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Large Scale Screening of Human Sera for HCV RNA and GBV-C RNA

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Abstract

North Carolina locates acute HIV cases by pooled nucleic acid testing of HIV- antibody negative serum samples. Here, 224 pools of 80 HIV-negative samples (N=17,920) were screened for viral RNA from HCV, GBV-C, and influenza A. No evidence of influenza A was found, but HCV and GBV-C were common (1.2% and 1.7% prevalence, respectively), demonstrating the utility of pooled testing in locating individuals that may remain undiagnosed otherwise. By sequencing positive pools, potential transmission clusters may be located as well.

Keywords

Hepatitis C Virus; GB Virus C; Influenza A; Public Health; Emerging Pathogens; Acute HIV Infection

Introduction

Emerging viruses with the potential to expand into a widespread epidemic are the subject of vigorous research and surveillance measures. Serum pooling strategies offer the opportunity to screen efficiently for people experiencing viremia, either from chronic or acute infection [Morandi et al., 1998; Roth et al., 1999]. In particular, locating persons with chronic Hepatitis C Virus (HCV) infection [Center for Disease Control and Prevention, 1998; Smith et al., 2012] could prevent future clinical sequelae including cirrhosis and carcinoma in light of newer, potentially curative treatments [Lok et al., 2012].

A centralized screening program is already in place for acute HIV in North Carolina, where cases are located by testing pools of HIV antibody negative samples for HIV RNA [Pilcher et al., 2005]. This strategy minimizes cost while maximizing the potential to locate persons with acute HIV in a low prevalence context [Quinn et al., 2000]. A subset of HIV-negative serum pools was obtained and tested using quantitative real time PCR for RNA from HCV, which is prevalent in the US and establishes chronic infection [Armstrong et al., 2006]; GB virus C (GBV-C), which is common [Gutierrez et al., 1997], is cleared among immune competent individuals [Thomas et al., 1998], and has been linked to increased survival among individuals with chronic HIV-1 infection, [Zhang et al., 2006]; and influenza A, which is associated with transient viremia [Carrat et al., 2008] and expected to be common given the timing of sample collection [Viboud et al., 2006].

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Materials and Methods

A total of 224 HIV-1-negative de-identified serum pools of 80 samples each were acquired from the North Carolina State Laboratory of Public Health, representing ≤17,920 adults (depending on repeat testing) seeking HIV testing in North Carolina from January – February 2010. This study was reviewed and approved by the North Carolina State Laboratory of Public Health.

From each pool, RNA was extracted from 140 μL of serum (QIAmp, QIAGEN, Hilden, Germany). Half of the eluted RNA (30 μL) was used for cDNA synthesis (in 60 μL total) with random primers (SuperScript III, Life Technologies, Carlsbad, CA). cDNAs were screened in triplicate parallel reactions (5 μL cDNA/reaction) for HCV (family *Flaviviridae*, genus *Hepacivirusm*, species *Hepatitis C virus*), GBV-C (family *Flaviviridae*, species *GC virus C*), and influenza A (family *Orthomyxoviridae*, genus *Influenzavirus A*, species *Influenza A virus*) by real time PCR (Universal Master Mix II, Life Technologies). Primers and probes targeting the 5′UTR of HCV and GBV-C were: HCV1a5′UF: TCTGCGGAACCGGTGAGT, HCV1a5′UR: AGCGGGTTTATCCAAGAAAGG, HCV1a5′Uprobe: 6FAM-CACCGGAATTGCCAGGACGACC-TAMRA; GBVC5′UF: TGTTGGCCCTACCGGTGTTA, GBVC5′UR: CGTACGTGGGCGTCGTTT, GBVC5′Uprobe: 6-FAM-CTCGTCGTTAAACCGAGCCCGTCA-TAMRA. HCV and GBV-C primers and probes were designed using Primer Express version 3.0 (Life Technologies) with the exception of the GBV-C probe, which was developed by Souza and colleagues [Souza et al., 2006]. The primers and probe targeting influenza A M2 have been described elsewhere [Hourfar et al., 2007].

To create standards for real time PCR, three plasmids were constructed by sub-cloning a 564 bp region of HCV H77 1a 5′UTR (nt 50–613), a 265 bp region of GBV-C pAF121950 5′UTR (nt 136–400), and an 864 bp region of influenza A M2 (nt 25–817 into pT7Blue (EMD Millipore, Darmstadt, Germany). Inserts were transcribed in vitro using the T7 RiboMAX kit (Promega, Madison, WI). RNA transcripts were purified using the RNeasy Minelute kit (QIAGEN), quantified by agarose gel electrophoresis with an RNA standard (RiboRuler, Fermentas, Vilnius, Lithuania) using a Gel Logic 212 Pro imager (Carestream Health Inc., Rochester, NY), and spiked with 2 μg of carrier RNA prior to cDNA synthesis. To confirm RNA concentration, purified RNA were diluted prior to the addition of carrier RNA and quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). cDNA standards were diluted over 6 $\log_{10}$ copies based on RNA concentration, aliquoted into silicone-coated tubes, and stored at −20°C.

Sequence diversity of HCV and GBV-C-positive samples was characterized by sequencing HCV or GBV-C NS5B using published primers [Muerhoff et al., 1997; van Asten et al., 2004]. Sequences were inspected for quality using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI). Trimmed sequences were aligned using Clustal W [Chenna et al., 2003], Neighbor-Joining trees were resolved in MEGA 5.0 using 1,000 bootstrap replicates [Tamura et al., 2011], and evolutionary differences were estimated using the Kimura 2-parameter method [Kimura, 1980]. Pooled serum sequences were compared to isolates from NCBI (GBV-C, http://www.ncbi.nlm.nih.gov) or Los Alamos (HCV, http://www.hcv.lanl.gov).

Results

A subset of serum pools collected in early 2010 was screened for the RNA genomes of viruses of public health interest, specifically HCV, GBV-C, and influenza A. The North Carolina HIV testing pooling strategy and the estimated amount of serum screened per

sample per pool is shown in Figure 1A. To establish the sensitivity of real time PCR, known amounts of viral RNA was reverse transcribed into cDNA and diluted based on the RNA concentration to serve as standards for real time PCR. A detectable signal was observed when cDNA was diluted down to an estimated 10 copies for each assay, with ≥26 of 27 replicates positive at this dilution. Because of the dilution effect of pooling and testing, the expected limit of detection for an individual sample was approximately 150,000 RNA copies/mL per sample assuming 10 copies of RNA per reaction could be detected.

Of 224 serum pools screened, 138 (62%) were positive for HCV RNA, 168 (75%) were positive for GBV-C RNA, and none were positive for influenza A RNA. About 44% of pools were positive for both HCV and GBV-C RNA (N=99). Assuming the number of positive samples per pool followed a Poisson distribution, with 37% of pools negative for HCV an estimated 0.96 (95% CI 0.94–0.97) samples were HCV-viremic per pool of 80, with 37% having 1 positive sample, and 25% having >1 positive sample. Similarly, 25% of pools had no evidence of GBV-C, yielding an estimated 1.39 (95% CI 1.37–1.40) GBV-C positives per pool, with about 35%, 24%, and 16% having 1, 2, or >2 positive samples per pool. Given the dilution factor for each sample (Figure 1A) and the estimated positives per pool, the median viral load among positives was estimated to be 6.4 $\log_{10}$ HCV RNA copies/mL serum (inter-quartile range (IQR): 5.9–7.0) and 6.4 $\log_{10}$ GBV-C RNA copies/mL serum (IQR: 5.7–6.9). These assays had good reproducibility in that a repeat of six positive samples gave titers within two-fold between two runs (data not shown).

To increase specificity of these assays, samples were considered positive if all 3 real time PCR replicates yielded a signal before 40 cycles. For HCV, 11 of 86 (13%) negative samples had 1 or 2 replicates with a signal. The median cycle threshold of these 11 negative calls was 37.6 cycles (IQR: 36.4–38.7), which was above the median 36.0 cycles (IQR: 35.3–36.3) for 10 HCV positive calls with the lowest HCV copy number (p=0.01). For GBV-C, 21 of 56 (37%) negative calls had ≥1 replicate with a signal. These 21 negative calls had a median cycle threshold of 37.9 (IQR: 37.3–38.7) versus a median of 36.1 cycles (IQR: 36.0–36.3) for the 10 GBV-C-positive samples with the lowest copy numbers (p=0.0002).

If real time PCR is detecting extant viral infections, the viral genomes among positive pools would be expected to display sequence diversity. To test this, NSB5 amplicons from a sample of GBV-C and all HCV positive pools were sequenced. A total of 21 GBV-C and 106 HCV NSB5 sequences were obtained from 8 separate real time PCR runs of up to 32 pools at a time, with each sequence representing the consensus sequence from a single positive pool. Sequences were highly diverse across real time PCR runs, with GBV-C sequences having 0.047 (SE 0.007) substitutions per position and HCV sequences having 0.067 (SE 0.007) substitutions per position (Figures 2A; 2C). The only exception to this observed variability was a cluster of 2 HCV NS5B sequences (pools 062 and 111), which differed by a single nucleotide in this region, indicative of a recent common ancestor. This observation was confirmed by repeated sequencing from RNA extraction forward. Heterogeneity of serum pool NS5B sequences was observed when compared to published sequences, as evidenced by sequence branching patterns on the phylogenetic trees for both viruses (Figures 2B; 2D). When compared to downloaded HCV 1a and HCV 1b sequences, 17 of the HCV sequences were more consistent with HCV 1b (Figure 2D).

Discussion

After screening 224 HIV-negative pools of serum from ≈18,000 adults at risk for HIV infection, no evidence of influenza A was found. However, 62% and 75% of tested pools had detectable HCV and GBV-C RNA, with an estimated 1.2% (0.96/80) and 1.7%

(1.39/80) of samples having actively replicating HCV or GBV-C and median viral loads exceeding 10^6 copies/mL sera. These figures agree with national surveillance data from blood donors, which estimate that 1.3% and 1.7% of the population is actively infected with HCV or GBV-C [Gutierrez et al., 1997; Armstrong et al., 2006]. As expected, estimates among this HIV-negative population are lower than among persons with HIV infection, where up to 15% and 24% of the population have evidence of HCV or GBV-C viremia, respectively [Lefrere et al., 1999; Sherman et al., 2002]. However, inferred estimates of the titers of these viruses in plasma are limited by the accuracy of using purified RNA as the standard curve for the real time PCR rather than spiking serum samples. Good reproducibility was observed, however, in that when pools were rerun they gave comparable results within two-fold.

Although no influenza A was detected in this population, a negative result is limited by the relatively high detection limit (>150,000 copies/mL). There have been cases of influenza RNA detection in patient blood [Likos et al., 2007], but a proof of concept study among blood donor plasma pools failed to detect influenza A in >10,000 samples [Hourfar et al., 2007]. The success of the North Carolina program in locating persons at high risk of HIV-1 transmission and the high burden of HCV and GBV-C viremia in this same population demonstrate the utility of the pooling strategy to screen for other viruses. For HCV in particular, as the possibility of potentially curative therapy draws closer, existing infrastructure may be exploited to identify persons with chronic HCV infection in a test-and-treat strategy.

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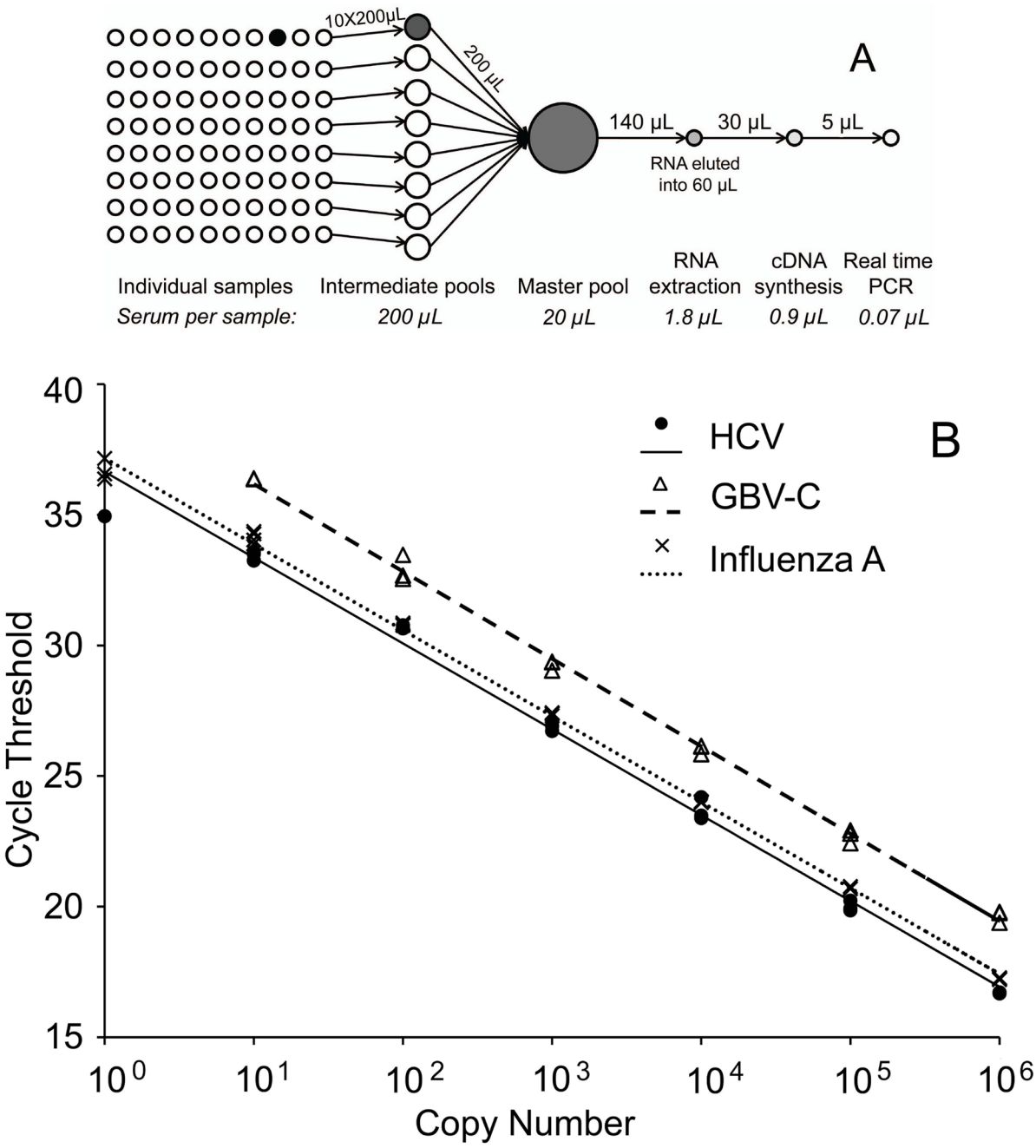
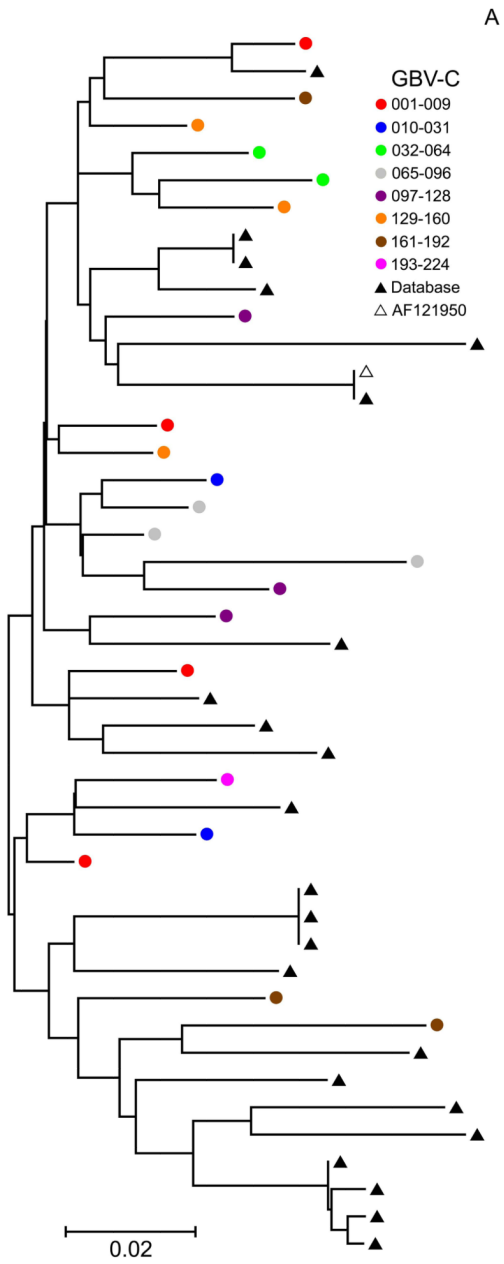


Figure 1.

Sensitivity of real time PCR assays to detect Hepatitis C Virus (HCV), GB Virus C (GBV-C), or influenza A RNA in pools of serum composed of 80 samples each. (A) Schematic of the pooling process used by the North Carolina State Laboratory of Public Health program for HIV-1 RNA testing of HIV antibody-negative samples. The approximate volume of individual sample serum tested for HCV, and GBV-C, and influenza A with real time PCR is shown in italic type. A single positive sample is represented as a single black circle, and as the sample is pooled with 79 negative samples, the positive pool becomes lighter as it is diluted for real time PCR testing. By the time each master pool is tested, the positive sample's viral titer (in copies/mL sera) has been diluted 13,714-fold (RNA extraction:

140/1600 × 20 μL = 1.75 μL serum/sample; cDNA synthesis = 30/60 × 1.75 μL = 0.875 μL serum/sample; real time PCR = 5/60 × 0.875 μL = 0.073 μL serum/sample; dilution factor 1/0.0723 × 1000 = 13,714). (B) Standard curves generated for HCV (solid line with solid circles), GBV-C (dashed line with open triangles), and influenza A (dotted line with Xs) real time PCR assays. Known quantities of standards were run in separate reactions to generate each standard curve; symbols and lines for each curve may overlap. The equations for each standard curve are as follows: (1) HCV: $Y = 3.42(X) + 38.31$, $r^2 = 0.992$, where X is $\log_{10}$ copies/5 μL; (2) GBV-C: $Y = 3.35(X) + 39.52$, $r^2 = 0.997$; and (3) influenza A: $Y = 3.29(X) + 37.17$, $r^2 = 0.998$.



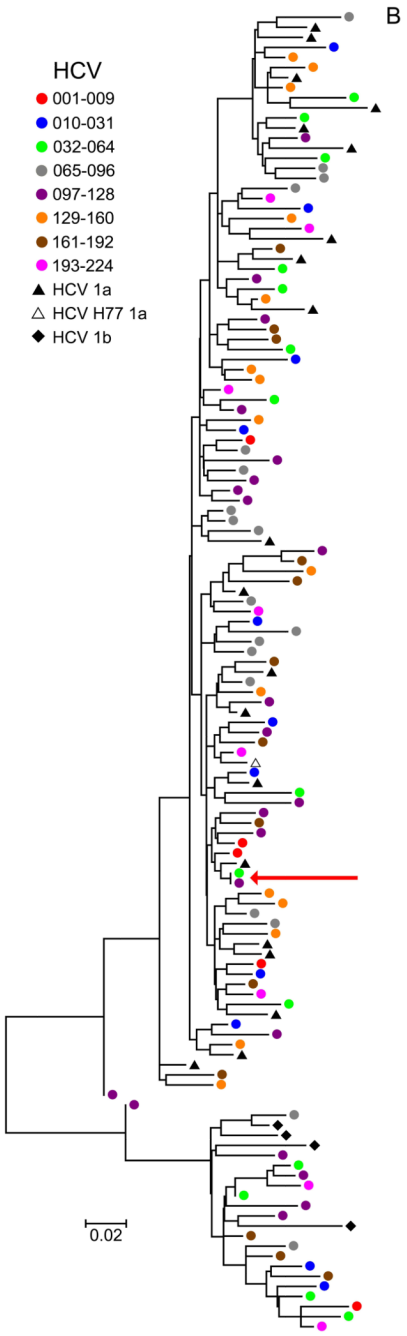


Figure 2.

Phylogenetic comparison of GB virus C (GBV-C) or Hepatitis C Virus (HCV) NS5B sequences from positive sera pools and published isolates (403 and 336 bp, respectively). (A) Neighbor-Joining tree of 21 GBV-C NS5B sequences obtained from GBV-C positive serum pools (color-coded by real-time PCR plate run) and 24 NCBI Nucleotide Database GBV-C NS5B sequences (black triangles, GenBank accession numbers U44402, U45966, U36380, U63715, U75356, AX338086, FB707087, U96117, U96118, U96119, U96120, U96121, U96122, U96124, U96125, U96126, AY196904, CQ972014, U94695, D90600, D90601, D87708, AF104403). The reference strain (AF121950) used to construct GBV-C real time PCR standards is shown as an open black triangle. (B) Neighbor-Joining tree of

106 HCV NS5B sera pool sequences obtained from HCV-positive serum pools (color-coded by real time PCR plate run), 21 HCV 1a Los Alamos (black triangles, GenBank accession no. EU660383, EU155216, EU155339, EU255984, EU155288, EU155299, EU155249, EU155272, EU255939, EU155314, EU256053, EU155237, EU256019, EF407428, EF407433, EF407456, EU781758, EU781766, EU781779, EU781794), and 4 HCV 1b Los Alamos NS5B sequences (black diamonds, GenBank accession no. FJ024277, EF407482, EU255962, GU133617). The cluster of HCV sequences is highlighted with a red arrow. The reference HCV 1a strain (AF009606) used to construct real time PCR standards is represented by an open black triangle.