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Development and validation of a real-time, reverse transcription PCR assay for rapid and low-cost genotyping of hepatitis C virus genotypes 1a, 1b, 2, and 3a

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Hepatitis C virus (HCV) infection affects millions of people and leads to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Treatment regimen selection requires HCV genotype (Gt) and Gt 1 subtype determination. Use of a laboratory developed, reverse transcription (RT)-PCR assay was explored as a low-cost, high-throughput screening approach for the major HCV genotypes and subtypes in North America. A commercial line probe assay (LiPA) was used for comparison. Sequencing and/or an alternative PCR assay were used for discordant analyses. Testing of 155 clinical samples revealed that a paired, duplex real-time RT-PCR assay that targets Gts 1a and 3a in one reaction and Gts 1b and 2 in another had 95% overall sensitivity and individual Gt sensitivity and specificity of 98–100% and 85–98%, respectively. The RT-PCR assay detected mixed HCV Gts in clinical and spiked samples and no false-positive reactions occurred with rare Gts 3b, 4, 5, or 6. Implementation of the RT-PCR assay, with some reflex LiPA testing, would cost only a small portion of the cost of using LiPA alone, and can also save 1.5 h of hands-on time. The use of a laboratory developed RT-PCR assay for HCV genotyping has the potential to reduce cost and labour burdens in high-volume testing settings.

1. Introduction

The burden of hepatitis C virus (HCV) infection is estimated at 80 million people worldwide (Gower et al., 2014). Chronic HCV infection can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. HCV is classified into 7 genotypes and 67 subtypes (Smith et al., 2014). In North America, the majority of HCV infections are caused by genotype (Gt) 1, 2, and 3 (Zein et al., 1996; Nainan et al., 2006; Messina et al., 2015). Although new, highly effective, direct acting antivirals (DAA) against HCV are now available (GSCTP, 2016; Kamal-Yanni, 2015) prescribing the optimal HCV treatment regimen still requires pre-determination of the infecting HCV genotype and Gt 1 subtype (1a *versus* 1b). HCV can be genotyped using various methods including sequencing (the gold standard), real-time reverse transcriptase (RT)-PCR, and reverse hybridization line probe assays (LiPA) (Verbeeck et al., 2008; Larrat et al., 2013; Yang et al., 2014; Nakatani et al., 2010; Avó et al., 2013; Gao, 2012). The HCV 5′ untranslated region (UTR), Core, and polymerase (NS5B) regions are the most common targets of genotyping assays (Larrat et al., 2013; Jacka et al., 2013; Mallory et al., 2014; Hara et al., 2013).

As of December 2013, anti-HCV antibodies have been detected in 73,500 individuals within British Columbia (BC), Canada (Communicable Disease Prevention and Control Services, 2013). The major HCV genotypes identified (61.4% Gt 1, 12.6% Gt 2, 24.3% Gt 3, 1.7% other) mirror those that predominate across North America (Janjua et al., 2016). The BC Ministry of Health approval of HCV DAA coverage in 2015 resulted in a 78% increase in genotype test-

Abbreviations: BCCDC PHL, British Columbia Centre for Disease Control Public Health Laboratory; Ct, cycle threshold; DAA, direct acting antiviral; EDTA, ethylenediamine-tetraacetic acid; Gt, genotype; RT, reverse transcription; HCV, hepatitis C virus; NS, non-structural; PCR, polymerase chain reaction; E1, Envelope-1; LiPA, line probe assay; UTR, untranslated region.

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ing at the BC Centre for Disease Control Public Health Laboratory (BCCDC PHL) (2015/16 fiscal year *versus* 2014/15).

A laboratory developed, real-time RT-PCR genotyping assay was explored as an approach for low-cost, high-throughput screening of major HCV genotypes and subtypes. Various primer/probe combinations targeting the HCV Core, E1, and NS5B genomic regions were tested. A paired, duplex real-time RT-PCR assay that targets HCV Gt 1a and 3a in one reaction and Gt 1b and 2 in another reaction was ultimately validated using samples genotyped by LiPA.

2. Materials and methods

2.1. Clinical samples

Specimens were previously submitted to the BCCDC PHL for HCV RNA quantification and/or genotyping. Nucleic acid extracts from 57 HCV-positive, EDTA-blood samples (Gts 1a (19), 1b (16), 2 (5), 3a (17)) were used for initial primer/probe screening. For validation of the RT-PCR assay, extracts from EDTA-blood ($n = 178$) or serum ($n = 31$) were used (Gts 1a (74), 1b (25), 2 (17), 3a (39), 3b (3), 4 (18), 5 (4), 6 (14), 1 and 2 (5), 1 and 3 (5), 1 and 4 (5)). HCV RNA detection and viral load quantification was previously performed using the pan-genotype COBAS AmpliPrep/COBAS TaqMan^{TM} 48 system (Roche) for 163/209 samples. Where samples used for genotyping were not tested for RNA, another patient sample collected at a similar time confirmed RNA-positive status.

Nucleic acids were extracted using the MagMAX^{TM} Express-96 Deep Well Magnetic Particle Processor (Life Technologies). For EDTA-blood, HCV genotype was determined using the VERSANT HCV Genotype 2.0 assay (LiPA) (Siemens Healthcare). For serum, HCV genotyping was performed by NS5B gene Sanger sequencing (Murphy et al., 2007; Montoya et al., 2015). Procedures were performed according to manufacturer instructions.

2.2. Control samples

Custom-designed gBlock oligomers representing HCV Gt 1a, 1b, 2, and 3a (Integrated DNA Technologies (IDT)) diluted in HCV RNA negative extract were used as positive controls in each RT-PCR run. Additional positive controls included low and high copy number HCV armoured RNA used during extraction and RT-PCR (Asuragen). A negative, no template control was also included in each RT-PCR run.

2.3. Primers and probes

HCV Gt 1a, 1b, 2, and 3a primers and probes were designed using Geneious v.6.1.7 (Biomatters) and purchased from IDT or Life Technologies Inc. Conserved regions were identified using Core, E1, and NS5B sequences from the Los Alamos National Laboratory (LANL) database (Kuiken et al., 2005) and BCCDC (Olmstead et al., 2015). The total number screened were: six primers and three probes for Gt 1a, 12 primers and 13 probes for Gt 1b, 10 primers and six probes for Gt 3a, and two primers and one probe for Gt 2.

2.4. Real-time RT-PCR

Laboratory developed RT-PCR assays were performed on an ABI 7500 FAST enabled real-time PCR system with 1× Fast Virus 1-Step PCR Master Mix (Life Technologies Inc), 1× primer/probe mix (Table 1), and 5 μl nucleic acid. Thermocycling conditions were: 50 °C for 5 min, 95 °C for 20 s, then 40 cycles of 95 °C for 3 s and 60 °C for 30 s.

2.5. Specificity, sensitivity, and precision

For *in silico* specificity of probes, sequences were aligned to HCV sequences from LANL and BCCDC using Geneious 6.1.5 at 100% match. An NCBI BLAST (Madden, 2003) search of probe sequences was performed to identify matches to non-target HCV genotype sequences, defined as <3 nucleotide mismatches between sequences with melting temperature differences of <8 °C.

Overall sensitivity of the RT-PCR assay as well as individual genotype sensitivity and specificity was calculated using 155 single HCV genotype positive samples (Gt 1a, 1b, 2, or 3a). For individual genotype specificity calculations, samples positive for one genotype were used as true negatives of another genotype. Overall specificity could not be calculated as no true HCV-negative samples were tested. For discrepant results between RT-PCR and LiPA, where possible, the Abbott Realti *m*e HCV genotyping assay was used according to manufacturer instructions. The HCV NS5B gene was also sequenced for four discordant samples (Murphy et al., 2007; Montoya et al., 2015).

RT-PCR assay performance was also evaluated using 36 rare HCV genotype samples (Gt 3b (3), Gt 4 (18), Gt 5 (4), Gt 6 (14)), 15 mixed HCV genotype samples (Gt 1 and 2 (5), Gt 1 and 3 (5), Gt 1 and 4 (5)), and HCV RNA-negative EDTA-blood samples spiked with a mix of two gBlocks (Gt 1a and 3a or Gt 1b and 2b) diluted to 100 copies/μl or 10^6 copies/μl in the PCR reaction (run in triplicate).

For precision testing, gBlocks (Gt 1a, 1b, 2b, and 3a) were diluted to 5 copies/μl and 5×10^4 copies/μl in the PCR reaction and tested in six runs over 3 days by two different technologists. The standard deviation and coefficient of variance were calculated using cycle threshold (Ct) values.

2.6. Cost and time requirements

Consumable costs were calculated in Canadian dollars (CAD) based on catalogue pricing at the time of study. Costs assumed runs of 40 samples, as LiPA reagent kits are sold for 40 samples/kit. Consumables for the in-house RT-PCR assay included plastics for PCR (plates and microfilms), primers and probes, water, TE buffer, and Fast virus master mix. Consumables for LiPA included the VERSANT Genotype 2.0 assay detection kit and HCV Amplification 2.0 kit. Labour costs were excluded from calculations because they vary greatly among institutions, while extraction costs were excluded, as they would be equivalent for both methods.

Labour time calculations for the RT-PCR assay were based on hands-on time for reagent preparation, plate set-up, sample loading, materials storage, and result analysis (checking curves and control results). Labour time calculations for LiPA included preparing master mix, plate set-up, preparation of the Auto-LiPA Instrument and tray, sample loading, materials storage, and result analysis (reading of strips by two technologists). Calculations did not include nucleic acid extraction (equivalent for both methods) or events without manual involvement (instrument run times); these, combined with hands-on time, are captured in turn-around times.

2.7. Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated with 95% confidence intervals (CI) using MedCalc for Windows, version 15.0 (MedCalc Software).