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Efficient Extraction of Virus DNA by NucliSens Extractor Allows Sensitive Detection of Hepatitis B Virus by PCR

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The NucliSens Extractor is an automated nucleic acid isolation system based on guanidinium thiocyanate (GuSCN)-silica extraction technology. The system has been validated for the isolation of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) RNAs from human samples in combination with nucleic acid sequence-based amplification- and reverse transcription-PCR-based methods. We evaluated the extractor for hepatitis B virus (HBV) DNA extraction from human samples using a noncommercial HBV DNA PCR. Several sample pretreatment procedures in combination with the extractor were compared with the Qiagen extraction method, and the impact of the sample volume used in the extraction on the sensitivity was investigated. Heating of the lysed sample prior to extractor isolation and the use of a large sample volume resulted in highly sensitive detection of HBV DNA. Incubation of a 1-ml sample in GuSCN at 80°C (10 min) and at 37°C (30 min) allowed detection of 4 and 40 HBV genome equivalents/ml, respectively, in standard dilution panels. Sample lysis in GuSCN at room temperature and proteinase K treatment prior to use of the extractor were less efficient procedures. All clinical samples that were PCR positive after Qiagen extraction and/or that were HBsAg positive were also PCR positive after extractor isolation. HBV DNA, HCV RNA, and HIV type 1 RNA were efficiently coextracted from a single sample, allowing reliable detection of viral genomes.

Efficient extraction of nucleic acids from clinical samples is highly determinant for the reliability and performance of any molecular diagnostic assay. Moreover, as many extraction procedures are labor-intensive and time-consuming, the need for automated systems is emerging. The NucliSens Extractor (Organon Teknika, Boxtel, The Netherlands) is an automated nucleic acid isolation system based on the guanidinium thiocyanate (GuSCN)-silica extraction method as described by Boom et al. (4). Thus far, the system has been validated for the isolation of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) RNAs from human plasma and serum in combination with nucleic acid sequence-based amplification (NASBA)- and reverse transcription (RT)-PCR-based nucleic acid amplification methods (3, 6, 7, 11, 12, 15, 22).

Hepatitis B virus (HBV) is an important cause of chronic hepatitis, which can deteriorate into liver cirrhosis or hepatocellular carcinoma (2). The virus has a circular DNA molecule that is partially double stranded and consists of a long complete (minus) and a short incomplete (plus) strand. An RNA-dependent DNA polymerase responsible for hepadnaviral reverse transcription (21) is covalently linked to the 5′ end of the long DNA strand (9). Sensitive detection and quantification of HBV genomic DNA seem to constitute a promising method to monitor HBV infections and the efficacy of antiviral treatment (1, 10) and can be considered for routine screening of pooled blood donations in order to increase the safety of the blood supply (16, 18, 19).

In this study we investigated the suitability of the NucliSens Extractor to isolate HBV DNA from human plasma and serum samples in order to allow sensitive detection of the viral genome by a noncommercial PCR method.

Several sample pretreatment procedures were evaluated in combination with the extractor and compared with the Qiagen (Westburg, The Netherlands) extraction method. The impact of the sample volume used in the extraction on the sensitivity of the extractor and its suitability to coextract several viral genomes was also investigated.

MATERIALS AND METHODS

Samples. Commercial Virological Quality Control and European Expert Group on Viral Hepatitis HBV DNA plasma standard dilution panels (PELICHECK; CLB, Amsterdam, The Netherlands), 10-fold serial dilutions of 3 patient serum samples with known HBV viral loads (previously determined by means of the Digene HBV DNA hybrid capture assay), and 20 serum samples obtained from patients with diagnosed HBV infections were tested to assess analytical and clinical sensitivity. A PELISPY multimarker control (CLB) containing 375 genome equivalents (geq) of HBV DNA/ml, 375 geq of HIV type 1 (HIV-1) RNA/ml, and 380 geq of HCV RNA/ml was used in the coextraction experiments. Four negative controls (human sera) per test run were included.

Pretreatment of samples prior to HBV DNA isolation by the NucliSens Extractor. According to the standard manufacturer's instructions (NucliSens Extractor Operator Manual), 100-μl samples were lysed in 900 μl of a GuSCN buffer (NucliSens Lysis Buffer; Organon Teknika) for 10 min at room temperature (which is the standard lysis procedure for low-volume samples up to 200 μl), and 1-ml samples were lysed in 9 ml of GuSCN buffer for 30 min at 37°C (which is the standard lysis procedure for high-volume samples from >200 μl to 2 ml).

Two additional pretreatment procedures were investigated in combination with the extractor. (i) samples (100 μl) were treated with proteinase K (Boehringer Mannheim, Almere, The Netherlands) in the presence of 0.5% sodium dodecyl sulfate at 60°C for 1 h prior to lysis in 900 μl of GuSCN buffer; (ii) samples (100 μl and 1 ml) were lysed in 900 μl and 9 ml of GuSCN buffer, respectively, and heated to 80°C for 10 min.

NucliSens Extractor. After lysis of the sample, HBV DNA isolation was carried out with the NucliSens Extractor according to the manufacturer's instructions. Briefly, nucleic acids in the lysed pretreated sample were bound to silica

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particles, which were immobilized on a filter in a disposable cartridge and were subsequently washed with several solvents (a GuSCN-containing wash buffer, 70% ethanol, and acetone) in consecutive steps. After being dried, the nucleic acids were released from the silica particles in an elution buffer. Twenty microliters of the eluate was used in the HBV DNA PCR.

QIAamp blood kit. The QIAamp spin column procedure (Qiagen) was performed according to the manufacturer's instructions and was preceded by a 10-min protease (Qiagen) sample pretreatment at 56°C.

HBV DNA PCR. The sense primer V058 (5′ GGG AGG AGA TTA GGT TAA 3′) and the biotinylated antisense primer V059 (5′ bio GGC AAA AAM GAG AGT AAC TC 3′) were used to amplify a sequence of 216 bp in the precore region of the HBV genome (17), and detection of the amplicons was performed by hybridization with a specific digoxigenin-labeled probe, V060 (5′ dig TAG GAG GCT GTA GGC ATA A 3′) in a microwell format assay. To exclude inhibition of the PCR, all samples with a negative result were again analyzed after an HBV spike, was added. The cutoff value of the assay was arbitrarily set at three times the background optical density, which was the mean optical density of four negative controls included in each test run. This PCR method is applied for routine diagnostic procedures and performs very well in proficiency panels of the European Union Quality Control Concerted Action (Second QCCA hepatitis B virus proficiency panel summary of results).

Commercial assays for HIV-1 viral-load quantitation and HCV RNA detection. HIV-1 viral-load quantitation and HCV RNA detection in the multimarker control were performed by means of the NASBA-based NucliSens HIV-1 QT (Organon Teknika) and the RT-PCR-based Cobas HCV Amplicor 2.0 (Roche, Almere, The Netherlands), respectively. The HIV-1 RNA quantitation by the NucliSens HIV-1 QT is based on coextraction and coamplification of known concentrations of three in vitro-generated RNA calibrators (23); the qualitative Cobas HCV Amplicor assay uses an internal control. The NucliSens calibrator mix (20 μl) and the Amplicor internal control (6 μl) were added to each individual sample prior to extraction in the NucliSens Extractor, after lysis and pretreatment.

RESULTS

Preliminary sensitivity study. In a preliminary experiment, 100 μl of 10-fold dilutions of an in-house HBV DNA positive control (human plasma) was extracted in duplicate according to four different protocols: (i) by the extractor after standard sample lysis (10 min in GuSCN buffer at room temperature), (ii) by the extractor after proteinase K treatment of the sample, (iii) by the extractor after the lysed sample was heated to 80°C, and (iv) by the QIAamp extraction method with protease sample treatment. This experiment demonstrated that proteinase K and heat treatment prior to extractor isolation increased the sensitivity of the PCR method compared to the standard sample lysis procedure (data not shown).

Analytical sensitivity using 100-μl samples. As the standard 10-min at room temperature lysis procedure prior to use of the extractor seemed less efficient for HBV DNA extraction, we continued with the proteinase K and 10 min at 80°C extractor protocols and the QIAamp method and compared them in more detail using VQC and EUROHEP HBV DNA standard dilution panels. A sample volume of 100 μl was used as the input in extraction with the three methods. With the 10 min at 80°C extractor protocol, we were able to detect 300 (VQC panel) and 400 (EUROHEP panel) geq/ml; with the proteinase K extractor protocol, we could detect 3,000 and 4,000 geq/ml, respectively; and with the QIAamp extraction, we could detect 91 and 121 geq/ml, respectively (Table 1).

Analytical sensitivity using 1-ml samples. To investigate the influence of the sample volume used in the extraction on the sensitivity of the PCR, we tested the panels in duplicate according to the standard 30 min at 37°C and the 10 min at 80°C extractor protocols using a sample input of 1 ml. As shown in Table 2, we were able to detect 40 geq/ml in the EUROHEP panel and 91 geq/ml in the VQC panel by applying the standard method and 4 geq/ml in the EUROHEP panel and 30 geq/ml in the VQC panel by the latter method.

TABLE 1. Number of HBV DNA PCR-positive samples following extraction by three different methods in EUROHEP and VQC standard dilution panels, using 100-μl sample input

Panel (geq/ml)	No. positive[a]		
	Proteinase K + extractor	10 min 80°C + extractor	Q1Aamp extraction
EUROHEP			
40,000,000	1/1	1/1	1/1
40,000	1/1	1/1	1/1
4,000	2/2	3/3	3/3
1,212	0/2	1/3	3/3
400	0/2	2/3	2/3
121	0/2	0/3	1/3
40	0/2	0/3	0/2
4	0/1	0/1	0/1
0.4	0/1	0/1	0/1
VQC			
30,000,000	1/1	1/1	1/1
300,000	1/1	1/1	1/1
30,000	1/1	1/1	1/1
9,090	2/2	3/3	3/3
3,000	1/2	3/3	3/3
909	0/2	2/3	3/3
300	0/2	2/3	2/3
91	0/2	0/3	1/3
30	0/1	0/1	0/1
9	0/1	0/1	0/1
3	0/1	0/1	0/1
0.3	0/1	0/1	0/1

[a] Number positive/number tested.

In addition, 1-ml samples of 10-fold dilutions in negative citrate plasma of three patient samples with high HBV viral loads (329, 380, and 391 pg/ml, as previously measured by the Digene hybrid capture assay) were tested following both pretreatment protocols. For two patient samples, the highest dilutions tested ($1/10^8$ and $1/10^9$) were still PCR positive with both procedures. One patient sample, on the contrary, showed a discrepancy between the methods: using the 30 min at 37°C protocol, the last positive dilution was $1/10^7$, whereas with the 10 min at 80°C protocol, the limiting dilution was $1/10^5$. Since it was based on a single observation, the latter result should be considered with caution, as such a large sensitivity difference in favor of the 30 min at 37°C procedure is not in line with the results of our other experiments.

Clinical sensitivity. Twenty samples from patients with diagnosed HBV infections were tested by PCR after extraction from 1 ml of serum in the extractor according to the 10 min at 80°C protocol and after QIAamp extraction from 200 μl of serum.

Consecutive samples ($n = 10$) were available from two patients: patient A, with known HBV viral loads (previously quantitated by the Digene hybrid capture assay) and HBsAg serology (Axsym; Abbott), and patient B, with known HBsAg and a-HBs (the titer of antibodies to hepatitis B surface antigen) serology (Axsym). All samples with a detectable HBV viral load and/or positive for HBsAg were also positive in the PCR after extractor isolation; two HBsAg-positive samples

TABLE 2. Sensitivity of PCR in EUROHEP and VQC standard dilution panels with the 30 min at 37°C and 10 min at 80°C extractor protocols using 1-ml sample input

Panel(geq/ml)	No. positive[a]	
	10 min 80°C + extractor	30 min 37°C + extractor
EUROHEP		
40,000,000	2/2	NT
40,000	2/2	NT
4,000	2/2	2/2
1,212	2/2	2/2
400	2/2	1/2
121	2/2	2/2
40	1/2	1/2
4	1/2	0/2
0.4	0/2	0/2
VQC		
30,000,000	2/2	NT
300,000	2/2	NT
30,000	2/2	NT
9,090	2/2	NT
3,000	2/2	2/2
909	2/2	2/2
300	2/2	2/2
91	2/2	1/2
30	1/2	0/2
9	0/2	0/2
3	0/2	0/2
0.3	0/2	0/2

[a] Number positive/number tested. NT, not tested.

were PCR negative after QIAamp extraction (Table 3). For patient B, the PCR after extractor isolation became negative simultaneously with HBsAg, at the moment that the patient displayed full-blown immunity (>1,000 IU of a-HBs antibodies/ml).

Individual samples from six patients (sample numbers 7, 9, 10, 15, 17, and 18) that were previously positive in the PCR after QIAamp extraction from 200 μl of serum were retested by PCR after 1-ml extraction in the extractor according to the 10 min at 80°C protocol. Four other individual samples (numbers 19, 20, 21, and 22) that were positive for HBsAg (Axsym)

TABLE 3. HBV DNA PCR reactivity in consecutive patient serum samples compared to serology

Sample no.	Result[a]				
	HBV viral load (pg/ml)	HBsAg	a-HBs (IU/ml)	QIAamp + PCR[b]	Extractor + PCR[c]
Patient A					
1	1,427	NT	NT	Pos	Pos
2	42	Pos	NT	Pos	Pos
3	3.5	NT	NT	Pos	Pos
4	0.9	Pos	NT	Pos	Pos
5	Neg	Pos	NT	Pos	Pos
6	Neg	Pos	NT	Neg	Pos
Patient B					
11	NT	Pos	NT	Pos	Pos
12	NT	Pos	402	Neg	Pos
13	NT	Neg	>1,000	Neg	Neg
14	NT	Neg	>1,000	Neg	Neg

[a] Pos; positive; Neg, negative; NT, not tested.
[b] Qiagen extraction using 200-μl sample input.
[c] 10 min at 80°C extractor protocol using 1-ml sample input.

TABLE 4. HBV DNA PCR results for 10 individual HBV-positive serum samples

Sample no.	Result[a]		
	HBsAg	QIAamp + PCR[b]	Extractor + PCR[c]
7	Pos	Pos	Pos
9	Pos	Pos	Pos
10	NT	Pos	Pos
15	NT	Pos	Pos
17	NT	Pos	Pos
18	Pos	Pos	Pos
19	Pos	Pos	Pos
20	Pos	Neg	Pos
21	Pos	Neg	Pos
22	Pos	Neg	Pos

[a] Pos, positive; Neg, negative; NT, not tested.
[b] Qiagen extraction using 200-μl sample input.
[c] 10 min at 80°C extractor protocol using 1-ml sample input.

were tested by PCR after QIAamp extraction (200 μl of sample) and after extractor isolation (according to the 10 min at 80°C protocol with 1 ml of sample input). All 10 samples were positive in the PCR after extractor isolation, whereas the PCR method did not detect HBV DNA in patient samples 20, 21, and 22 after QIAamp extraction (Table 4).

HBV DNA, HIV-1 RNA, and HCV RNA coextraction by means of the NucliSens Extractor. To investigate the influence of the heat pretreatment on the quantification and detection efficiencies of assays for HIV-1 viral load quantitation and HCV RNA detection after coextraction of HBV DNA, HIV-1 RNA, and HCV RNA, we compared the standard GuSCN-mediated sample lysis procedure for large sample volumes (30 min at 37°C) prior to extractor isolation (which is currently used with NucliSens HIV-1 QT and the qualitative Cobas HCV Amplicor assay) with extraction according to the 10 min at 80°C protocol. One milliliter of a multimarker control containing 375 geq of HBV DNA, 375 geq of HIV-1 RNA, and 380 geq of HCV RNA was tested nine times according to both methods. Of each extractor eluate, 20 μl was used in the HBV DNA PCR, 25 μl was used in the Cobas HCV Amplicor assay, and 5 μl was used in the NucliSens HIV-1 QT assay. Irrespective of the extraction procedure used, all the multimarker replicates tested positive for HBV DNA, HCV RNA, and HIV-1 RNA. The mean NucliSens HIV-1 QT RNA copy numbers were comparable (2.81 ± 0.37 and 2.84 ± 0.37 log copies/ml with the 30 min at 37°C and the 10 min at 80°C protocols respectively). The HCV Amplicor internal control (about 84 copies in the extraction) always tested positive no matter which method was used.

DISCUSSION

The NucliSens Extractor is an automated GuSCN-silica-based nucleic acid extraction method which was able to efficiently isolate HBV DNA from pretreated plasma and serum samples, allowing sensitive detection of HBV by a noncommercial PCR method. In particular, heating of the lysed sample prior to nucleic acid extraction by the extractor and the use of a large sample volume in the extraction resulted in high sensitivity: incubation of a 1-ml sample in a GuSCN buffer at 80°C (for 10 min) and at 37°C (for 30 min) allowed detection of 4

and 40 HBV geq/ml in the Pelicheck panels. On the other hand, sample lysis in GuSCN at room temperature and proteinase K treatment seemed to be less efficient procedures in combination with the extractor.

Boom et al. (5) hypothesized that the protein covalently linked to the 5′ end of the long strand of the HBV genome is not removed during standard GuSCN-mediated lysis (4) and probably interferes with the elution of the HBV DNA. As proteinase K or protease treatment can be used to separate this protein from the genome (13, 14), they treated serum samples with proteinase K prior to the manual GuSCN-silica extraction method or during the elution phase of the method. They showed by means of a Southern blot technique that this treatment increased the HBV DNA yield dramatically compared to the standard extraction method with a 10-min sample lysis in GuSCN buffer at room temperature. The experiments of Boom et al. (5) demonstrated that by applying the standard lysis-extraction method HBV DNA remains partly bound to the silica particles, resulting in inefficient elution of HBV DNA. Their data thus suggested a beneficial effect of the proteinase K treatment on the sensitivities of HBV DNA PCR methods. Using the extractor, we observed in our preliminary experiments (data not shown) a better sensitivity of the PCR method when 100-μl samples were extracted after proteinase K treatment compared to the standard lysis procedure (10 min at room temperature). However, heating of the lysed specimen to 80°C prior to extraction by the extractor resulted in superior PCR sensitivity compared to the proteinase K method, as is shown in Table 1. The high efficiency of the 10 min at 80°C protocol was confirmed by our experiments using a 1-ml sample input (Tables 2, 3, and 4). Incubation of 1-ml samples in GuSCN buffer at 37°C for 30 min prior to extractor isolation (which is the standard lysis procedure for high-volume samples) also allowed sensitive detection of HBV DNA. Using the 30 min at 37°C protocol, the sensitivity of the PCR method seemed slightly lower in the Pelicheck panels than after application of the 10 min at 80°C protocol (Table 2). However, in the specimen dilution series of two patients, the sensitivities of the PCRs were comparable for both pretreatment methods, whereas for one patient, the 30 min at 37°C protocol seemed even more sensitive (although the latter result should be considered with caution). To what extent the protein covalently linked to the 5′ end of the long DNA strand hampers the GuSCN-silica extraction and by which mechanisms are not clear. Gerlich and Robinson (9) demonstrated that heating to 80°C alone does not separate this protein from the DNA but does convert the circular HBV DNA to a linear form. We incubated HBV-positive samples at 80°C in the presence of GuSCN, a chaotropic agent with protein-denaturing properties. Although hypothetical, a synergistic effect is probably obtained by combining two denaturing activities which change the structures of both the protein and the DNA simultaneously, thus resulting in unfavorable energy conditions for a covalent bond and, finally, in the separation of both molecules. The reason proteinase K sample treatment (applied according to the manufacturer's instructions) in combination with the extractor was less effective than heat treatment is not clear. Although Drosten et al. (8) reported an increase in HBV DNA PCR sensitivity after proteinase K sample digestion prior to QIAamp extraction, they observed at the same time a decrease in RT-PCR sensitivity for HIV and HCV detection. This makes the proteinase K treatment even more questionable if the same extraction eluate is used for detection of multiple viral genomes.

Other authors (3, 7, 12, 15, 22) underlined the benefit of the ability to use large sample volumes in the extractor in order to increase the sensitivity of NASBA- and RT-PCR-basedamplification methods for HIV-1 and HCV detection and quantification. Our study confirmed this benefit for the detection of HBV DNA, as the sensitivity of the PCR method was increased at least 10-fold by using 1 ml of a sample in the extraction compared to the use of 100 μl. As the extractor is able to process samples up to 2 ml, it should be possible to increase the sensitivity of our method even more. Although the Qiagen extraction procedure seemed slightly more sensitive in our comparison with equal sample inputs (Table 1), the sensitivity of the method is limited by the sample volume that can be used in the extraction (up to 200 μl). Previous studies (3, 6, 7, 12) demonstrated the superior sensitivity of the NucliSens Extractor-Cobas Amplicor HCV 2.0 combination for detection of HCV RNA, making this system suitable for testing for HCV RNA in plasma pools of up to 96 blood donations, in conformance with the sensitivity requirements of European regulatory authorities. Our data suggest that the extractor can also optimize the sensitivity of PCR-baseddetection of HBV DNA in such a way that HBV DNA screening of pooled plasma can possibly be considered in order to increase the safety of the blood supply by narrowing the preseroconversion HBV window.

The high analytical sensitivity of the PCR method after extractor isolation from 1-ml samples according to the 10 min at 80°C protocol was confirmed by a very good clinical sensitivity: this PCR method detected HBV DNA in all the patient samples which were PCR positive after Qiagen extraction and/or were HBsAg positive. Five HBsAg-positive samples were missed by the PCR method after Qiagen extraction but could be detected after extraction by the extractor. These results also suggest that the extractor could be used to increase the sensitivity of quantitative HBV nucleic acid amplification methods in order to allow more-efficient monitoring of patients under treatment. Additionally, the extractor allows combined clinical testing of the same target. As was already demonstrated for HIV-1 (D. R. McClernon, M. Stocum, and M. St. Clair, Abstr. 6th Conf. Retrovir. Opportunistic Infect., abstr., 1999), it should also be possible to use the extractor eluate to simultaneously perform an HBV viral load and an HBV genotyping assay in order to discover therapy-resistant viral strains and to be able to adequately adapt antiviral treatment if necessary (20).

Finally, the extractor efficiently coextracted HBV DNA, HCV RNA, and HIV-1 RNA from a single plasma sample, allowing reliable detection of the viral genomes in the eluate by means of our HBV DNA PCR, Cobas HCV Amplicor 2.0, and NucliSens HIV-1 QT, respectively. Heating of the sample to 80°C as a special pretreatment for HBV DNA amplification and detection did not influence the detectability of HCV and HIV-1 RNAs. These observations open perspectives with regard to screening for several viral nucleic acids in pooled blood donations and combined monitoring of patients with multiple

viral infections by using the NucliSens Extractor as a front end for nucleic acid amplification tests.

The reproducibility of the system with regard to HBV DNA detection should be further substantiated in large-scale multicenter trials, analogous to the validation studies previously performed for HCV and HIV RNA detection (6, 7, 22).

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