UK NEQAS
International Quality Expertise

How do we investigate an EQA failure?
Lessons learnt from a Hepatitis C RNA detection distribution

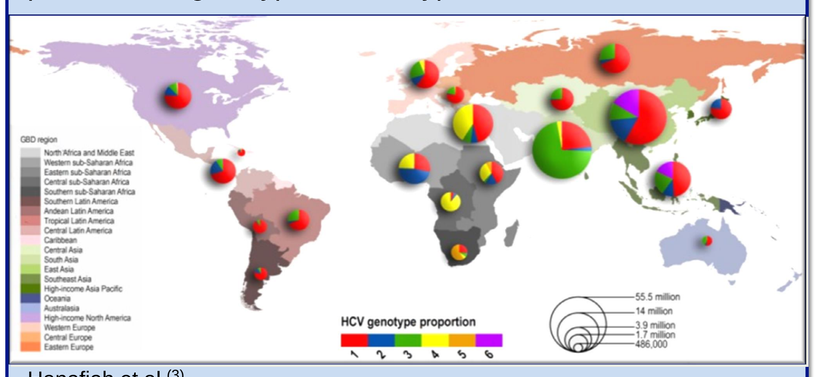
B. Kele[1], P. Jovanovic[2], E. Elenoglou[1], H. Seyedzadeh[1], S. Whitmore[1] and S. Rughooputh[1]
[1] **UK NEQAS** for Microbiology, Public Health England, 61 Colindale Avenue, London, NW9 5EQ, UNITED KINGDOM
[2] Blood Transfusion Centre of Slovenia, Slajmerjeva 6, Ljubljana, SLOVENIA

Background

In 2016, the World Health Organisation (WHO) through the Global Health Sector Strategy (GHSS) called for elimination of viral hepatitis as a major public health threat by 2030 (i.e. 90% reduction in incidence and 65% in mortality). The World Health Organization estimates that 71 million people worldwide are chronically infected with hepatitis C(1).

NHS England's ambition is to eliminate hepatitis C by 2025 at the latest, five years before the World Health Organisation target. Crucially, hepatitis C is preventable, treatable and curable for the vast majority of people. Since 2015, treatments with short durations, limited side-effects and cure rates upwards of 95% have been widely available.

HCV genotype 1 is the most prevalent worldwide, comprising 83.4 million cases. Genotype 3 is the most prevalent globally; genotypes 2, 4 and 6 are responsible for a total of 22.8% of all cases and genotype 5 comprises the remaining <1%. At present, the duration of treatment, cure rates and the need for adjuvant interferon and ribavirin with the new direct-acting antiviral (DAAs) therapies remain dependent in part on HCV genotype and subtype(2).



Hanafiah et al (3)

Introduction

The United Kingdom National External Quality Assessment Service (UK NEQAS) for Microbiology provides external quality assessment (EQA) for Hepatitis C RNA detection and quantification. Three distributions are dispatched in each year containing two specimens in each distribution. The scoring is based separately on the relevant markers reported.

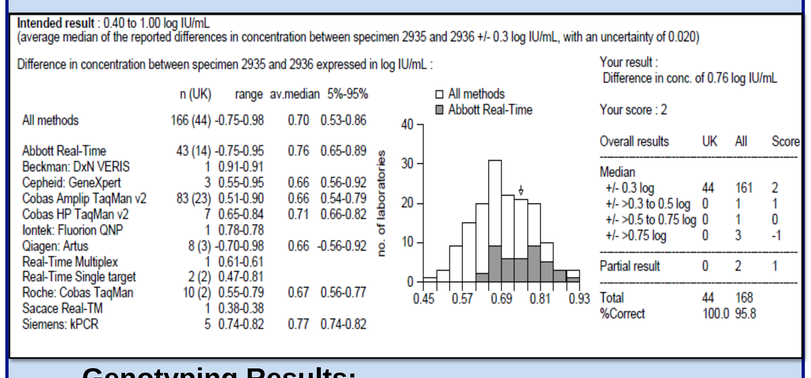
- **Qualitative Detection**: scoring is based on detection/non-detection of HCV RNA in the specimen
- **Quantitative Detection**: scoring is based on the log difference in viral load between the specimen pair
- **Genotyping**: scoring is based on the type and subtype reported

Materials and Methods

In January 2016, a freeze-dried human plasma specimens pair was dispatched with a request to report on HCV RNA qualitative detection, quantification and genotyping. Specimens **2935** and **2936** consisted of a single donation of HCV RNA positive plasma (genotype **1a**) diluted 1:1.8 and 1:12 respectively in human plasma negative for HCV RNA.

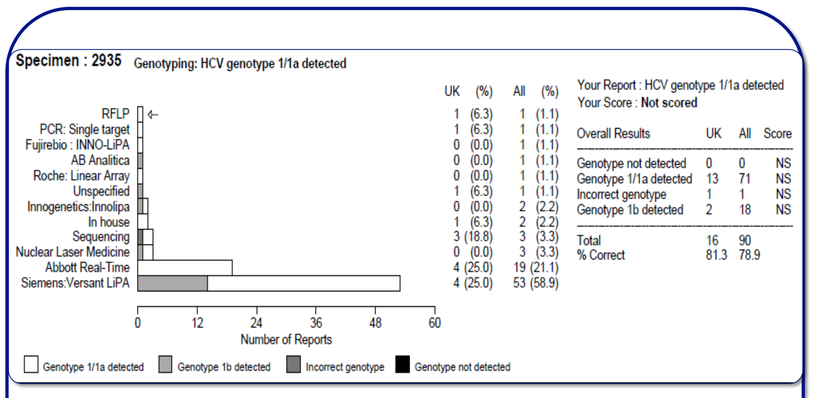
Results

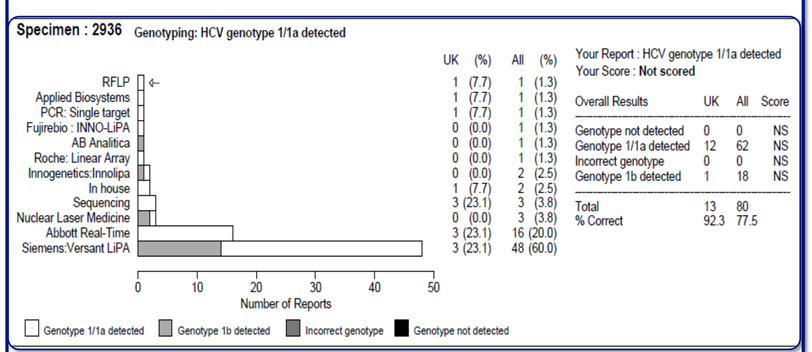
- Qualitative detection: a correct result was reported by 100% and 98.4% of participants
- Quantitative results: The overall performance for this specimen pair was good with 95.8% of participants reporting difference in concentration to within 0.3log IU/mL.



Genotyping Results:

- ✓ Genotype 1/1a was reported by 71 participants (78.9%) and genotype 1b was reported by 18 participants (20%) for specimen 2935.
- ✓ Genotype 1/1a was reported by 62 participants (77.5%) and genotype 1b was reported by 18 participants (22.5%) for specimen 2936.





Out of the 90 participants reporting genotype results, 51 tested the specimens with the Siemens LiPA assay. Out of the 51 users, 37 (73%) reported correct type and subtype results for the specimen pair. The second most popular assay was the Abbott: Real Time test. 19 participants tested the specimen pair with this assay and 100% of them reported correct results for the specimen pair. Test results by assay are shown in the table below

Assay name	Number of participants reported genotype 1a result	Number of participants reported genotype 1b result	Number of participants reported different genotype result for the sample pair	Number of participants
Siemens: Versant LiPA	37 (73%)	12 (24%)	2 (3%)	51
Abbott Real-Time	19 (100%)	0	0	19
Nuclear Laser Medicine	1 (33%)	1 (33%)	1 (33%)	3
Fujirebio: INNO-LiPA	2 (67%)	1 (33%)	0	3
In house	2 (genotype 1) (100%)	0	0	2
Sequencing	2 (67%)	0	1 (33%)	3
Roche: Linear Array	1 (genotype 1) (50%)	1 (50%)	0	2
AB Analitica	0	1 (100%)	0	1
Applied Biosystems	1 (100%)	0	0	1
PCR: Single target	1 (genotype 1) (100%)	0	0	1
RFLP	0	1 (100%)	0	1
Sacace Real-TM	1 (100%)	0	0	1
Unspecified	0	1 (100%)	0	1
Versant HCV	0	1 (100%)	0	1
Σ	67 (74%)	19 (21%)	4 (5%)	90

The original, neat donation was tested with PCR and genotyped in 2003 with RFLP and was quantified 8180 IU/mL with a genotype 1b.

What actions were taken by UK NEQAS?

- Laboratory that performed the RFLP typing was contacted to confirm the typing results
- Original donation has been sent away for sequencing
- Siemens was contacted and informed about the issue
- Siemens LiPA assay users were asked to provide their genotyping results (scanned version of line assay and interpretation).

Outcomes

- Whole genome sequencing using PCR-based method which amplifies the HCV genome in 6 overlapping regions
- Genotype specific primers for Gt1a and 1b have been used and both sets have been used independently on the sample
- Positive result for 5'UTR-E1 and NS34A fragments using Gt1a primers and NS5A fragment using Gt1b primers.
- Genome fragments were generated for
- ✓ 2 out 6 amplicons using the genotype 1a-specific WGS assay, these being 5'UTR-E1 and NS34A fragments
- ✓ 1 out of 6 amplicons using the genotype 1b-specific WGS assay, this being the NS5A fragment

Regions covered by overlapping amplicons for PCR-based HCV WGS assay for genotype 1a and 1b

Regions covered by amplicons (nucleotide positions relative to reference strain H77)					
5'UTR-E1	E2-NS2	NS34A	NS4B	NS5A	NS5B
43-1612[a]	1429-3532[b]	3298-5712	5167-6333	6217-7807	7419-9372
149-1612	1425-3641	3279-5529	5396-6422	6133-7794	7637-9424

[a] green shading indicates amplicon for which positive amplification result was generated
[b] red shading indicates amplicon for which negative amplification result was generated

- 58% of the HCV genome covered (5,629/9,626 bp relative to HCV reference strain H77).
- A nucleotide BLAST analysis using the NCBI database showed that the amplicons were closely related to genotype 1a sequences with the top 100 hits having a 93-95% similarity.
- Phylogenetic reconstruction using an alignment containing the concatenated sequence together with closely related sequences and genotype-specific reference sequences clearly showed that the subject sequence formed a monophyletic cluster with genotype 1a sequences with a maximum bootstrap support value of 100%.
- Out of 51 Siemens LiPA user participants 16 provided genotyping results for specimen 2935 and 2936. Reported results are shown in the table below.

	Positive bands	Genotype
1.	3, 4, 6	1b
2.	3, 4, 6, 23, 25	1a *
3.	3, 4, 6, 23, 25	1a
4.	3, 4, 6, 23, 25	1a
5.	3, 4, 6, 23, 25	1a
6.	4, 7, 24	
7.	3, 4, 6, 23, 25	1a
8.	3, 4, 6, 23, 25	1a
9.	3, 4, 6, 23, 25	1a
10.	3, 4, 6, 23, 25	1a
11.	3, 4, 6, 23, 25	1a
12.	3, 4, 6, 23, 25	1a
13.	3, 4, 6, 23, 25	1a
14.	3, 4, 6, 23, 25	1a
15.	3, 4, 6, 23, 25	1a
16.	3, 4, 6, 23, 25	1a

*** Based on the result of the bands in the 5' UTR genotype would be 1b but based on the core region genotype is 1a. The differentiation between subtypes 1a and 1b is best characterised by the core region according to Siemens specifications**

- Those Siemens LiPA users, that typed and subtyped the specimens based on both NS5' and core region reported genotype 1 subtype a
- Those Simens LiPA users that reported a 1b result have been analysed the NS5' region only

Conclusions

- Specimens 2935 and 2936 were genotype 1 subtype a
- Current methods for HCV genotyping can still result in mis-assigment of genotype or subtype of infecting HCV
- 5' UTR sequences from genotypes 1 and 6 are closely related and therefore methods relying on this region are more liable to mis-assign the genotype or subtype
- WGS should be used if possible for genotyping and subtyping HCV as this provides a more refined genotyping/subtyping assignment
- Manufacturer's instructions must be followed

Summary

As an EQA provider we always have our participants' interests in mind by ensuring they work and deliver to very high standards, therefore we occasionally provide challenging and educational samples so our participants can reflect and learn, as well as help in evaluating their testing methodologies.

Acknowledgements

We would like to thank to Tamyo Mbisa, Richard Tedder and Maria Zambon (Public Health England-National Infection Services, Virus Reference Department) for their help in sequencing the specimens and for providing us scientific advise regarding this specimen pair.

References

1. WHO:(http://www.who.int/news-room/fact-sheets/detail/hepatitis-c)

2. Global distribution and prevalence of hepatitis C virus genotypes. *Messina JP* et al. **Hepatology**, 2015 Jan; 61(1):77-87

3. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hanafiah* et al. **Hepatology,** 2013; 57:1333-1342