Journal of Clinical Virology 76 (2016) 20–23

Contents lists available at ScienceDirect

Journal of Clinical Virology

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

Short communication

Evaluation of a commercial real-time PCR kit for the detection of the Q80K polymorphism in plasma from HCV genotype 1a infected patients

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form
18 December 2015
Accepted 8 January 2016

Keywords:
Drug resistance assay
Natural polymorphisms
HCV
Q80K
Simeprevir

ABSTRACT

Background: Screening of the natural HCV NS3 polymorphism Q80K is required prior to simeprevir administration due to the reduced susceptibility of genotype 1 viruses carrying this amino acid variant. A simple, rapid and robust test for Q80K screening would be advisable in routine diagnostic laboratories.
Objectives: The aim of this study was to evaluate a commercial NS3 Q80K real-time PCR kit (Q80K Polymorphism Kit, Clonit srl, Milan, Italy).
Study design: Forty-three plasma samples obtained from untreated HCV genotype 1a-infected patients and previously sequenced at a reference laboratory, were sent to two public clinical virology laboratories for blinded Q80K screening with the kit under evaluation. The sample panel included 25 cases with the wild type 80Q, 17 with the mutant 80K and 1 with the mutant 80L.
Results: Laboratory 1 identified 22/25 (88.0%) 80Q and 17/17 (100.0%) 80K cases. Laboratory 2 identified 23/25 (92.0%) 80Q and 16/17 (94.2%) 80K cases. All of the unidentified cases were scored as negative, with no mutant/wild type miscalling. The 80L variant was scored as indeterminate by Laboratory 1 and as negative by Laboratory 2. Overall, sensitivity and specificity for detection of 80K were 97.1% (95% C.I., 82.9–99.8%) and 100.0% (90.2–100.0%), respectively. However, the system did not provide any result for 6/84 cases (7.1% failure rate), not including the 80L variant which is not expected to be detected as stated in the kit package insert. Global inter-laboratory concordance was 93.0%.
Conclusions: Despite good specificity, this Q80K detection system needs improvements in amplification success rate and robustness.

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1. Background

Several direct-acting agents targeting hepatitis C virus (HCV) NS3, NS5A or NS5B proteins are currently available [1]. Among NS3 protease inhibitors, simeprevir is recommended for treatment of HCV genotype (GT) 1a, 1b and 4 in combination with pegylated interferon and ribavirin (PR) or with the NS5B polymerase inhibitor sofosbuvir [2,3]. The NS3 Q80K variant shows decreased susceptibility to simeprevir in vitro compared with the wild type 80Q [4]. Phase III trials of simeprevir/PR in HCV GT1 and GT4 infected patients showed sustained virologic response (SVR) rates of approximately 80% [5–8] but SVR rates were reduced in HCV GT1 patients with Q80K compared with HCV GT1 patients without this polymorphism [5,7,9,10]. The Q80K polymorphism is mainly associated with HCV GT1a [10], prevalence values being close to zero in HCV GT1b with the only exception of France where 11% of GT1b have Q80K [11,12]. In addition, two distinct clades of GT1a (clade 1 and clade 2) have been identified by phylogenetic analysis of full-length HCV genomes [13] or distinct genomic regions [11,14–16]. The Q80K polymorphism is strongly associated with clade 1 [17], however the two clades cannot be distinguished by commonly used rapid HCV genotyping assays. On the basis of these data, European and US guidelines [2,3] recommend the Q80K screening in HCV GT1a patients candidate to simeprevir administration. The gold standard for detection of Q80K is sequence analysis of the

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http://dx.doi.org/10.1016/j.jcv.2016.01.006
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NS3 region [18], however easier yet accurate systems should be advisable in clinical practice.

2. Objective

The aim of this study was to evaluate a recently developed commercial kit (Q80K Polymorphism Kit, Clonit srl, Milan, Italy; http://www.clonit.it/repofiles/160-RT-14%20_ENG__rev1.pdf), currently labelled as Research Use Only and based on a qualitative real time PCR allowing allelic discrimination of the NS3 Q80K polymorphism in plasma and serum from HCV positive samples.

3. Study design

The Department of Medical Biotechnology of the University of Siena, Italy has been providing HCV NS3 Sanger sequencing since 2012, for clinical and research use [14]. Plasma samples were retrospectively obtained from patients followed at different clinics in central Italy and included in the Antiviral Response Cohort Analysis (ARCA), an observational study approved by the South-East Tuscany Ethical Committee, following written informed consent allowing use of residual specimens derived from clinical practice. A panel of 43 plasma samples was collected including 25 cases with wild type 80Q (12 with CAA and 13 with CAG codon), 17 cases with mutant 80K (15 with AAA and 2 with AAG codon) and one case with the rare 80L variant (CTG codon). All the chromatograms had only one peak at each position of codon 80, indicating a pure population within the limits of Sanger sequencing. Two 750-μL aliquots of each sample were sent in dry ice to two public clinical virology laboratories (Laboratory 1 and 2) for blind HCV NS3 Q80K screening by the system under evaluation. Both laboratories have long-standing experience with molecular diagnostics and have been using real time PCR for years. Data analysis included sensitivity and specificity for Q80K detection, percentage of cases without result (i.e. neither wild type 80Q nor mutant 80K) and inter-laboratory reproducibility as the percentage of concordant cases, whether correct or not.

Both laboratories extracted plasma RNA from the whole 750-μL aliquot of plasma and performed amplification and analysis by using the VERSANT Sample Preparation 1.0 assay and the VERSANT kPCR Amplification/Detection Module, respectively (Siemens Healthcare Diagnostics, Milan, Italy). The Q80K Polymorphism Kit (Clonit srl, Milan, Italy) is a qualitative real time PCR assay for allelic discrimination of the 80Q and 80K variants based on differently labelled probes (FAM and HEX, respectively). Other polymorphisms such as 80R or 80L are not detected and no amplification signal is expected in this case.

Table 1
Composition of the sample panel and results obtained at the two laboratories. Nucleotides at NS3 codon 80 were identified by Sanger sequencing. Discordant and negative or indeterminate results are in bold.

Sample code	Nucleotides at codon 80	Amino acid at codon 80	HCV RNA (IU/mL)	Laboratory 1 result	Laboratory 2 result
1	CAA	Q	15268	**Negative**	**Wild type Q**
2	CAA	Q	NA	Wild type Q	Wild type Q
3	CAA	Q	NA	Wild type Q	Wild type Q
4	CAA	Q	NA	Wild type Q	Wild type Q
5	CAG	Q	NA	Wild type Q	Wild type Q
6	CAA	Q	1681555	Wild type Q	Wild type Q
7	CAA	Q	NA	Wild type Q	Wild type Q
8	CAA	Q	NA	Wild type Q	Wild type Q
9	CAA	Q	1048616	Wild type Q	Wild type Q
10	CAA	Q	165605	Wild type Q	Wild type Q
11	CAG	Q	NA	Wild type Q	Wild type Q
12	CAA	Q	2321500	Wild type Q	Wild type Q
13	CAA	Q	6760000	Wild type Q	Wild type Q
14	CAA	Q	8410000	Wild type Q	Wild type Q
15	CAG	Q	9600000	**Negative**	**Negative**
16	CAG	Q	NA	Wild type Q	Wild type Q
17	CAG	Q	NA	Wild type Q	Wild type Q
18	CAG	Q	NA	Wild type Q	Wild type Q
19	CAG	Q	NA	Wild type Q	Wild type Q
20	CAG	Q	NA	Wild type Q	Wild type Q
21	CAG	Q	11300000	**Negative**	**Negative**
22	CAG	Q	1130000	Wild type Q	Wild type Q
23	CAG	Q	23200000	Wild type Q	Wild type Q
24	CAG	Q	4451882	Wild type Q	Wild type Q
25	CAG	Q	1090498	Wild type Q	Wild type Q
26	AAG	K	164000	Mutant K	Mutant K
27	AAA	K	4070	**Mutant K**	**Negative**
28	AAA	K	12000000	Mutant K	Mutant K
29	AAA	K	NA	Mutant K	Mutant K
30	AAA	K	NA	Mutant K	Mutant K
31	AAA	K	NA	Mutant K	Mutant K
32	AAA	K	NA	Mutant K	Mutant K
33	AAA	K	NA	Mutant K	Mutant K
34	AAA	K	NA	Mutant K	Mutant K
35	AAA	K	NA	Mutant K	Mutant K
36	AAA	K	14000000	Mutant K	Mutant K
37	AAA	K	NA	Mutant K	Mutant K
38	AAA	K	10912	Mutant K	Mutant K
39	AAA	K	NA	Mutant K	Mutant K
40	AAA	K	NA	Mutant K	Mutant K
41	AAA	K	NA	Mutant K	Mutant K
42	AAG	K	26000	Mutant K	Mutant K
43	CTG	L	1140000	**Indeterminate**	**Negative**

NA, Not available.

4. Results

Table 1 shows the complete results for the individual samples. HCV viremia was unknown in 23/43 (53.5%) samples, however all the patients were naïve for anti-HCV therapy and all the same samples were suitable for HCV NS3 sequencing at the reference laboratory. Laboratory 1 identified 22/25 (88.0%) 80Q and 17/17 (100.0%) 80K cases. Laboratory 2 identified 23/25 (92.0%) 80Q and 16/17 (94.2%) 80K cases. Failure to detect the wild type 80Q occurred with both the CAA and the CAG codon while the only failure to detect the mutant 80K occurred with the AAA codon which was predominant in this mutant panel. The 6 (7.1%) cases where the correct variant was not detected were not associated with low viremia and scored all as negative, i.e. neither 80Q nor 80K positive, thus there was no mutant/wild type miscalling. Also, there were no cases where both 80Q and 80K were detected. The only 80L variant present in the sample panel was scored as indeterminate by Laboratory 1 (80Q amplification signal crossing the threshold but flat slope) and as negative by Laboratory 2. Cumulative sensitivity and specificity for detection of 80K were 97.1% (95% C.I., 82.9–99.8%) and 100.0% (90.2–100.0%), respectively, not including the 80L case which is not expected to be detected as per manufacturer indications. Global inter-laboratory concordance was 93.0% when considering all paired data independently from accuracy. Of note, excluding the cases with negative result would translate into a 100% sensitivity, specificity and reproducibility.

5. Conclusions

The recommendation to screen for Q80K prior to simeprevir administration has made it desirable to develop simple, rapid and cost-effective assays for Q80K detection. In principle, detecting the C to A transversion (from CAA or CAG to AAA or AAG) can be accomplished by a real time PCR using discriminating primers or probes in a variety of formats. However, the variability of the Q80K surrounding region and the intrinsic variability of codon 80 (Q80K, Q80R and Q80L variants) make this task not straightforward. In this study, we have analyzed the performance and inter-laboratory reproducibility of a newly developed commercial real time PCR kit, based on discriminating probes. While there were no cases of incorrect calling for the 80Q and 80K samples, the amplification failure rate was not satisfactory because 2 samples were negative (no amplification signal for either 80Q or 80K) in both laboratories and another 2 samples were negative in one or the other laboratory. In addition, the only 80L case included in the panel scored negative in one laboratory, as expected according to the kit package insert, but was labelled as indeterminate in the other laboratory. However, confirming the absence of incorrect base calls in a larger sample panel would suggest safe use of simeprevir when this system scores the natural 80Q variant.

Inter-laboratory reproducibility was good but not excellent. While amplification failures were equally distributed in the two laboratories, there were 3 cases where the results generated at the two sites were in disagreement (93.0% concordance), despite the perfectly matched use of the same plasma RNA extraction and real time PCR platforms. This suggests that the assay robustness should be improved. However, it is reassuring to note that there were no cases with complete disagreement, i.e. 80K at one laboratory and 80Q at the other. In addition, it is important to note that amplification failures could theoretically have derived from issues during RNA extraction rather than during PCR. However, RNA extraction was fully automated with the same long used system at both laboratories making this hypothesis unlikely.

A major limitation of this study is the small number of samples tested, as reflected by quite large sensitivity and specificity confidence intervals. This was mainly due to the retrospective nature of the sample collection and the need for relatively large plasma volumes to be sent to the the two laboratories running the Q80K real time PCR assay. For example, availability of a larger sample panel could have allowed inclusion of the rarely occurring 80Q/K mixtures and simeprevir resistant 80R variant [19,20]. A further limitation of the clinical sample panel is the possibility that minority species at NS3 codon 80 were present which were not detected by Sanger sequencing but could have been detected by the real time PCR assay. However, there were no cases of simultaneous detection of both the 80Q and 80K variant by PCR as it would have been expected in case of mixtures. Indeed, next generation sequencing studies have shown that minority species at NS3 codon 80 are uncommon in vivo [20].

While a rapid and practical Q80K detection assay is desirable, HCV NS3 sequencing remains the gold standard method. An inherent information generated by NS3 sequencing is the nucleotide composition of the region surrounding codon 80. This can be useful for designing primers and probes for rapid assays which are less affected by nucleotide variability, preventing the likely cause for the amplification failures shown in this study. Moreover, sequence analysis provides a complete description of other mutations impacting susceptibility to simeprevir itself and to other licensed NS3 protease inhibitors, possibly contributing to treatment decisions.

Conflict of interest

None.

Funding

None.

Ethical approval

The study was conducted in compliance with the permission to use residual samples derived from clinical practice based on informed consent from patients included in the Antiviral Response Cohort Analysis (ARCA), an observational cohort study approved by the South-East Tuscany Ethical Committee.

Acknowledgments

We wish to thank Clonit and Siemens for providing reagents for this study.

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