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A novel quantitative microarray antibody capture (Q-MAC) assay identifies an extremely high HDV prevalence amongst HBV infected Mongolians

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Abstract

Hepatitis delta virus (HDV) causes the most severe form of human viral hepatitis. HDV requires a hepatitis B virus (HBV) co-infection to provide HDV with HBV surface antigen envelope proteins. The net effect of HDV is to make the underlying HBV disease worse, including higher rates of hepatocellular carcinoma (HCC). Accurate assessments of current HDV prevalence have been hampered by the lack of readily available and reliable quantitative assays, combined with the absence of an FDA-approved therapy. We sought to develop a convenient assay for accurately screening populations and to use this assay to determine HDV prevalence in a population with abnormally high rates of HCC. We developed a high throughput quantitative microarray antibody capture (Q-MAC) assay for anti-HDV IgG wherein recombinant HDV delta antigen is printed by microarray on slides coated with a noncontinuous, nanostructured plasmonic gold film, enabling

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quantitative fluorescent detection of anti-HDV antibody in small aliquots of patient serum. This assay was then used to screen all HBV-infected patients identified in a large randomly selected cohort designed to represent the Mongolian population. We identified two quantitative thresholds of captured antibody that were 100% predictive of the sample either being positive on standard western blot, or harboring HDV RNA detectable by qPCR, respectively. Subsequent screening of the HBV-positive cohort revealed that a remarkable 57% were RNA positive and an additional 4% were positive on western blot alone.

Conclusions—The Q-MAC assay's unique performance characteristics make it ideal for population screening. Its application to the Mongolian HBsAg+ population reveals an apparent ~60% prevalence of HDV co-infection amongst these HBV-infected Mongolian subjects, which may help explain the extraordinarily high rate of HCC in Mongolia.

Keywords

HDV; protein microarray; plasmonic gold; anti-HDV

Hepatitis delta virus (HDV) causes the most rapidly progressive human viral hepatitis leading to accelerated rates of cirrhosis and hepatocellular carcinoma (1, 2). HDV has a unique 1.7 kB single-stranded circular RNA genome that encodes for one protein, the viral coat like protein, hepatitis delta antigen (HDAg). Together, these are encapsidated by a lipid envelope containing hepatitis B virus (HBV) surface antigen (HBsAg) envelope proteins that HDV acquires from HBV that replicates in the hepatocytes simultaneously with HDV (3). This requirement for HBV envelope proteins is the only helper function provided by HBV, but explains why HDV can only infect subjects with a co-existing HBV infection due either to the simultaneous transmission of the two viruses or superinfection in an established HBV carrier (4). Approximately 5% of the global HBV infected population, or 15–20 million people worldwide, are infected with HDV, although HDV prevalence rates are not uniform, with higher rates of HDV co-infection reported in the Mediterranean basin, parts of Africa, Middle East and South America (5). In a study of 249 apparently healthy individuals living in and around the capital city of Mongolia, 10% were HBsAg+, with 83% of those having detectable HDV RNA (6), prompting calls for a larger nationwide survey.

The usual first step in the diagnosis of HDV infection is testing HBsAg-positive individuals for the antibody to the HDAg (anti-HDV). The anti-HDV antibody is not protective; it is present in all immunocompetent patients with HDV infection (7). Total antibodies to HDV can be detected with an enzyme-linked immunosorbent assay (ELISA). In anti-HDV-positive patients, the ideal next step is testing for HDV RNA in serum to confirm the presence of active HDV infection. With the advent of reverse transcription polymerase chain reaction (RT-PCR) techniques, HDV RNA has been measured with qualitative or semiquantitative RT-PCR assays (8). Sensitivity has markedly improved, with current detection limits of 1000 genome/mL for simple PCR and 10 genome/mL for nested PCR (8). Unfortunately, the results from different laboratories are often not comparable due to the diverse sensitivity of the assays; variance is caused by the use of different primers sets and by the variability of the RNA region amplified (9, 10). A WHO international RNA standard is now available, enabling reporting results in International Units (IU), although no quantitative HDV RNA assay is commercially available currently in the US.

Here, we present a new quantitative microarray antibody capture (Q-MAC) assay for detecting anti-HDAg antibodies in human sera. This platform is constructed on noncontinuous, nanostructured plasmonic gold films with enhanced near-infrared (NIR) fluorescence detection that we hypothesized would have high sensitivity ideal for high-throughput antibody capture screening. Indeed, similar technology was previously demonstrated to have vastly improved sensitivity over peptide arrays on glass, with the limit of detection (LOD) down to the 10 femtomolar (pg/mL) range (11, 12). For the anti-HDV Q-MAC assay, recombinant full length HDV small delta antigen was arrayed on a plasmonic gold substrate for sensitive profiling of antibodies in the sera of HDV patients. We first determined the performance characteristics of this new assay format using reference HDV RNA positive and negative control sera. We then used the assay to determine the prevalence of HDV co-infection amongst HBV infected individuals identified in a national survey sampling the population of Mongolia.

Materials and Methods

Patient serum samples

De-identified unique serum samples from the following collections were used for this study: 82 historical HDV RNA positive patient sera; 30 samples from HBV monoinfected patients; 30 samples from HCV monoinfected patients; 10 samples from pregnant women and 10 samples from healthy control patients; 123 HBsAg-positive samples from a national survey study recently conducted in Mongolia. Briefly, for the latter, study subjects were chosen based on a three-stage cluster sampling method to reflect the gender, age and geographical origin of the Mongolian population. Participants were randomly selected from the adult general population in 16 different locations, representative of the country (13). A total of 1,158 subjects were enrolled in the study. 123 of them tested positive for HBsAg, using a commercial ELISA kit (DiaSorin, srl., Saluggia, Italy).

Preparation of internal standard anti-HDV IgG reference antibody

An internal standard of purified anti-HDV IgG was prepared from a HDV positive sample with high titer anti-HDV antibody using a protein G column. The concentration of purified IgG was determined using an Easy-Titer IgG Assay kit according to the manufacturer's instructions.

Expression and purification of recombinant full-length small hepatitis delta antigen (S-HDAg)

The HDV small delta antigen was expressed in and purified from *E. Coli*, and stored in single-use aliquots of >90% purity until use, as described in the Supplementary Materials and Methods.

Antigen microarray printing

Plasmonic gold (pGOLD) slides were purchased from Nirmidas Biotech, Inc (Palo Alto, CA) containing a functionalized coating of polyethylene glycol and terminal activated carboxylic acid groups for amine coupling of proteins. The pGOLD slides were loaded into a microarray printing robot (Bio-Rad) where S-HDAg (100 μ M) was printed using solid pins

(Arrayit) at 25°C and 60% humidity, resulting in microarray feature diameters of ~2mm. The microarray layout was designed using the microarray printer software. The antigen was printed into 16 areas with 6 replicate spots each (Figure 1A). The slides were dried in a desiccator, vacuum-sealed in a bag and stored at 4°C.

Quantitative microarray antibody capture (Q-MAC) assay

The microarray printed slides were blocked with fetal bovine serum (FBS) for 1 hour, followed by washing three times with PBST (Phosphate buffered saline containing 0.5% tween-20). Up to 13 individual serum samples were analyzed per slide. 1.0 µl of each human serum sample was diluted to a total of 50µl with FBS and applied to one well of the array for 1 hour. On each slide, the following controls were each applied to separate wells: blank control (FBS), negative control (HCV patient sera), and internal standard positive control (purified IgG antibody from HDV patient sera). The slides were washed three times with PBST and IRDye800 labeled donkey anti human IgG (Rockland Immunochemicals Inc. PA, USA) diluted 1:1000 in FBS solution was applied to each array set for 1hour in the dark. Slides were then washed three times with PBST, once with deionized water and dried in the dark.

Slides were scanned using a Licor Odyssey scanner with the 800nm channel. Image Studio Lite Ver 4.0 was used to automatically identify features above a composite pixel intensity of 5. The fluorescence intensity of 100ng/ml internal standard purified anti-HDV IgG was defined as one Unit, and used to normalize the intensity of tested samples using the following formula: Value of fluorescence intensity (Unit) = (mean sample's exact intensity value – mean blank intensity value)/internal standard's intensity value – mean blank intensity value).

Anti-HDV IgG ELISA detection

Anti-HDV IgG testing was performed using a commercial HDV IgG ELISA Kit (GenWay Biotech, Inc, CA) according to the manufacturer's instructions. 50 microliters of patient serum was diluted 1:1 for the ELISA assays. The optical density (OD) 450 values were ascertained using photometry (Tecan Group Ltd, Switzerland).

Western blot detection

Purified recombinant protein (S-HDAg) was subjected to 12% SDS-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), probed with 10 microliters of patient serum diluted 1:100 diluted patient sera followed by detection with IRDye800 labeled goat anti-human IgG (diluted 1:20, 000) and visualization using a Licor Odyssey scanner, as described in the Supplementary Materials and Methods.

HDV RNA Extraction and full-length genomic HDV RNA calibration standard preparation

HDV RNA was extracted from 140 µL of plasma using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). In vitro transcription of full-length HDV RNA from plasmid pT7GM that contains 1679 bp of the HDV genome was used as HDV RNA reference standard for the quantification of HDV RNA by real-time PCR (14), as described in the Supplementary Materials and Methods.

Quantitation of HDV RNA by Taqman-based one step real-time PCR

In order to enable detection of all known eight genotypes, the highly conserved ribozyme region of the HDV genome was selected for design of the primers and probes (15) and subsequent PCR assays, as described in the Supplementary Materials and Methods. Standards for the calibration curve were prepared using a tenfold dilution series of full-length HDV RNA to cover the range 1.6×10^7 – 1.6 IU/ml. A normal human serum negative control and a HDV-positive serum positive control (1×10^4 RNA IU/ml) were included in each assay. The WHO HDV RNA international standard was used to normalize the results to international unit (IU) of HDV RNA.

The linearity of the PCR ranged from 1.6×10^1 to 1.6×10^7 IU/ml.

Statistical analyses

Statistical analyses were performed using the Student t-test. Receiver operating characteristic (ROC) curve analysis was used to assess assay sensitivity/ specificity. Linear regression analysis was used to evaluate the correlation between fluorescence intensity and HDV RNA level. All data are reported as means $\pm$ standard deviation. A p-value <0.05 was considered as significant.

Results

Detection of anti-HDV reference serum by standard western blot

Full-length S-HDAg was expressed in BL 21(DE3) bacteria cells and purified to yield a source of recombinant delta antigen for use in anti-HDV antibody detection (Supplementary Figure. S1A). The recombinant antigen was subjected to western blot analysis and probed with serial dilutions of an anti-HDV purified IgG internal reference standard in order to determine the limit of detection (Supplementary Figure. S1B). The lowest detected concentration was 10ng/ml purified anti-HDV IgG.

Dynamic range, reproducibility, sensitivity and specificity of the anti-HDV quantitative microarray antibody capture (Q-MAC) assay

Recombinant S-HDAg protein was printed on pGOLD microarray slides by a microarray printing robot such that six replicate spots were printed per future assay area (see Figure 1A). Following placement of the slide's partitions so as to create individual assay wells, serial dilutions of anti-HDV reference IgG were added to separate wells for one hour. Wells were then washed and bound anti-HDV antibody was detected with IRDye800 labeled anti-human IgG and measurement of the fluorescence intensity associated with each spot of printed S-HDAg (Figure 1B). The linear range of fluorescence intensity detection extended down to 1 ng/ml anti-HDV IgG, and the lower limit of detection was 10 pg/ml for this quantitative microarray antibody capture assay termed Q-MAC (Figure 1C). The fluorescence intensity of 100ng/ml internal standard purified anti-HDV IgG was defined as one Unit, and used to normalize fluorescence intensity determinations as detailed in Materials and Methods.

To determine the reproducibility of the new assay, the normalized fluorescence intensities of sera from 5 negative controls (healthy humans) and 5 HDV RNA+ patients were screened on microarray slides in 6 replicate spots in 3 independent experiments (Table 1). The mean intensity of each HDV RNA+ sample ranged from 1.742 to 8.135 Units and the coefficient of variation (CV) ranged from 6.7% to 11.1%. The mean intensity of each negative control ranged from 0.011 to 0.029 Units and the CV ranged from 9.1% to 28.6%.

The performance characteristics of the microarray assay were determined using sera from 80 negative controls (30 HBV monoinfected, 30 HCV monoinfected, and 10 each from pregnant women and healthy human subjects) and 82 historical HDV RNA+ patients on pGOLD microarray slides printed with S-HDAg. All negative controls' intensities were below 0.090 Units and this value was set as the microarray cutoff value (Figure 2A). The intensities of HDV RNA+ samples were all above this cutoff value (Figure 2A). The mean intensity was 6.221 Units. The median intensity was 5.683 Units, with a range from 0.166 to 16.91 Units.

To correlate the performance of Q-MAC at detecting anti-HDV antibodies with western blot assay results, all 162 samples were subjected to standard western blot analysis. The delta antigen-specific antibody in the sera of the 82 HDV RNA + patients was detectable on westerns, while no such signal appeared for the 80 negative controls. As shown in Figure 2B, for this sample set, a fluorescence intensity above 0.164 Units correlated with a positive western blot assay. This intensity value could be set as the western blot cutoff value.

Comparison of Q-MAC sensitivity to standard ELISA and western blot assays

The high sensitivity, broad dynamic range and easy adaptability of pGOLD microarray slides provides a new assay format with which to screen anti-HDV IgG accurately. To compare Q-MAC's sensitivity to other assays for detecting anti-HDV, we tested aliquots of serially diluted anti-HDV reference IgG in Q-MAC, a commercial ELISA, and western blot assays. As can be seen from their respective limits of detection presented in Table 2, the Q-MAC assay's sensitivity was 10^6 and 10^3 -fold higher than commercial anti-HDV IgG ELISA kit and western blot analysis, respectively (Table 2).

Analysis of HDV prevalence in Mongolia using the Q-MAC assay

We next used the HDV antibody capture assay to determine the prevalence of HDV infection among the 123 samples identified to be HBsAg-positive in a recently collected cohort of 1158 samples from a national surveillance study recently conducted in Mongolia to determine prevalence rates of hepatitis virus infections (13).

The HBsAg-positive samples were also analyzed independently by anti-HDV western blot, commercial anti-HDV ELISA kit (DiaSorin), and quantitative RT-PCR HDV RNA assays (see Table 3 and Supplementary Table 1). 39 samples were below the 0.09 U Q-MAC cutoff. All of these were negative by both western blot and HDV RNA assays. 21% (8/39) of these samples tested by ELISA were positive, which were interpreted as false positive. 61% (75/123) of the samples were above the previously defined threshold in the Q-MAC assay for predicting western blot positivity (0.164 U) (Figure 3A). All of these samples were confirmed to be positive on western blot. Thus, using the Q-MAC threshold of 0.164 U had

a 100% sensitivity, and 100% specificity, for predicting positivity on anti-HDV western blot. Although originally designed to accurately substitute for the more laborious western blot assay, the Q-MAC assay also performed quite well for predicting HDV RNA positivity as well.

Indeed, most of the samples with Q-MAC values of 0.164 U and above--93% (70/75)--were also found to be HDV RNA+, representing 57% (70/123) of the HBsAg-positive subjects. While all of the RNA+ samples in this cohort were scored as HDV+ by Q-MAC assay, 7.1% (5/70) of the RNA+ subjects had a false negative result by ELISA kit (Supplementary Table 1).

ROC analysis identified a Q-MAC threshold of 1.659 U as having 100% sensitivity for predicting HDV RNA positivity, with a specificity of 94.3% (AUC = 0.9978; $p < 0.0001$) (Figure 3B). 66 subjects had Q-MAC values above 1.659, and all were confirmed to be HDV RNA+. 4 subjects with fluorescence intensities above 0.164, but slightly below 1.659, were also found to be HDV RNA+. The overall distribution of Q-MAC values is graphically presented in Figure 3C, and tabulated in Supplementary Table 1.

Of note, 5 of the HDV RNA negative patients met the Q-MAC threshold for western blot positivity (fluorescence intensity ≥ 0.164 U), and all were confirmed to be positive by western blot. Finally, 9 samples were above the 0.09 U Q-MAC cutoff value, but below the 0.164 U threshold for western blot positivity.

Discussion

We describe here a new methodology, Q-MAC, for detecting infection with hepatitis delta virus that is sensitive, rapid, requires very small volumes of serum, and is high throughput in nature. Its quantitative nature and empiric relationship to the results of standard western blot and HDV RNA analyses enables prediction of clinically meaningful virologic status. The results allowed us to define a quantitative threshold of captured anti-HDV antibody above which 100% of the samples are positive for HDV RNA. Together, these attributes make it ideal for analyzing patient cohorts. Indeed, we have used this assay to determine the prevalence of HDV amongst HBV-infected subjects in the largest and most comprehensive sampling to date of the Mongolian population. Most striking was the finding that ~60% of Mongolian patients with chronic hepatitis B have evidence of HDV co-infection, that is provocatively related to the extremely high rate of HCC in Mongolia (16).

Peptide antigens have been recently described for detection of anti-HDV antibodies (17). These were limited, however, in their ability to detect all of the various genotypes that have been described for HDV (18). Use of full length recombinant HDV delta antigen, such as described here, appears to not suffer from this limitation. Instead, using full length recombinant HDV delta antigen provides a genotype-independent assay. Indeed, we have successfully used this assay in populations with diverse HDV genotypes. Moreover, this assay has allowed the detection of samples that had falsely been deemed negative by a genotype-1 specific HDV RNA assay (manuscript in preparation).

Peptide microarrays on plasmonic gold substrate coating on glass affords hundreds of fold NIR fluorescence enhancement when compared with commercial streptavidin coated glass (12). Our Q-MAC assay is based on the above plasmonic gold platform and exhibits detection capability down to 10pg/ml anti-HDV IgG concentrations. Moreover, the Q-MAC assay's sensitivity was 10^6 and 10^3 -fold higher than a commercial anti-HDV IgG ELISA kit, and western blot analysis, respectively.

Comparing the results of the Q-MAC assay to standard HDV western blot and quantitative RNA assays enabled the assignment of very practical empiric quantitative thresholds when using the Q-MAC assay that can be quite useful when analyzing new patient cohorts, such as the one described here from Mongolia. For example, the 0.164 Unit threshold for predicting positivity on western blot was confirmed to be accurate in this cohort. Indeed, 75/75 (100%) patients with fluorescence intensity determinations at or above this value were positive on western blot, and 48/48 (100%) patients with fluorescence intensity determinations below this value were negative on western blot. Moreover, most, but not all, of the patients with fluorescence intensity above 0.164 Unit were HDV RNA+.

One patient in the Mongolian cohort had an intensity of 0.163. This sample was negative by both western blot (and HDV RNA) assays, suggesting that indeed the 0.164 intensity is empirically close to a cutoff value for predicting positivity on standard western blots. Of note, we have recently screened two large cohorts from the United States and Africa containing a combined over 500 samples—all also analyzed by standard western blot--and this cutoff continues to indicate the threshold for predicting positivity on western blot (manuscripts in preparation).

This Mongolian cohort of HBsAg-positive patients was also screened by a commercial anti-HDV ELISA kit. Somewhat alarmingly, 7.1% of patients screened by ELISA were false negative using the HDV RNA assay as the gold standard. All of these patients were predicted to be RNA positive based on the results of the Q-MAC assay.

Interestingly, one can define a fluorescence intensity cutoff—1.659 Unit—that is predictive for 100% of patients being HDV RNA positive. Thus, the simple, relatively high throughput Q-MAC assay could prove useful for prospectively identifying patients who have active HDV replication, or whom could benefit from subsequent reflex testing for HDV RNA.

Although there is a correlation between fluorescence intensity of the Q-MAC assay and HDV RNA level (Supplementary Fig. 2), determination of HDV RNA remains the assay of choice for monitoring response to therapy.

5 patients in the Mongolian cohort were HDV RNA negative, but clearly antibody positive, as indicated by both Q-MAC and standard western blot assays. Such RNA negative/antibody positive patients have been described before and may represent false-negative RNA determination (due to RNA degradation during storage, assay inaccuracy) or patients who have lost active RNA replication, but have residual antibody levels.

Finally, due to the increased sensitivity of the Q-MAC, there is another category of patients whose fluorescence intensity unit values are above the negative control threshold (0.090

Unit) but below the cut off associated with western blot positivity. The clinical significance of these low but detectable levels of anti-HDV antibodies, which might reflect distant infection, is at present uncertain.

In addition to its small sample volume requirement, ease of use and relatively high throughput nature, the Q-MAC assay offers several convenient and practical quantitative readouts. If a patient is below the Q-MAC assay 0.09 U cutoff, one can say with certainty that the patient has no evidence of [current or past] HDV infection. If the patient is at or above the 0.164 U western cutoff, the patient definitely has been infected with HDV. If above the RNA cutoff of 1.659 U, there is a 100% chance of being RNA positive.

Various prior studies in selected Mongolian populations have indicated a range of HDV prevalences, with the latter being consistently higher than typical of Western populations, although this may be limited by suboptimal assays or sampling bias. For example, a study on 249 apparently healthy individuals in and around Ulaanbaatar (age 48.4+/- 13.9 years) detected HDV RNA in 8.0% (20/249) of the participants and in 83% (20/24) of the HBsAg positive subjects (6), although the details of how these subjects were chosen are not clear. Among 289 first-time blood donors at a single center in Ulaanbaatar (age 28.9 ± 9.6 years), HDV-RNA was detected in 26 (9%) of the total donors, and in 87% of the HBsAg positive subjects (19). In a study of 207 patients with known liver disease, 144 were HBsAg positive, including 117 (81% of HBsAg positives) with detectable HDV RNA (20). Among 655 apparently healthy children (0.3–15 years), 64 (9.8%) were infected with HBV and of these 13 (20.3%) were HDV RNA positive (21). Focusing on apparently healthy 7–12 year old children born after 1991 (when universal vaccination against HBV was introduced into the country), 59 of 1182 (5%) children were found to be HBsAg positive, and 8 (13.6%) of these were HDV-RNA positive (22). An apparent beneficial effect of vaccination is encouraging, yet its successful implementation appears to be incomplete. Equally concerning are the above studies pointing to a very high prevalence of chronic HDV infection amongst the HBsAg positive adult population, and the question of whether it extends beyond the capital city's environs.

The present study sought to address this by determining the prevalence of HDV infection within the largest cohort to date of a prospectively randomly sampled population throughout Mongolia. As such, the results likely represent the most accurate representation of the true prevalence rates for the assessed important human viral pathogens. While the full description of the HCV and HBV prevalence rates in this cohort is described elsewhere (13), we report here on the prevalence of HDV in Mongolia, which is astonishing. Indeed, while the average global HDV co-infection rate among HBV infected subjects is estimated at 5% (23), an approximately 60% of HBV-infected patients are co-infected with HDV. Extrapolation to the general population results in an estimated prevalence among all Mongolian adults of 6.4 ± 0.7% anti-HDV positivity and 6.1 ± 0.7% with detectable HDV-RNA (Table S2). Reasons for this much higher prevalence may include a relative isolation of this population combined with inadequate control of horizontal transmission associated with dental/medical procedures and sexual activity (13, 22). In any case, these results have important implications for both public health and the by agencies and institutions concerned with crafting and implementing appropriate responses.

In summary, we developed a new quantitative microarray anti-HDV capture (Q-MAC) assay. Defining in the new assay a quantitative threshold of captured anti-HDV above which 100% of the samples are positive for anti-HDV, or HDV RNA, allowed for prospective prediction of both western blot positivity and HDV RNA positivity, respectively. This assay allowed confirmation of a strikingly high ~60% prevalence of HDV co-infection among HBsAg-positive subjects, and a general HDV prevalence of 6.4% among all adults in Mongolia. The extremely high prevalence rate for this hepatitis virus associated with increased cancer risk (24) may help contribute to the alarming incidence of HCC in Mongolia, which ranks among the highest in the world (25). This serves to only underscore the urgent need for improved therapies for HDV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CV	coefficient of variation
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HDV, HDV	hepatitis D virus
IgG	Immunoglobulin G
S-HDAg	small hepatitis delta antigen
Q-MAC	quantitative microarray antibody capture

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Figure 1. Schematic of anti-HDV quantitative microarray antibody capture assay (Q-MAC) and lower limit of detection using delta antigen printed on plasmonic gold (pGOLD) microarray slide (A) S-HDAg was printed on a pGOLD microarray slide by a microarray printing robot such that six identical spots of ~2 mm diameter are contained within each partitioned area of the slide. (B) Microarray imaging results of serial dilutions of purified anti-HDV IgG incubated on a pGOLD microarray slide, as detected by fluorescence intensity. Blank = fetal bovine serum. (C) Calibration curve for captured anti-HDV antibody quantification. Mean fluorescence intensity of IRDye800-labelled anti-human secondary antibody emission from the 6 replicate microarray spots at each antibody concentration on the pGOLD microarray slides. Error bars represent the standard deviation of the mean over the 6 replicate assay features within each partition of the slide.

Figure 2. Anti-HDV IgG detection in the sera of 162 HDV RNA+ and negative control patients using Q-MAC and western blot assays

(A) The normalized fluorescence intensities of HDV negative samples (normal, HCV, and HBV controls) are below the Q-MAC cutoff value (0.090 Units). All of HDV RNA+ samples are above this cutoff value. (B) The sensitivity and specificity of antibody detection by Q-MAC were confirmed by western blot. The fluorescence intensity of 0.164 corresponds to a cutoff at and above which all samples are positive by western blot.

Figure 3. Analysis of anti-HDV IgG and HDV RNA in the sera of 123 HBsAg+ samples from Mongolia by Q-MAC, western blot and qRT-PCR assays
 (A) Q-MAC assay of samples categorized by western blot status. Note all samples below the Q-MAC 0.164 U predictive cutoff value for western blot positivity were negative on western blot, and all samples above this cutoff were positive on western blot. (B) ROC analysis of Q-MAC assay fluorescence intensity for predicting HDV RNA positivity in sera of HBsAg+ patients from Mongolia. The fluorescence intensity value of 1.659 Unit was identified as the optimal cutoff value. (C) Q-MAC assay of samples categorized by HDV RNA status. The fluorescence intensity cutoff values above which 100% of samples are predicted to be

positive on western blot, or qRT-PCR for HDV RNA, respectively, are indicated, along with the Q-MAC assay's negative control cutoff.

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Table 1
Reproducibility of the Q-MAC assay. (Mean microarray fluorescence intensity Units)

Q-MAC assay fluorescence intensity units determined in three independent assays performed on separate days on five HDV positive samples (Positive 1 to 5) and five HDV negative samples (Negative 1 to 5) are displayed, along with the corresponding mean and standard deviation values.

Patients	1 st Exp	2 nd Exp	3 rd Exp	Mean	SD	%CV
Positive 1	5.369 (9.7)	4.712 (8.5)	4.314 (7.7)	4.798	0.533	11.1
Positive 2	6.227 (4.2)	7.450 (7.2)	6.260 (7.3)	7.186	0.780	10.9
Positive 3	8.199 (6.2)	8.704 (11.2)	7.502 (12.4)	8.135	0.603	7.4
Positive 4	1.646 (4.1)	1.871 (8.8)	1.709 (7.6)	1.742	0.116	6.7
Positive 5	7.751 (9.1)	7.518 (2.8)	6.641 (2.3)	7.303	0.585	8.0
Negative 1	0.009 (8.5)	0.011 (9.4)	0.012 (6.3)	0.011	0.001	9.1
Negative 2	0.009 (9.4)	0.011 (11.3)	0.012 (8.8)	0.011	0.001	9.1
Negative 3	0.029 (6.9)	0.032 (11.6)	0.027 (10.8)	0.029	0.003	10.3
Negative 4	0.034 (8.8)	0.033 (2.6)	0.021 (7.1)	0.029	0.007	24.1
Negative 5	0.027 (3.4)	0.021 (10.5)	0.015 (7.7)	0.021	0.006	28.6

Note: as indicated in Figure 1, each value in the first three columns represents the mean fluorescence intensity of 6 replicate spots, with the intra-run variability (%CV) between each of the six spots indicated in parentheses.

Exp, experiment; SD, standard deviation; %CV, coefficient of variation.

Intra-run variability (% CV) is indicated in parentheses.

Table 2
Comparisons of the lower limit of detection associated with different methods used to detect purified anti-HDV IgG

The indicated serial dilutions of purified anti-HDV IgG reference standard were assayed by Q-MAC, western blot, and ELISA assays, respectively.

Detection method	Positive Control Concentration							
	100µg/ml	10µg/ml	1µg/ml	100ng/ml	10ng/ml	1ng/ml	10pg/ml	1pg/ml
Q-MAC	+	+	+	+	+	+	+	-
Western blot	+	+	+	+	+	-	-	-
ELISA	+	+	-	-	-	-	-	-

“+”, “-”, and “-” indicate a value above, or below, respectively, of each assay’s negative control cutoff.

Table 3
Summary of HDV markers amongst Mongolian cohort of 123 HBsAg+ patients

123 of the total 1158 Mongolian cohort samples were positive for HBsAg. The HBsAg+ samples were tested for anti-HDV antibody and HDV RNA, by Q-MAC and RT-qPCR assays, respectively. The total number (and percent) of the 123 samples that were positive for anti-HDV antibody and HDV RNA is indicated. See Table S1 and text for additional details.

	Number	Percent
Samples positive for anti-HDV antibody: (above Q-MAC western blot positivity threshold of 0.164 U)	75	61%
Samples positive for HDV RNA: (by RT-qPCR)	70	57%