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

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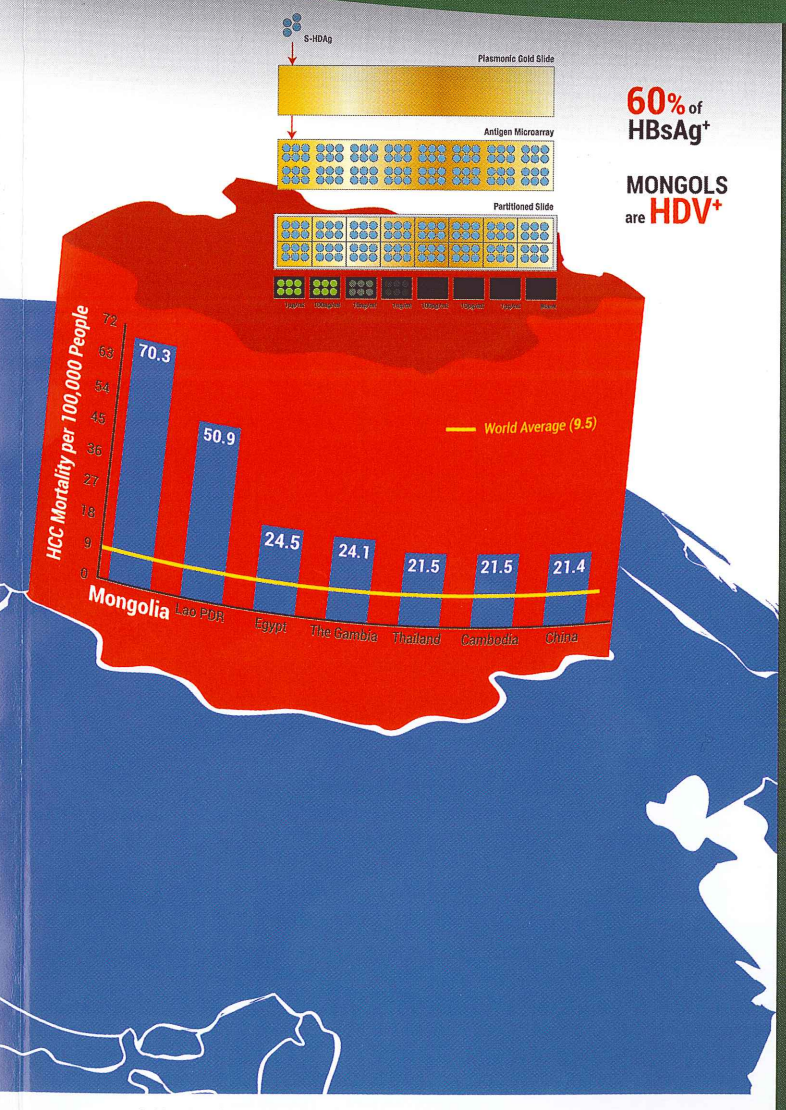

High HDV prevalence amongst HBV infected Mongolians

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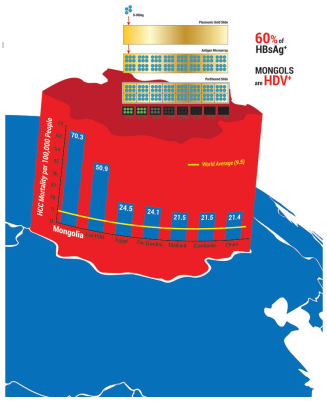

Cover Figure: Novel Quantitative Micro Array Capture (Q-MAC) assay reveals extremely high prevalence rate of hepatitis delta virus (HDV) in Mongolia, contributing to the world's highest hepatocellular carcinoma (HCC) rate. See article on page 1739.

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HEPATOLOGY

HEPATOLOGY, VOL. 66, NO. 6, 2017

Delta Hepatitis: Toward Improved Diagnostics

SEE ARTICLE ON PAGE 1739

Hepatitis D virus (HDV), the etiologic agent of hepatitis D or delta hepatitis, is a unique human virus that requires the hepatitis B surface antigen (HBsAg) of hepatitis B virus (HBV) for its replication and to establish infection. HDV, the only member of its own separate genus, *Deltavirus*, is the smallest known infectious viral agent of humans.[1] The HDV virion, which measures 35-37 nm in diameter, contains a single-stranded circular ~1,700-nucleotide RNA genome of negative polarity, which encloses only a single functional open reading frame encoding the sole viral protein, the hepatitis delta antigen (HDAg). The HDAg is associated with the genomic RNA to form a ribonucleoprotein complex which is encapsulated by a lipid envelope containing the small, middle, and large HBsAg proteins.[2] HDV exhibits a high degree of genetic heterogeneity, with estimated mutation rates between 3×10^{-2} and 3×10^{-3} base substitutions per genomic site per year.[1] The virus has a wide geographic distribution with eight distinct genotypes. HDV genotype 1 is the most common genotype, being prevalent in North America, Europe, the Middle East, and North Africa; genotypes 2 and 4 are found predominantly in East Asia; genotype 3 is found exclusively in the Amazon basin in South America; and genotypes 5-8, also known as African genotypes, are predominantly found in West and central Africa.[3]

HDV is a highly pathogenic virus, and clinical presentation of hepatitis D ranges from fulminant hepatitis, exacerbation of the course of underlying HBV infection, and acceleration of progression to cirrhosis, leading to early decompensation of liver function and hepatocellular carcinoma in the majority of patients.[4] However, a benign course of HDV infection has also been observed in Greece, Samoa, and the Far East; whether this is related to various viral characteristics such as the infecting genotype or host genetics remains to be determined.[4] Outbreaks of severe and fulminant hepatitis D have been reported from Brazil, Russia, Greenland, and Mongolia.[3] The laboratory diagnosis of coinfection or superinfection with HDV is based on simultaneous detection of various serologic markers of HBV and HDV infection. The markers of acute HDV infection include, along with the markers of HBV infection, HDAg, HDV RNA, and immunoglobulins M and G (IgM and IgG, respectively) antibodies to HDV (anti-HDV). These markers of HDV infection are present only transiently and disappear during early convalescence; IgM anti-HDV and even IgG anti-HDV also disappear with time in acute resolving HDV infection.[3] Superinfection of HBsAg carriers, which almost always leads to chronic hepatitis D, is marked by absence of IgM antibody to hepatitis B core antigen and presence of all the other markers of HBV and HDV infections (Fig. 1). However, markers of HBV replication, especially HBV DNA, may be suppressed during the acute phase of HDV infection and remain undetectable.[1] Chronicity of HDV infection is associated with persistence of HDAg, HDV RNA, and IgM and IgG anti-HDV. HDV RNA is the gold standard for diagnosis of current HDV infection because assays for detection of HDAg are fraught with sensitivity and specificity issues. Quantitative assays of HDV RNA are useful for monitoring response to antiviral therapy, and the recent availability of a World

Abbreviations: anti-HDV, antibody to HDV; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDAg, hepatitis delta antigen; HDV, hepatitis D virus; IgG/IgM, immunoglobulins G/M; Q-MAC, quantitative microarray antibody capture.

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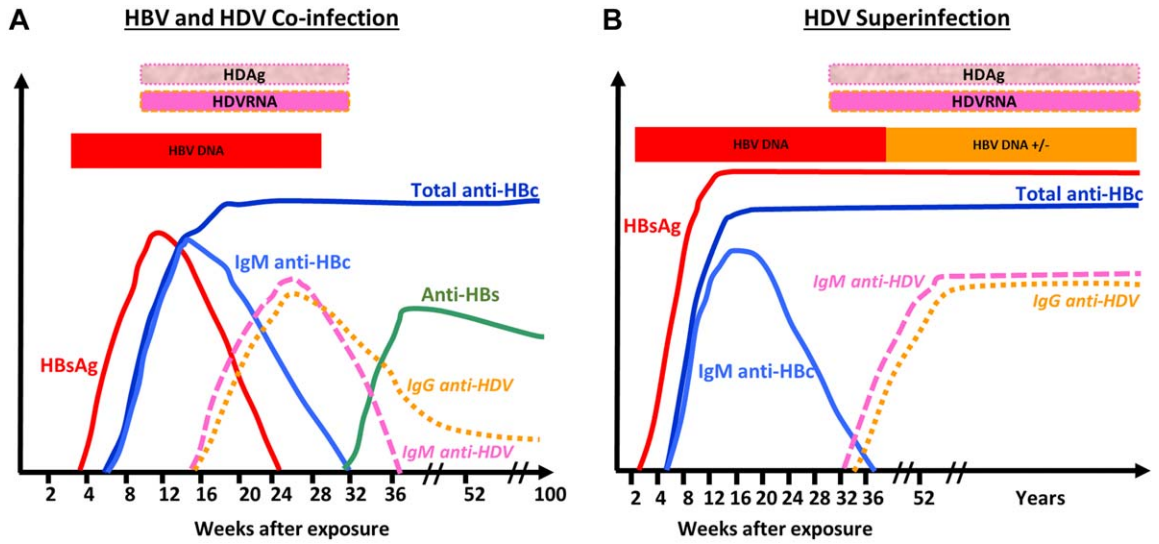

FIG. 1. Serologic course of acute resolving (A) and chronic (B) HDV infection. Abbreviations: anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg.

Health Organization standard has further helped in optimizing assays across various centers and laboratories. Sequencing of HDV RNA–positive samples is a reliable way to determine the HDV genotype.[5]

The prevalence of HDV is a measure of anti-HDV positivity among HBsAg positive carriers. It is estimated that 10 million to 20 million individuals (~5% of chronic HBV patients) worldwide are coinfected with HDV.[6] Vaccination against hepatitis B, which is also the default vaccination against HDV, resulted in a decrease of HDV prevalence in industrialized countries with the implementation of routine hepatitis B immunization of children and other populations.[7] However, in several European countries (e.g., Germany, Italy, and England), which have large and increasing numbers of migrants from HDV-endemic areas (e.g., eastern Europe and Turkey), HDV prevalence rates have remained unchanged.[2] High-risk populations like injection drug users continue to get impacted by superinfection with HDV, as has been observed in Europe and recently in the United States.[7,8] Based on cross-sectional studies, high rates of HDV infection, ranging from 10% to 70%, in HBsAg carriers have been reported from Nigeria, Gabon, India, Pakistan, Iran, the western Brazilian Amazon, Tajikistan, and Mongolia.[6] A number of developing countries have reported a high endemicity of HDV, and prevalence rates of >20% have been reported; these include central Africa, mountainous regions of Venezuela and Colombia, Romania, Pakistan, Iran, the Amazon basin in South America, and Mongolia. However, reliable data on the accurate prevalence of HDV are not available from all regions due to either lack of testing of HBsAg carriers for HDV infection or lack of availability of anti-HDV antibody assays with proven performance characteristics. In this context, the article by Chen et al. published in this issue of HEPATOLOGY[9] is of major significance. The authors found a substantially higher prevalence (~60%) of HDV infections among HBV-infected individuals identified in a national survey sampling of the Mongolian population. Of a total of 1,158 individuals chosen based on a three-stage cluster sampling method to reflect the gender, age, and geographical origin representative of the entire country, 123 tested positive for HBsAg, of whom 75 (60%) tested anti-HDV-positive by a newly developed high-throughput quantitative microarray antibody capture (Q-MAC) assay that the authors describe.[9] Modern diagnostic approaches, such as protein microarray–based Q-MAC assays, allow for improvement in the sensitivities of various diagnostic assays. Protein microarrays have become an important tool in studies of protein–protein interactions, protein detection, and other applications in quantitative and functional proteomics and beyond. Protein microarrays are arrays of protein targets, frequently antibodies and/or antigens, immobilized on a solid support such as a glass slide. Plasmonic substrates, such as nanostructured plasmonic gold film, have been

developed to improve the sensitivity and dynamic range of protein detection on microarrays and have been shown to detect cancer biomarkers and integrated human antibodies and antigens down to femtomolar ranges.[10] Chen et al. have used this protein microarray technology to develop a Q-MAC assay for sensitive quantitative fluorescent detection of anti-HDV IgG from patient sera. The authors not only convincingly demonstrated the excellent performance characteristics of their Q-MAC assay but also established quantitative thresholds of captured HDV antibody predictive of HDV RNA positivity. However, as the authors have righty stated, despite a good correlation between fluorescence intensity of the Q-MAC assay and HDV RNA levels, determination of HDV RNA remains the gold standard for monitoring of treatment against hepatitis D. The Q-MAC assay is rapid, requires a small volume of patient serum, and can easily be scaled to high-throughput screening of large cohorts of HBsAg carriers for HDV infection.

A true seroepidemiologic gauge of the prevalence of hepatitis D is to test HBsAg carriers only and estimate the prevalence rates using the HBsAg positives as the denominator. With this approach, Chen et al.[9] found an alarming proportion of their HBsAg-positive population in Mongolia superinfected with HDV. Compared to other HBsAg carriers, persons superinfected with HDV are at the highest risk for hepatocellular carcinoma, and HDV superinfection undoubtedly contributes to Mongolia's high rate of hepatocellular carcinoma. Assessments of HDV as a cause of excess morbidity and mortality among persons with HBV infection globally are limited by the lack of serologic studies. The data from Chen et al.[9] highlight the importance of conducting such seroprevalence studies among HBsAg carriers in HDV-endemic countries and high-risk populations in developed countries. Given that an estimated 248 million are chronically infected with HBV worldwide and thus susceptible to superinfection with HDV, concerted efforts, like the one undertaken by the Hepatitis Delta International Network (http://hepatitis-delta.org/), are needed for the determination of accurate estimates of hepatitis D disease burden, evidence-based policies for HDV testing, and research to understand the biological mechanisms of HDV infection and find efficacious therapies for treatment of hepatitis D.

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HEPATOLOGY

HEPATOLOGY, VOL. 66, NO. 6, 2017

A Novel Quantitative Microarray Antibody Capture Assay Identifies an Extremely High Hepatitis Delta Virus Prevalence Among Hepatitis B Virus–Infected Mongolians

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Hepatitis delta virus (HDV) causes the most severe form of human viral hepatitis. HDV requires a hepatitis B virus (HBV) coinfection 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. Accurate assessments of current HDV prevalence have been hampered by the lack of readily available and reliable quantitative assays, combined with the absence of a Food and Drug Administration–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 hepatocellular carcinoma. We developed a high-throughput quantitative microarray antibody capture assay for anti-HDV immunoglobulin G wherein recombinant HDV delta antigen is printed by microarray on slides coated with a noncontinuous, nanostructured plasmonic gold film, enabling 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 real-time quantitative PCR. Subsequent screening of the HBV^+ cohort revealed that a remarkable 57% were RNA^+ and an additional 4% were positive on western blot alone. *Conclusion:* The quantitative microarray antibody capture assay's unique performance characteristics make it ideal for population screening; its application to the Mongolian HBV surface antigen–positive population reveals an apparent ~60% prevalence of HDV coinfection among these HBV-infected Mongolian subjects, which may help explain the extraordinarily high rate of hepatocellular carcinoma in Mongolia. (HEPATOLOGY 2017;66:1739-1749).

SEE EDITORIAL ON PAGE 1716

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

Abbreviations: anti-HDV, antibody to HDV; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDAg, hepatitis delta antigen; HDV, HDV, hepatitis delta virus; IgG, immunoglobulin G; pGOLD, plasmonic gold; Q-MAC, quantitative microarray antibody capture; S-HDAg, small hepatitis delta antigen.

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that HDV acquires from HBV and 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 coexisting HBV infection due either to the simultaneous transmission of the two viruses or to superinfection in an established HBV carrier.[4] Approximately 5% of the global HBV-infected population, or 15 million to 20 million people worldwide, are infected with HDV, although HDV prevalence rates are not uniform, with higher rates of HDV coinfection reported in the Mediterranean basin, parts of Africa, the 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 $HBsAg^+$ individuals for antibody to HDV (anti-HDV). Anti-HDV 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^+ patients, the ideal next step is testing for HDV RNA in serum to confirm the presence of active HDV infection. With the advent of 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 1,000 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 primer sets and by the variability of the RNA region amplified.[9,10] A World Health Organization international RNA standard is now available, enabling the reporting of results in international units, although no quantitative HDV RNA assay is commercially available in the United States.

Here, we present a new quantitative microarray antibody capture (Q-MAC) assay for detecting anti-HDV in human sera. This platform is constructed on noncontinuous, nanostructured plasmonic gold (pGOLD) films with enhanced near-infrared fluorescence detection that we hypothesized would have high sensitivity and would be 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 down to the 10 femtomolar (picograms per milliliter) range.[11,12] For the anti-HDV Q-MAC assay, recombinant full-length HDV small delta antigen (S-HDAg) was arrayed on a pGOLD 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 coinfection among HBV-infected individuals identified in a national survey sampling the population of Mongolia.

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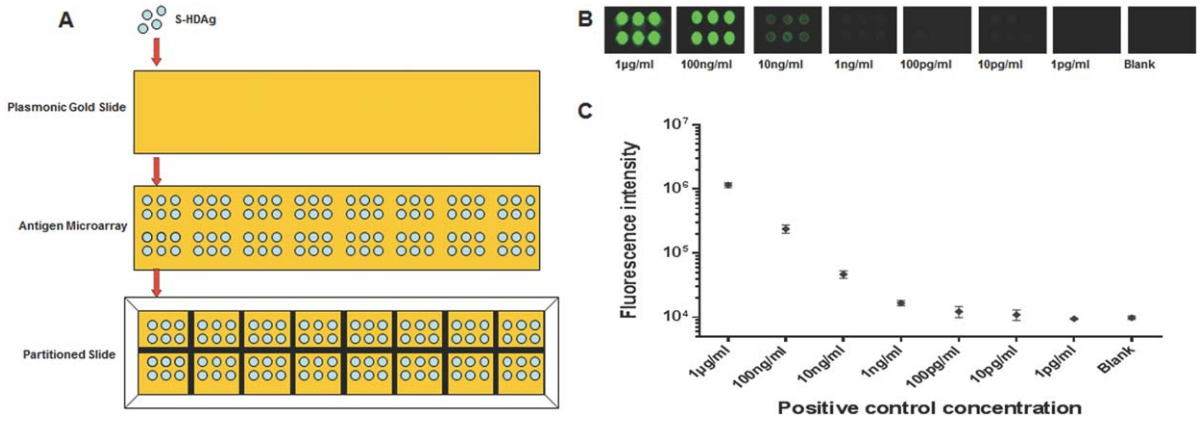

FIG. 1. Schematic of anti-HDV Q-MAC assay and lower limit of detection using delta antigen printed on pGOLD microarray slide. (A) S-HDAg was printed on a pGOLD microarray slide by a microarray printing robot such that six identical spots of ~2 mm diameter are contained within each partitioned area of 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 = FBS. (C) Calibration curve for captured anti-HDV quantification. Mean fluorescence intensity of IRDye800-labeled antihuman secondary antibody emission from the six replicate microarray spots at each antibody concentration on the pGOLD microarray slides. Error bars represent the standard deviation of the mean over the six replicate assay features within each partition of the slide.

Materials and Methods

PATIENT SERUM SAMPLES

Deidentified unique serum samples from the following collections were used for this study: 82 historical HDV RNA$^+$ patient sera, 30 samples from HBV-monoinfected patients, 30 samples from hepatitis C virus (HCV)–monoinfected patients, 10 samples from pregnant women and 10 samples from healthy control patients, and 123 HBsAg$^+$ 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, and 123 of them tested positive for HBsAg, using a commercial ELISA kit (DiaSorin, srl., Saluggia, Italy).

PREPARATION OF INTERNAL STANDARD ANTI-HDV IMMUNOGLOBULIN G REFERENCE ANTIBODY

An internal standard of purified anti-HDV immunoglobulin G (IgG) was prepared from an HDV$^+$ sample with high-titer anti-HDV 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 S-HDAg

S-HDAg was expressed in and purified from *Escherichia coli* and stored in single-use aliquots of >90% purity until use, as described in the Supporting Information.

ANTIGEN MICROARRAY PRINTING

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. pGOLD slides were loaded into a microarray printing robot (Bio-Rad) where S-HDAg (100 μM) was printed using solid pins (Arrayit) at 25°C and 60% humidity, resulting in microarray feature diameters of ~2 mm. The microarray layout was designed using the microarray printer software. The antigen was printed into 16 areas with six replicate spots each (Fig. 1A).

The slides were dried in a desiccator, vacuum-sealed in a bag, and stored at 4°C.

Q-MAC ASSAY

The microarray printed slides were blocked with fetal bovine serum (FBS) for 1 hour, followed by washing three times with phosphate-buffered saline containing 0.5% Tween-20. Up to 13 individual serum samples were analyzed per slide. One microliter of each human serum sample was diluted to a total of 50 μL 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). Slides were washed three times with phosphate-buffered saline containing 0.5% Tween-20 and IRDye800-labeled donkey antihuman IgG (Rockland Immunochemicals, Inc.) diluted 1:1,000 in FBS solution and applied to each array set for 1 hour in the dark. Slides were then washed three times with phosphate-buffered saline containing 0.5% Tween-20 and once with deionized water and dried in the dark.

Slides were scanned using a Licor Odyssey scanner with the 800-nm channel. Image Studio Lite, version 4.0, was used to automatically identify features above a composite pixel intensity of 5. A fluorescence intensity of 100 ng/mL internal standard purified anti-HDV IgG was defined as 1 U and used to normalize the intensity of tested samples with the following formula: Value of fluorescence intensity (unit) = (mean sample exact intensity value – mean blank intensity value)/(internal standard 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.) according to the manufacturer's instructions. Fifty microliters of patient serum was diluted 1:1 for the ELISAs. Optical density 450 values were ascertained using photometry (Tecan Group Ltd., Switzerland).

WESTERN BLOT DETECTION

Purified recombinant protein (S-HDAg) was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Millipore), probed with 10 μL of patient serum diluted 1:100, followed by detection with IRDye800-labeled goat antihuman IgG (diluted 1:20,000) and visualization using a Licor Odyssey scanner, as described in the Supporting Information.

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 1,679 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 Supporting Information.

QUANTITATION OF HDV RNA BY TAQMAN-BASED ONE-STEP REAL-TIME PCR

In order to enable detection of all eight known 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 Supporting Information. Standards for the calibration curve were prepared using a 10-fold dilution series of full-length HDV RNA to cover the range 1.6×10^1 to 1.6×10^7 IU/mL. A normal human serum negative control and an HDV^+ serum positive control (1×10^4 RNA IU/mL) were included in each assay. The World Health Organization HDV RNA international standard was used to normalize the results to international units 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 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 ± standard deviation. $P < 0.05$ was considered significant.

TABLE 1. Reproducibility of the Q-MAC Assay (Mean Microarray Fluorescence Intensity Units)

Patients	1st Experiment	2nd Experiment	3rd Experiment	Mean	Standard Deviation	Coefficient of Variation
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%

Q-MAC assay fluorescence intensity units determined in three independent assays performed on separate days on five HDV^+ samples (Positive 1-5) and five HDV^- samples (Negative 1-5) are displayed, along with the corresponding mean and standard deviation values. Each value in the first three columns represents the mean fluorescence intensity of six replicate spots, with the intra-run variability (% coefficient of variation) between each of the six spots indicated in parentheses (as indicated in Fig. 1).

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 detection (Supporting Fig. 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 (Supporting Fig. S1B). The lowest detected concentration was 10 ng/mL purified anti-HDV IgG.

DYNAMIC RANGE, REPRODUCIBILITY, SENSITIVITY, AND SPECIFICITY OF THE ANTI-HDV 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 Fig. 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 1 hour. Wells were then washed, bound anti-HDV was detected with IRDye800-labeled antihuman IgG, and the fluorescence intensity associated with each spot of printed S-HDAg was measured (Fig. 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 Q-MAC assay (Fig. 1C). A fluorescence intensity of 100 ng/mL internal standard purified anti-HDV IgG was defined as 1 U 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 six replicate spots in three independent experiments (Table 1). The mean intensity of each HDV RNA^+ sample ranged from 1.742 to 8.135 U, and the coefficient of variation ranged from 6.7% to 11.1%. The mean intensity of each negative control ranged from 0.011 to 0.029 U, and the coefficient of variation ranged from 9.1% to 28.6%.

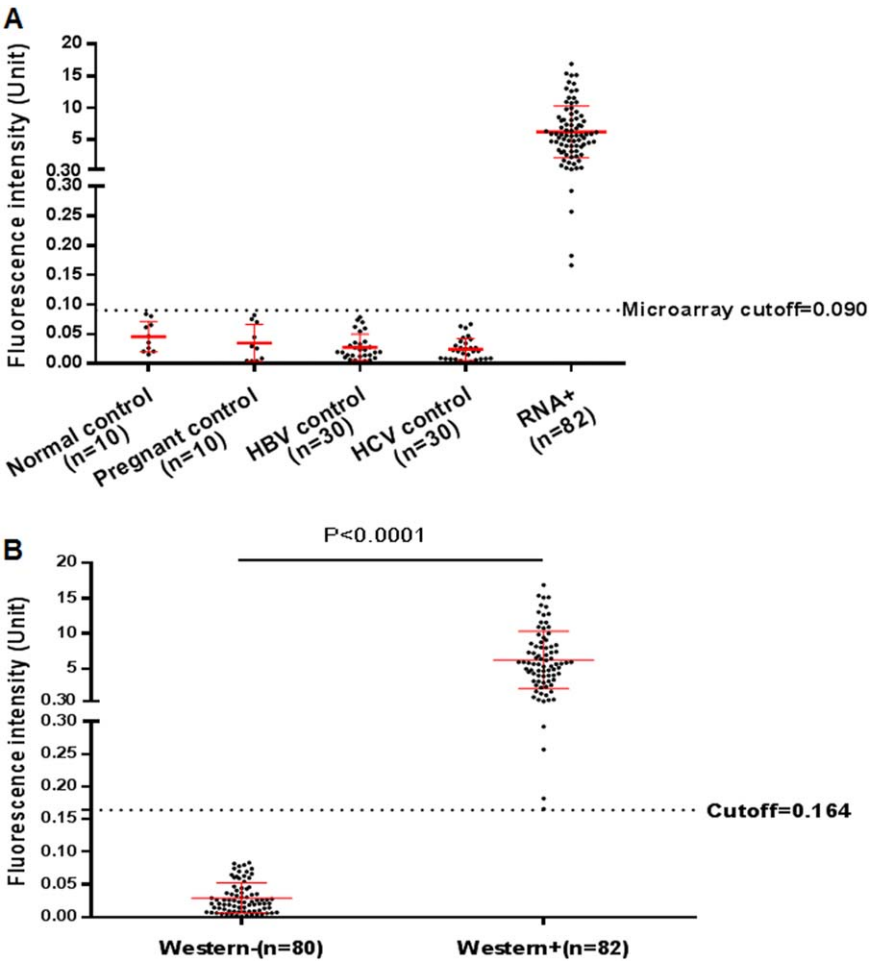

FIG. 2. Anti-HDV IgG detection in the sera of 162 HDV RNA$^+$ and negative control patients using Q-MAC and western blot assays. (A) Normalized fluorescence intensities of HDV$^-$ samples (normal, HCV, and HBV controls) are below the Q-MAC cutoff value (0.090 U). All of HDV RNA$^+$ samples are above this cutoff value. (B) 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.

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 control intensities were below 0.090 U, and this was set as the microarray cutoff value (Fig. 2A). The intensities of HDV RNA$^+$ samples were all above this cutoff value (Fig. 2A). The mean intensity was 6.221 U. The median intensity was 5.683 U, with a range from 0.166 to 16.91 U.

To correlate the performance of Q-MAC at detecting anti-HDVs with western blot assay results, all 162 samples were subjected to standard western blot analysis. Anti-HDV in the sera of the 82 HDV RNA$^+$ patients was detectable on westerns, while no such signal appeared for the 80 negative controls. For this sample set, a fluorescence intensity above 0.164 units correlated with a positive western blot assay (Fig. 2B). 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 provide 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, the Q-MAC assay's sensitivity was 10^6-fold and 10^3-fold

TABLE 2. Comparisons of the Lower Limit of Detection Associated With Different Methods Used to Detect Purified Anti-HDV IgG

Detection Method	Positive Control Concentration							
	100 μg/mL	10 μg/mL	1 μg/mL	100 ng/mL	10 ng/mL	1 ng/mL	10 pg/mL	1 pg/mL
Q-MAC	+	+	+	+	+	+	+	–
Western blot	+	+	+	+	+	–	–	–
ELISA	+	+	–	–	–	–	–	–

+ and – indicate a value above or below, respectively, each assay's negative control cutoff.

higher than the 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 $HBsAg^+$ in a recently collected cohort of 1,158 samples from a national surveillance study conducted in Mongolia to determine prevalence rates of hepatitis virus infections.[13]

$HBsAg^+$ samples were also analyzed independently by anti-HDV western blot, a commercial anti-HDV ELISA kit (DiaSorin), and quantitative RT-PCR HDV RNA assays (see Table 3; Supporting Table S1). Thirty-nine 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 positives. 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) (Fig. 3A). All of these samples were confirmed to be positive on western blot. Thus, using the Q-MAC threshold of 0.164 U had 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.

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 $HBsAg^+$ 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 (Supporting Table S1).

Receiver operating characteristic 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% (area under the curve = 0.9978, $P < 0.0001$) (Fig. 3B). Sixty-six subjects had Q-MAC values above 1.659, and all were confirmed to be $HDV\ RNA^+$. Four 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 Fig. 3C and tabulated in Supporting Table S1.

Of note, 5 of the $HDV\ RNA^-$ 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, nine 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 HDV that is sensitive, is 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 enable prediction of clinically meaningful virologic status. The results allowed us to define a quantitative threshold of captured anti-HDV 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 among 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 coinfection that is provocatively related to the extremely high rate of hepatocellular carcinoma in Mongolia.[16]

Peptide antigens have been recently described for detection of anti-HDV.[17] These were limited, however, in their ability to detect all of the various

TABLE 3. Summary of HDV Markers Among the Mongolian Cohort of 123 HBsAg$^+$ Patients

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

Of the total 1,158 Mongolian cohort samples, 123 were positive for HBsAg. The HBsAg$^+$ samples were tested for anti-HDV and HDV RNA by Q-MAC and quantitative RT-PCR assays, respectively. The total number and percent of the 123 samples that were positive for anti-HDV antibody and HDV RNA are indicated. See Supporting Table S1 and text for additional details.

genotypes that have been described for HDV.[18] Use of full-length recombinant HDAg, as described here, appears to not suffer from this limitation. Instead, using full-length recombinant HDAg 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 pGOLD substrate coating on glass afford hundreds of fold near-infrared fluorescence enhancement when compared with commercial streptavidin-coated glass.[12] Our Q-MAC assay is based on the above pGOLD platform and exhibits a detection capability down to 10 pg/mL anti-HDV IgG concentrations. Moreover, the Q-MAC assay's sensitivity was 10^6-fold 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 U 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 U 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 over 500 samples combined—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$^+$ 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$^+$ based on the results of the Q-MAC assay.

Interestingly, one can define a fluorescence intensity cutoff—1.659 U—that is predictive for 100% of patients being HDV RNA$^+$. Thus, the simple, relatively high-throughput Q-MAC assay could prove useful for prospectively identifying patients who have active HDV replication or who 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 (Supporting Fig. S2), determination of HDV RNA remains the assay of choice for monitoring response to therapy.

Five patients in the Mongolian cohort were HDV RNA$^-$ 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 U) but below the cutoff 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

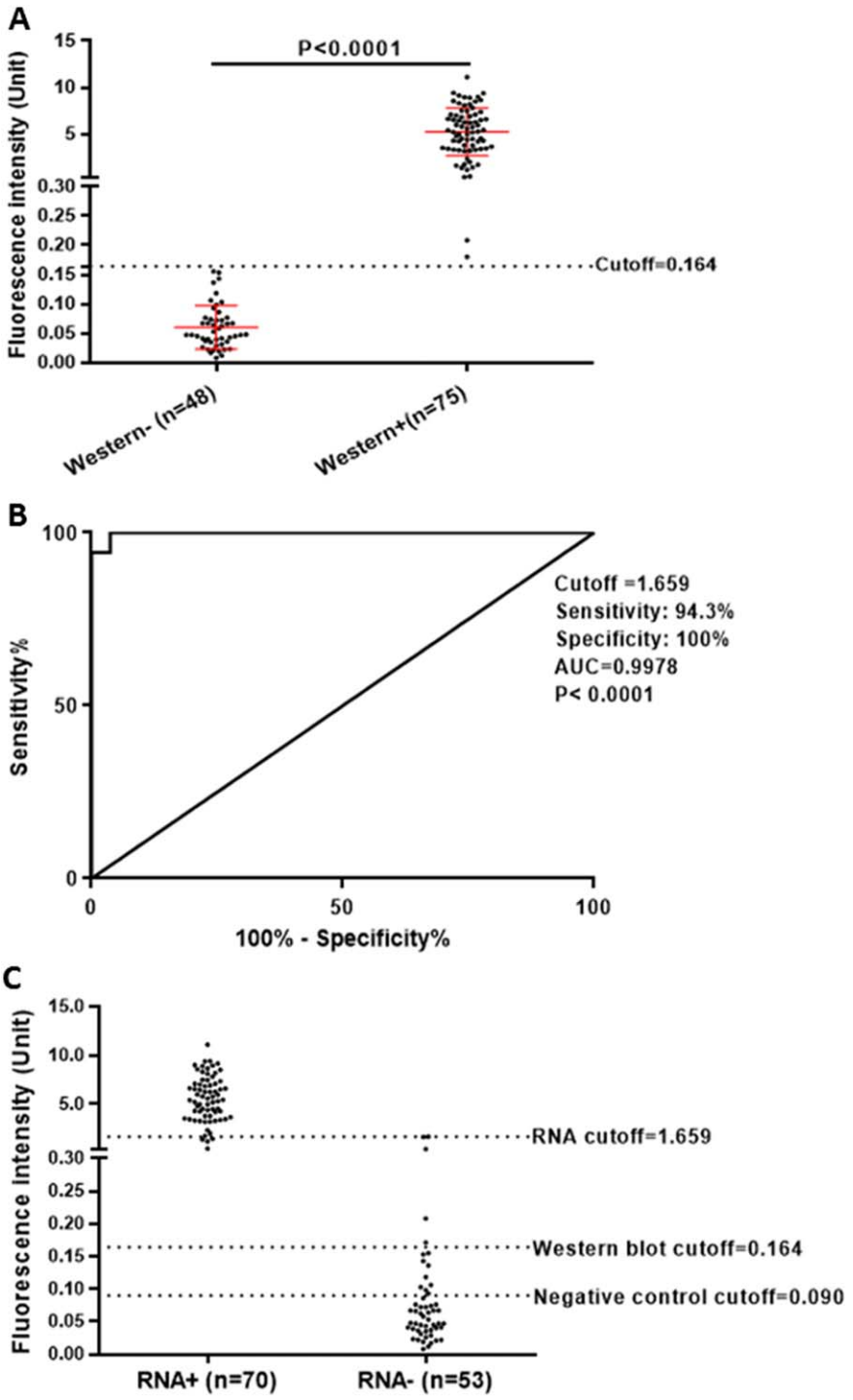

FIG. 3. Analysis of anti-HDV IgG and HDV RNA in the sera of 123 $HBsAg^+$ samples from Mongolia by Q-MAC, western blot, and qRT-PCR assays. (A) Q-MAC assay of samples categorized by western blot status. Note all samples below the Q-MAC 0.164 U predictive cutoff value for western blot positivity were negative on western blot, and all samples above this cutoff were positive on western blot. (B) Receiver operating characteristic 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 U was identified as the optimal cutoff value. (C) Q-MAC assay of samples categorized by HDV RNA status. Fluorescence intensity cutoff values above which 100% of samples are predicted to be positive on western blot or quantitative RT-PCR for HDV RNA, respectively, are indicated, along with the Q-MAC assay's negative control cutoff. Abbreviation: AUC, area under the curve.

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^+.

Various prior studies in selected Mongolian populations have indicated a range of HDV prevalences, with the latter being consistently higher than is 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 $HBsAg^+$ 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 $HBsAg^+$ subjects.[19] In a study of 207 patients with known liver disease, 144 were $HBsAg^+$, including 117 (81% of those $HBsAg^+$) 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^+.[21] Focusing on apparently healthy children 7-12 years old born after 1991 (when universal vaccination against HBV was introduced into the country), 59 of 1,182 (5%) children were found to be $HBsAg^+$, and 8 (13.6%) of these were HDV RNA^+.[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 among the $HBsAg^+$ 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 coinfection rate among HBV-infected subjects is estimated at 5%,[23] approximately 60% of HBV-infected patients are coinfected 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 (Supporting Table S2). Reasons for this much higher prevalence may include the 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 agencies and institutions concerned with crafting and implementing appropriate responses.

In summary, we developed a quantitative microarray anti-HDV capture (Q-MAC) assay. Defining in the new assay quantitative thresholds 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 coinfection among $HBsAg^+$ 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 contribute to the alarming incidence of hepatocellular carcinoma in Mongolia, which ranks among the highest in the world.[25] This serves to underscore the urgent need for improved therapies for HDV.

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Supporting Information

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