Journal of Virological Methods 248 (2017) 107–115

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Immiscible phase filter extraction and equivalent amplification of genotypes 1–6 of hepatitis C RNA: The building blocks for point-of-care diagnosis

Mário F. Neto[a,b], Matthew A. Butzler[a], Jennifer L. Reed[a], Xiang Rui[a], Mark J. Fisher[a], David M. Kelso[a], Sally M. McFall[a,*]

[a] Center for Innovation in Global Health Technologies, Northwestern University, Evanston, IL 60208, USA
[b] University of Illinois Incubator Lab Facility, Chicago, IL 60612, USA

ARTICLE INFO

Keywords:
HCV
Viral load monitoring
RT-qPCR
Sample prep

ABSTRACT

The lack of hepatitis C virus (HCV) diagnostic tests designed for use in decentralized settings is a major obstacle for providing access to treatment and prevention services particularly in low and middle income countries. Here we describe the development and validation of two building blocks of the HCV Quant Assay, a test in development for point-of-care use: 1) an RT-qPCR assay with noncompetitive internal control that equivalently detects the 6 major HCV genotypes and 2) an automated sample prep method using immiscible phase filter technology. This novel assay has wide dynamic range of HCV quantification and a limit of detection of 30 IU/ml with 200 µl specimen volume. In a preliminary study of 61 clinical specimens, the HCV Quant Assay demonstrated 100% sensitivity and specificity and gave comparable viral load results across 4 logs of IU/ml when compared to the Abbott RealTime HCV Assay.

1. Introduction

Globally, approximately 115 million people have serological evidence of current or past hepatitis C virus (HCV) with 80 million of those having active chronic infection as evidenced by detectable viral RNA (Gower et al., 2014). HCV infection is frequently asymptomatic and therefore the majority of those infected are unaware of their illness. Untreated HCV infection causes chronic inflammation and scarring of the liver which in turn leads to chronic liver disease and potentially hepatocellular carcinoma. It is estimated that 703,800 die annually as a result of HCV infection (Mortality, 2015).

Until recently, the treatment regime for HCV infection was complex, could take up to one year, had significant toxicity, and relatively poor efficacy (Cohn et al., 2015). HCV treatment is undergoing a dramatic transformation as new oral direct acting antivirals (DAA) are becoming available with high treatment success rates, treatment durations as short as 8 weeks and more manageable side effects (Gower et al., 2014; Ford et al., 2014; WHO, 2014; WHO, 2016). In 2014, the World Health Organization released its first set of global guidelines for HCV treatment including recommendations for the use of DAAs (Ford et al., 2014; WHO, 2014; WHO, 2016). Experts have predicted that HCV infection can be eliminated by using screen and treat strategies combined with transmission prevention services (Martin et al., 2013; Wedemeyer et al., 2014). Recently, it was reported that after one year of unrestricted DAA availability in the Netherlands, the incidence of new HCV infection in a high risk of infection population, HIV positive men who have sex with men (MSM), decreased by 52% demonstrating that treatment as prevention may indeed avert new infections (Boerekamps et al., 2017).

Generic forms of DAAs have been made available in some low and middle income countries (LMICs) reducing the cost per patient and leading dramatically to a need for the scale-up of treatment services. To bridge the gap from undiagnosed HCV infections to treatment and cure, affordable, point of care (POC) diagnostic tests are urgently required (Trianni et al., 2015). Currently, estimates show that less than 1% of the global population is aware of their status (Gower et al., 2014). Serological tests are frequently used in POC settings, but their impact is limited because they cannot be used to detect newly acquired infections as antibodies may not be detected for 2–3 months nor can they be used to distinguish patients with active infections from those that have spontaneously cleared the infection (Easterbrook et al., 2017).

Nucleic acid tests, however, can detect HCV in the acute phase of infection and can also be used in monitoring the effectiveness of antiviral therapy (Busch and Shafer, 2005). Until recently, HCV viral load monitoring has been confined to central laboratories, but recent reports

Abbreviations: HCV, hepatitis C virus; GT, genotype; IPF, immiscible phase filter; KF, KingFisher; LOD, limit of detection; IC, internal control; NA, nucleic acid

* Corresponding author at: Center for Innovation in Global Health Technologies, Biomedical Engineering Department, Northwestern University, Tech E-310, 2145 Sheridan Rd., Evanston, Illinois, 60208, USA.
E-mail address: s-mcfall@northwestern.edu (S.M. McFall).

http://dx.doi.org/10.1016/j.jviromet.2017.06.016
Received 21 April 2017; Received in revised form 28 June 2017; Accepted 29 June 2017
Available online 30 June 2017

describe potential for the use of dried blood spots to store and transport specimens without refrigeration to central laboratories for testing (Greenman et al., 2015). We propose to develop a platform for POC settings where patients present for diagnosis and treatment eliminating the need for sample transfer and the associated delay in delivering results to patients allowing for immediate treatment decisions thus reducing patient loss to follow-up (Trianni et al., 2015).

Detection of HCV is performed by isolating viral RNA from a plasma sample and performing reverse transcription quantitative polymerase chain reaction (RT-qPCR). HCV, like many other RNA viruses, is genetically variable due to its error-prone RNA polymerase; strains are classified into 6 major genotypes which share 70–80% nucleotide identity with numerous subtypes (Kuiken and Simmonds, 2009). In an infected individual, a population of closely related HCV variants with less than 10% variance at the nucleotide level can be detected (Simmonds, 2004). If such polymorphisms occur in the primer and/or probe sequences the nucleotide binding affinity may be reduced resulting in decreased analytical sensitivity and accuracy. Therefore, the RT-qPCR assay conditions must be designed to tolerate the viral genetic diversity (Cloherty et al., 2014).

A second impediment to HCV testing at the POC is the complex and labor-intensive sample prep required for nucleic acid testing (Dineva et al., 2007). Genetic targets present in plasma or serum must be concentrated and separated from contaminants that may interfere with qPCR. The isolation of nucleic acids via a solid phase coated with silica was first introduced by Boom (Boom et al., 1991), and this method has evolved over the years into the use of silica coated paramagnetic particles (PMPs) incorporated into many commercial nucleic acid extraction systems (Troiano et al., 2017). In this method, clinical specimens are exposed to chaotrope and alcohol to drive nucleic acids onto the silica coating of PMPs. Many commercial nucleic acid extraction systems process samples in a single well by repeatedly pelleting PMPs, aspirating the liquid, and adding the next solution. Numerous wash steps are often required to remove cell debris and other amplification inhibitors that adhere to tube surfaces, become entrapped in the magnetically-aggregated PMPs, or remain in the residual volume after the supernatant is removed by aspiration (Sur et al., 2010).

Recently, our lab (Sur et al., 2010; Kelso et al., 2012) and others (Beebe and Barry, 2013; Beebe et al., 2014; Berry et al., 2011; Berry et al., 2012; den Dulk et al., 2013; Uehara et al., 2016) have shown that instead of pipetting liquids in and out of a tube that contains the PMPs, there is an advantage to using stationary microfluidics whereby different buffers needed for extraction are located in fixed positions, and an external magnet transfers the PMPs between the fluids through an immiscible phase filter (IPF) made up of a layer of oil or liquid wax that is immiscible with both aqueous solutions. In addition to eliminating the need for fluid pumping or pipetting, moving PMPs instead of fluids simplifies the instrumentation and consumable test cartridge required for nucleic acid extraction. This concept of “stationary microfluidics” (den Dulk et al., 2013) allows for the development of an integrated system that automatically performs cell lysis and purification of nucleic acids and subsequent qPCR amplification in a closed cartridge.

In this report, we describe the development of our HCV Quant Assay that combines IPF HCV sample prep with an RT-qPCR assay that equivalently quantifies genotypes 1–6. HCV Quant Assay achieved a similar limit of detection and precision to commercial tests used in reference laboratories. The clinical performance of our HCV Quant Assay was verified by testing 61 clinical specimens yielding 100% sensitivity and 100% specificity, and good correlation ($r^2 = 0.91$) was observed between our HCV Quant Assay and the Abbott RealTime HCV Assay. The development of a simple and cost effective POC HCV viral load test would allow HCV treatment and monitoring to be extended into remote areas where advanced laboratory infrastructure is not available.

2. Materials and methods

2.1. Viral strains and sample sources

HCV- and HIV-negative plasma was obtained from ProMedDx (Norton, MA). HCV nucleic acid-positive, antibody-negative samples with known viral load and genotype were requested from the American Red Cross (Gaithersburg, MD). Genotype 1b sample (024GJ66260) was used as a reference for some of the following studies. The HCV Worldwide AccuSet™ Performance Panel was purchased from SeraCare Life Sciences (Milford, MA). Forty plasma samples were obtained from the A5294/BIRTH trial [A Prospective, Phase III, Open-Label Study of Boceprevir, Pegylated-Interferon Alfa 2b, and Ribavirin in HCV/HIV Coinfected Subjects of the AIDS Clinical Trials Group (ACTG)]. We requested 10 samples with viral loads (VL) between 100 and 1000; 10 with VL between 1000 and 10,000; 10 with VL between 10,000 and 100,000 and 10 with VL between 100,000 and 1,000,000. Twenty-one negative controls (blood plasma from HCV negative individuals) were acquired from Evanston Hospital.

2.2. Cloning of amplicon sequences and RNA transcript preparation

HCV genotype variation was analyzed by aligning 80 genome-representative sequences from the Los Alamos Hepatitis C Database (Kuiken et al., 2005) The HCV Sequence Alignments Genotype Reference tool (https://hcv.lanl.gov/content/sequence/NEWALIGN/align.html) was used to create an alignment of the highly conserved 5′UTR genomic region of approximately 4 representative DNA sequences from each genomic clade. From the alignment, 4 sequences were selected: Genbank sequence NC_004102.1 representing genotypes (GTs) 1, 5, and 6; D10077.1 representing GT 2; D28917.1 representing GT 3; and DQ295833.1 representing GT 4. The first 400 nucleotides of reference genotype NC_004102.1 were cloned into pIDTBlue plasmid (IDT; Corvallis, IA), with genotype-specific modifications in the target amplicon region only, nucleotides 60–180. An additional sequence representing GT 6, D37841.1, was later cloned following the same procedure in order to test a polymorphism in the qPCR probe-binding region.

RNA transcripts were synthesized from the pIDTBlue plasmids using the MEGAshortscript kit (Applied Biosystems; Foster City, CA). Briefly, linearized plasmid DNA containing sequences described above were mixed with 10 x reaction buffer, enzyme mix, and NTPs following the kit instructions and incubated at 37 °C for 3 h. Then, 1 µl of TURBO DNase from the kit was added to each reaction and incubated at 37 °C for 20 min to degrade parental DNA. The ~470 nucleotide transcripts were isolated via gel extraction. Samples (diluted 1:2 in 2 x TBE-Urea Sample Buffer) were run in a 10% Novex TBE-Urea Gel (Thermo Fisher Scientific; Waltham, MA) for 2 h at 180 V. After staining the gel with SYBR Green II Gel Stain (Thermo Fisher Scientific) for 30 min with gentle agitation, the gel was visualized with UV light and bands of the expected size were excised and placed in RNA gel elution buffer (20 mM Tris-HCL pH 7.5, 0.25 M sodium acetate, 1 mM EDTA pH 8, 0.25% SDS) (Nilsen, 2013). The tubes were frozen in dry ice for 15 min and then left at room temperature overnight to allow for the RNA to diffuse from the gel slice. The following day, tubes were centrifuged for 10 min at maximum speed in a tabletop centrifuge, and the supernatant was placed in a clean microcentrifuge tube. RNA was extracted with 1 vol of 1:10 phenol:chloroform and precipitated with 2 vols 100% ethanol. Finally, RNA was resuspended in 10 mM Tris pH 7.5 and quantified using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Isolation of a single product was confirmed by gel electrophoresis.

2.3. RT-qPCR primer and probe selection and PCR conditions

Primers were selected in the region of the 5′UTR with the least