, stool suspension, and cell culture supernatant) and a broad range of viral titers (Table 1). The amount of viral nucleic acid in each extraction was measured by quantitative PCR, where lower cycle threshold (Ct) values indicate more amplifiable nucleic acid in the sample (Fig. 1). All four extraction kits yielded detectable viral nucleic acid from all samples, except for sample HAV3, which could not be detected in the MagNA Pure Compact and the LC 2.0 TNA extractions (Fig. 1). The relative amount of

viral nucleic acid yielded by each extraction method (average Ct of the four methods minus the Ct value of the method of interest) was generally consistent across samples when analyzed by virus type (Fig. 2A). This consistency is found across the range of viral titers and sample source materials tested for each virus. For HAV nucleic acid extractions, The LC 2.0 TNA kit yielded significantly less detectable RNA than the LC 2.0 HP kit (one-way ANOVA with Tukey correction for multiple comparisons; adjusted $p = 0.0053$) and MagNA Pure 96 (adjusted $p = 0.015$). The MagNA Pure 96 yielded more amplifiable HBV DNA than the other three methods (adjusted $p < 0.0012$ for each comparison). Similar to its performance with HAV, the LC 2.0 TNA kit extracted less HCV RNA from samples than the other methods (adjusted $p = 0.028$ (compact), adjusted $p = 0.0027$ (96), adjusted $p = 0.0002$ (LC 2.0 HP)). The largest differences in nucleic acid extraction efficiency were observed with HDV; the LC 2.0 TNA kit and the MagNA Pure 96 both performed significantly better than either the MagNA Pure Compact or the LC 2.0 HP kit (adjusted $p < 0.0005$ for each comparison). Nucleic acid extractions of HEV showed no statistically significant differences in measurable RNA yield among the four methods tested. When method performance is aggregated across all five virus types, The MagNA Pure 96 performed the best on average, yielding greater than two-fold more measurable viral nucleic acid (more than 1 Ct difference) than any of the other three methods (Fig. 2B).

The lower limit of detection may be an important criteria to consider for certain nucleic acid extraction applications. We performed nucleic acid extractions of eight additional human plasma specimens containing HCV RNA titers below 1000 IU/mL using each of the four extraction methods to test if extraction efficiency correlates with detection limit. The LC 2.0 High Performance kit and the MagNA Pure Compact each yielded detectable HCV RNA for five of the eight samples, while the LC 2.0 TNA kit and the MagNA Pure 96 yielded detectable RNA for only two samples. Since detection is based on the amount of the nucleic acid analyte, we tested if increasing sample input while maintaining final elution volume (50 μL) could improve detection of the low-titer HCV samples. Increasing the sample input 2.5-fold (500 μL) for the LC 2.0 HP kit and 2-fold (400 μL) for the MagNA Pure Compact each improved the detection to six out of eight samples.

4. Discussion

Nucleic acid extraction is a key step in the detection of viruses in clinical specimens and for a variety of research laboratory applications. Automated platforms, which improve throughput and decrease hands-on time, are increasing in both their accessibility and the number of available options. Extraction methods based on different technologies are known to vary in efficiency when used on viruses (Verheyen et al., 2012; Chevaliez et al., 2008; Pyne et al., 2012; Kang et al., 2012; Yang et al., 2011). Here, we have evaluated four different automated methods based on magnetic-bead technology from Roche Life Sciences for their ability to extract nucleic acids from samples containing HAV, HBV, HCV, HDV, and HEV. We found that kits using similar extraction and purification procedures can differ significantly in their performance, and that the relative performance of these kits differ by the type of virus being detected. For example, compared with the other methods, the MagNA Pure LC 2.0 Total Nucleic Acid Isolation kit averaged higher recovery of nucleic acids for HDV, similar recovery for HEV, and lower recovery for HAV, HBV, and HCV. Users of

nucleic acids extraction applications that require high sensitivity, such as clinical diagnostics, may benefit from evaluating different kits, platforms, and/or sample input volumes to determine if they are using the best method for their work. This finding is especially evident in our results for HDV extraction where there is a greater than ten-fold (more than 3.32 Ct) difference in detectable nucleic acids among the best (96) and worst (LC 2.0 HP) methods. Similar to our results, others have found significant differences in HDV extraction efficiency among manual and automated nucleic acid extraction methods (Bremer et al., 2019). While two-fold to four-fold differences (1–2 Ct values) in nucleic acid yields may not affect results for samples with high titers, they could be the difference between a positive and a negative diagnostic result for a low-titer sample. Our results exhibit this possibility with HAV and HCV. We were unable to detect HAV RNA in the extractions of the diluted sample HAV3 using the MagNA Pure Compact or the LC 2.0 TNA kit, which averaged the least measurable HAV RNA of the methods tested. Additionally, the most efficient extraction method for HCV, the LC 2.0 HP, produced detectable RNA from three more low-titer HCV samples than the least efficient kit, the LC 2.0 TNA. We demonstrated that a possible way to increase analytical sensitivity is to increase the sample input volume. When we increased the input volumes of low-titer HCV samples with the MagNA Pure LC 2.0 HP kit 2.5-fold and MagNA Pure Compact 2-fold while retaining the same elution volume, we were able to improve detection from five to six out of the eight low-titer HCV samples. These results suggest that lower limits of detection can be mitigated by increasing the volume of the sample that is used for nucleic acid extraction (Germer et al., 2003).

The reason for the differences in nucleic acid extraction efficiency is uncertain. The purity of the extracted nucleic acid is a factor that could have affected our measurements of viral DNA and RNA. Proteins and other contaminants from the extraction process can interfere with PCR. We were unable to assess the purity of our extracted nucleic acids because of the low concentrations obtained from the serum, cell culture supernatants, and diluted stool suspension that were used in this study. Our results suggest that the architecture and structure of the virion and the viral genome may play a role in determining which extraction method works most efficiently. The High Performance kit on the MagNA Pure LC 2.0 yielded the most amplifiable nucleic acid from two viruses possessing linear single-stranded RNA genomes (HAV and HCV). The MagNA Pure 96 yielded the most measurable nucleic acids from HBV and HDV, which both have small circular genomes and lipid envelopes. While HBV has a partially double-stranded DNA genome and HDV has a single-stranded RNA genome, they both have the same surface antigen protein and lipid structure (Shirvani-Dastgerdi and Tacke, 2015). It is worth noting that one previous comparison of the MagNA Pure 96 with extraction methods from other manufacturers showed that it performed well with HBV, while another study found that it could produce false-negative results with low-titer HDV samples (Kang et al., 2012; Bremer et al., 2019). Further investigation is required to see how generalizable these trends are among other viruses with similar characteristics and to test extraction efficiencies for viruses with fully double-stranded, linear, and larger DNA genomes.

By comparing the efficiency of four automated nucleic acid extraction methods with five types of hepatitis virus representing diverse virion architecture and genome composition, we have shown that not all methods work equally well for all viruses. Selecting an extraction

platform and kit for use in a clinical or research laboratory often focuses on the cost, ease of use, and run time. While these are critical parameters to consider, our research sheds light on the importance of evaluating the extraction performance especially for applications that require high sensitivity. Often, extraction platforms have multiple available kits (for example the MagNA Pure LC 2.0 has the Total Nucleic Acid Isolation Kit and the Total Nucleic Acid Kit - High Performance) or allow variable sample input volumes that could be optimized for certain applications. Our observed differences in the nucleic acid extraction efficiency were among Roche Life Sciences manufactured platforms and kits that are based on magnetic-bead technology with similar reagents and automated protocols. With the diversity of manual and automated extraction methods currently on the market using different protocols and technologies, product selection could have important consequences for diagnostic and experimental work.

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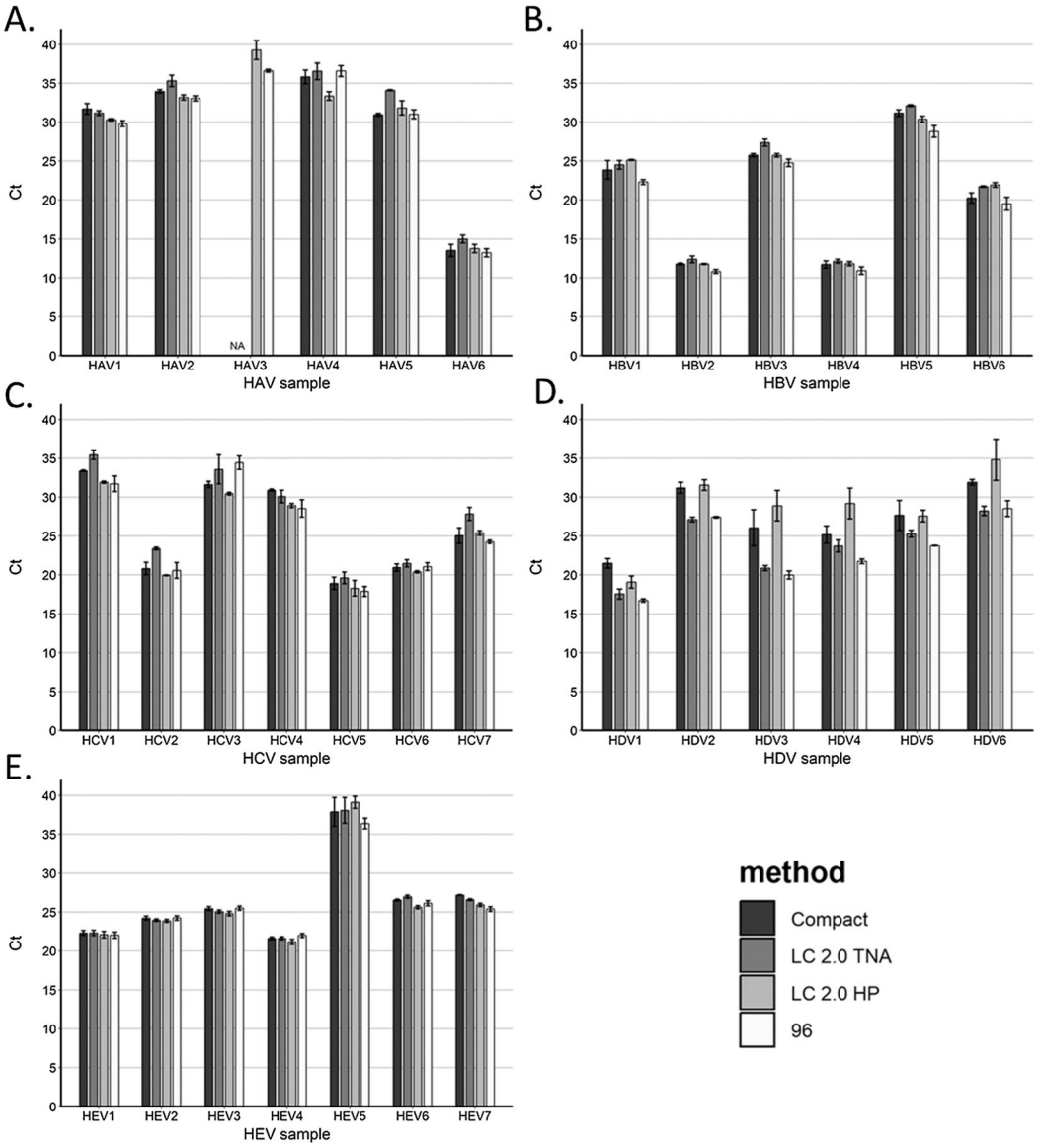

Fig. 1. Measurement of extracted hepatitis virus nucleic acids.

Nucleic acids were extracted from samples containing hepatitis viruses using a MagNA Pure Compact (black), MagNA Pure LC 2.0 with either a Total Nucleic Acid Isolation Kit (dark gray) or a Total Nucleic Acid Kit – High Performance (light gray), or MagNA Pure 96 (white). One-step reverse transcription qPCR was used to measure RNA from HAV (A), HCV (C), and HDV (D). B. DNA from HBV was measured by standard qPCR. E. RNA from HEV was measured using reverse transcribed cDNA as a template for standard qPCR. Bars show the mean Ct value ± the standard deviation of three qPCR technical replicates for each extracted nucleic acid sample. Lower Ct values indicate more nucleic acid in a sample. NA = no amplification.

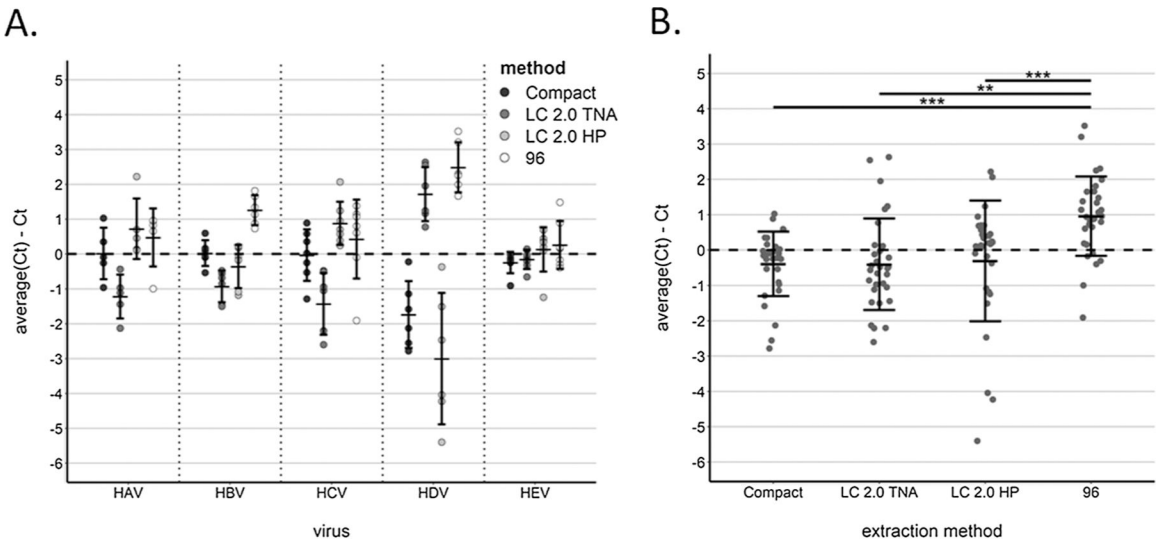

Fig. 2. Comparison of nucleic acid extraction methods.

The relative amount of nucleic acid extracted from each sample by each platforms was calculated by subtracting its Ct value from the average Ct value of the four methods (see Fig. 1 for raw data). Values for each extraction, along with the mean and standard deviation, are shown. Extractions with values above 0 yielded better than average amounts of amplifiable nucleic acid. One Ct value corresponds to a 2-fold difference in the amount of nucleic acid. **A**. Relative amounts of extracted nucleic acids arranged by virus (x-axis) and extraction method; MagNA Pure Compact (black), MagNA Pure LC 2.0 with either a Total Nucleic Acid Isolation Kit (dark gray) or a Total Nucleic Acid Kit – High Performance (light gray), or MagNA Pure 96 (white). **B**. Relative amounts of extracted nucleic acid from all samples for all viruses arranged by extraction method. Extraction methods were compared using a one-way ANOVA with a Tukey multiple comparison test. ** = $p < 0.01$, *** = $p < 0.001$.

Table 1

Hepatitis virus samples.

Sample	Source material	Viral titer log_{10}(IU/mL)[a]	Dilution[b]
HAV1	human plasma	ND	1:3
HAV2	human plasma	4.5	1:4
HAV3	human plasma	4.7	1:10
HAV4	human stool	4.8	–
HAV5	human plasma	4.0	–
HAV6	cell culture supernatant	ND	–
HBV1	human plasma	6.6	1:8
HBV2	human plasma	> 8.0	–
HBV3	human plasma	4.0	–
HBV4	human plasma	8.0	–
HBV5	human plasma	2.8	–
HBV6	human plasma	6.5	–
HCV1	human plasma	3.0	–
HCV2	human plasma	6.9	–
HCV3	human plasma	4.0	–
HCV4	human plasma	3.8	–
HCV5	human plasma	7.5	–
HCV6	human plasma	6.5	–
HCV7	human plasma	7.0	1:4
HDV1	human plasma	ND	1:2
HDV2	human plasma	4.4	1:6
HDV3	human plasma	6.9	1:6
HDV4	human plasma	5.9	1:6
HDV5	human plasma	5.1	1:6
HDV6	human plasma	4.0	1:6
HEV1	human plasma	6.5	–
HEV2	cell culture supernatant	ND	–
HEV3	human plasma	5.3	1:3
HEV4	human plasma	6.3	1:3
HEV5	human plasma	ND	–
HEV6	macaque feces	9.5	1:100
HEV7	macaque feces	10.0	1:1000

[a] Titers were determined independent of the extractions described in results. ND = not determined.

[b] Plasma samples were diluted in negative human serum and feces samples were diluted in PBS prior to nucleic acid extractions. - = no dilution/ neat.

Table 2

Nucleic acid extraction methods.

Extraction platform	Extraction kit	Samples per run	Price of reagents and consumables[a]	Prep time (min)	Run time (min)	Sample volume (µL)
MagNA Pure Compact	Nucleic Acid Isolation Kit I	1–8	$7.69 per sample	5	30	100–400
MagNA Pure LC 2.0	Total Nucleic Acid Isolation Kit (“LC 2.0 TNA”)	1–32	$132.23 per full run ($4.13 per sample[b])	20	90[c]	50–200
MagNA Pure LC 2.0	Total Nucleic Acid Kit - High Performance (“LC 2.0 HP”)	1–32	$97.19 per full run ($3.04 per sample[b])	20	180[c]	100–1000
MagNA Pure 96	DNA and Viral NA Small Volume Kit	1–96	$477.20 per full run ($4.97 per sample[b])	10	60	50–200

[a] Calculated based on August 2018 pricing.

[b] Price per sample increases when using fewer than the maximum number of samples per run.

[c] Run times are shorter with fewer samples (multiples of 8).

Table 3

Primers and probes.

Virus	Target	Sequence (5’ to 3’)	Function	Concentration (nM)	Reference
HAV	5’ UTR	TCA CCG CCG TTT GCC TA	primer	250	(Costafreda et al., 2006)
		GGA GAG CCC TGG AAG AAA G	primer	250	
		TTA ATT CCT GCA GGT TCA GGG TTC TT	probe	100	
HBV	S gene	TGT CCT GGY TAT CGC TGG AT	primer	300	(Mixson-Hayden et al., 2014)
		CCA ACA AGA AGA TGA GGC ATA GC	primer	300	
		TGC GGC GTT TTA TCA TAT TCC TCT TCA T	probe	200	
HCV	5’ UTR	AGY GTT GGG TYG CGA AAG	primer	400	(Mixson-Hayden et al., 2014)
		CAC TCG CAA GCR CCC T	primer	400	
		CCT TGT GGT ACT GCC TGA	probe	300	
HDV	upstream of δAg gene	TCT CCC TTW GCC ATC MGA G	primer	600	(Kodani et al., 2013, 2014)
		TCC TCT TCG GGT CGG	primer	600	
		CYC GCG GTC CGW CCT GGG C	probe	200	
HEV	Capsid gene	GGT GGT TTC TGG GGT GAC	primer	500	(Jothikumar et al., 2006; Kodani et al., 2014)
		AGG GGT TGG TTG GAT GAA	primer	500	