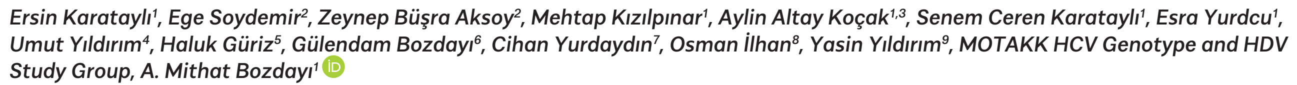
Evaluation of the results of MOTAKK hepatitis C virus RNA genotyping and hepatitis delta virus external quality assessment programs during 2015-2016



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ABSTRACT

Background/Aims: *To evaluate the HCV RNA genotyping and HDV RNA tests that are performed in molecular microbiology laboratories in Turkey as part of a national external quality assessment programme, MOTAKK (Moleküler Tanıda Kalite Kontrol) (English translation: Quality control in molecular diagnostics).*

Materials and Methods: *Plasmas having different HCV RNA genotypes were used to prepare HCV genotype control sera. The HDV RNA main stock was prepared from patients with chronic delta hepatitis who had a significant amount of viral load detected, as per the WHO reference materials on viral load studies that were compiled for the purpose of developing HDV RNA control sera. Samples with different viral loads were prepared from this main stock by dilution. The prepared controls were delivered to the registered laboratories. The laboratories carried out the relevant tests and entered their results via the MOTAKK web page. External quality assessment (EQA) reports of the participants were uploaded to the website as well.*

Results: *In total, there were 23 participating laboratories, out of which 20 exclusively performed HCV genotyping, and 15 and 16 only performed HDV RNA in 2015 and 2016, respectively. The success rate of the results of the HCV genotype was 56-96% in 2015 and 30-95% in 2016. The tube with a 30% success rate had a recombinant type of HCV, therefore, it could not be detected in most of the laboratories. The HDV RNA results were evaluated qualitatively. Accordingly, HDV RNA detection rates of participant laboratories were 71-100% in 2015 and 50-100% in 2016.*

Conclusion: *This study was the first national external quality control program in Turkey regarding HCV RNA genotyping and HDV RNA in the field of molecular microbiology, and it was implemented successfully.*

Keywords: *Hepatitis C virus, genotype, hepatitis delta virus, external quality assessment, quantification*

INTRODUCTION

Hepatitis C virus (HCV) infection affects approximately 71 million people (1%) in the world, according to a statistic recorded by the World Health Organization (WHO) (1). About 700,000 people die annually because of HCV-related diseases such as cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (2). After the use of various treatments for 25 years after the discovery of HCV, recently, new compounds with direct antiviral activity have been introduced in the medical market (3). These therapies have yielded over 95% cure rates in most patient populations (4).

According to various phylogenetic analyses, HCV has extensive genetic heterogeneity which has 7 major genotypes and 67 subtypes. Genotypes 1 and 3 are the most prevalent, comprising 46% and 30% of all infections, respectively (5). In Turkey, genotype 1b is the most prevalent genotype (6). Previous studies reported from different regions of Turkey showed that genotype

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1b (87.2%-97.4%) was the most prevalent subtype in Turkey, followed by genotype 1a (2.6%-11%), genotype 2 (0.9%-3%), genotype 3 (0.6%-2.7%), and genotype 4 (0.6%-1%) (7). Detecting the HCV genotype (including genotype 1 subtype 1a or 1b) is necessary to regulate the treatment and its duration (4). Recent recommendations reported that both the genotype and the subtype of the virus are critical for drug selection among those available. Thus, accurate genotyping now plays a key role in the management of HCV patients. Commercially kits for investigating HCV genotypes have limited availability (8). The most reliable and reference method used to determine genotypes is sequencing of a specific region of the viral genome, however, sequencing is impractical on a large scale because of its complexity (9).

According to WHO estimates, 240 million people are chronically infected with Hepatitis B virus at present, of which ≈20 million are also simultaneously infected with the satellite hepatitis delta virus (HDV) (10-13). Super- or co-infection of HDV causes more severe liver disease with more frequent occurrence of fulminant hepatitis and a more rapid evolution into cirrhosis and HCC (11). HDV is highly endemic in Mediterranean countries, Eastern Europe, and the Middle East. HDV is also highly prevalent in Turkey (14). Based mainly on studies in blood donors, the overall prevalence of chronic HBV infection has been reported to range from 4-5% in Turkey with considerable regional differences and a decline to 2% has been evident in recent years. Tozun *et al.* reported a detection rate of anti-HDV positivity in 0.1% of the overall study population and in 2.8% of HBsAg-positive participants (15).

Although there are currently no FDA-approved treatments for HDV, some promising drugs are being tested in early clinical trials. Regular monitoring of viral loads is necessary for follow-up appointments and evaluation of treatment success (16-18). The quantification of HDV RNA viral load is the only reliable marker of HDV replication and should be an integral part in the management of HDV patients worldwide (19,20). Several in-house or commercial assays have been developed to detect and quantify HDV RNA (19,21,22). However, it has been shown that most of the assays underestimate or fail to detect or quantify HDV RNA in positive samples, especially if they have been localized from patients infected with non-genotype 1 HDV. Therefore, providing standardized kits for quantification of HDV RNA is urgently needed in order to precisely evaluate the efficiency of these new HDV therapies in large multicenter trials. However, the current assays have not been sufficiently evaluated on a large panel of clinical samples of various genotypes and viral loads (19,20).

Several external quality assessment programs for HCV genotyping and HDV RNA have been carried out in Europe and some have also been used widely in Turkey (13,19,23-25). However, a national external quality assessment program for molecular microbiology in Turkey did not exist until 2015. In order to establish such a program for molecular microbiology, the MOTAKK (Moleküler Tanıda Kalite Kontrol) (English translation: Quality control in molecular diagnostics) National Program was initiated in 2015, in accordance with the guidelines of ISO 17043. The results of HCV RNA genotyping and HDV RNA quality control panels that were included in this program between 2015 and 2016 were evaluated in this paper.

MATERIAL AND METHODS

Preparation of quality control samples

To develop the main stock of the HCV genotype and HDV RNA quality control, the groups used were: HCV RNA positive plasmas (HBV, HIV-1, HAV, Parvovirus B19, CMV-negative) that had different HCV genotypes and the HDV RNA positive plasmas (HCV, HIV-1, HAV, Parvovirus B19, CMV negative) localized from patients with chronic delta hepatitis who had a substantial viral load detected according to WHO reference materials. Six samples that had several HCV genotypes and a viral load of 10.000 IU/mL HCV were prepared from the HCV RNA genotype main stock. Five samples (4 positives, 1 negative) with several HDV viral loads were prepared from the main stock of HDV RNA genotype 1 by dilution using negative plasma, which had negative hepatitis serological markers and viral loads. Prepared samples were distributed to 1.2 mL tubes with screw caps, as a result of which HCV genotype and HDV RNA panels were formed. A code number was created for all the different tubes, which contained the year of distribution, the number of the call, and the tube number (e.g., HCV-GTP1501.02 and HDV-K1601.03). The tubes were labelled according to their code numbers and were stored at -80°C until the dispatch time. Homogeneity and stability tests of the panels were performed before distribution (7,26).

Organization of the external quality control program

Contact between the participant and the program was established online via the web page and call organization, and the evaluation program developed within this project. The calls were announced on the MOTAKK web page (http://www.motakk.org/). All university and state hospi-

tals, private laboratories, and public health centres that used HCV genotyping and HDV RNA tests were invited to participate in the program. Requested panels were shipped together with user manuals to all the participating laboratories in 2015 and 2016. The panels were delivered to the registered laboratories in the cold chain. These selected laboratories carried out the relevant tests and entered their results via the MOTAKK web page.

Assessment of quality control results

Results of the participating laboratories were compared with the true results and success rates were calculated for HCV genotype and HDV RNA according to different methods. The frequencies of detected HCV genotypes were calculated. Finally, the external quality assessment reports for each test and each laboratory were created and uploaded to the online system.

RESULTS

The numbers of participating laboratories were 23 and 20 for HCV genotyping in 2015 and 2016, respectively. HCV genotype results success rate of all participants was 56-96% in 2015 and 30-95% in 2016 (Table 1, 2). HCV genotype panel consisted of 2 tubes of genotype 1a and 1 tube of genotype 1b, 2, 3, and 4 in 2015. Among 23 participating laboratories, genotype 2 (96%) and genotype 1b (91%) were the most frequent genotypes that were detected correctly. The genotype with the lowest true detection rate was one of the two genotype 1a's in the panel with a 57% success rate. The other genotype 1a was detected with a 74% success rate. Also, genotype 4 was reported as negative by 17% of the participants (Table 3). There was one genotype 4, two pairs of 1a and 1b, and one recombinant genotype consisting of 1b/2 in the panel in 2016. While genotypes 1a and 1b were detected with high rates (85-95%), the recombinant type was detected correctly by only 30% of the participants and was reported as genotype 1b by 55% of the laboratories (Table 4).

The numbers of participating laboratories were 15 and 16 for the HDV RNA test in 2015 and 2016, respectively.

Table 1. HCV Genotype Results - 2015

Code No	TRUE GENOTYPE RESULTS		ALL PARTICIPANTS SUCCESS RATES	COMMERCIAL METHODS SUCCESS RATES	REAL TIME PCR METHODS SUCCESS RATES	STRİP TEST METHODS SUCCESS RATES	IN HOUSE METHODS SUCCESS RATES	PCR DNA SEQUENCING METHODS SUCCESS RATES
	Genotype	Subtype						
HCV-GTP1501.01	-	1a	17/23 (74%)	11/15 (73%)	8/12 (67%)	3/3 (100%)	6/8 (75%)	6/8 (75%)
HCV-GTP1501.02	4	4	17/23 (74%)	13/15 (87%)	10/12 (83%)	3/3 (10%0)	3/8 (38%)	3/8 (38%)
HCV-GTP1501.03	2	2	22/23 (96%)	15/15 (100%)	12/12 (100%)	3/3 (100%)	7/8 (88%)	7/8 (88%)
HCV-GTP1501.04	-	1b	22/23 (96%)	14/15 (93%)	11/12 (92%)	3/3 (100%)	8/8 (100%)	8/8 (100%)
HCV-GTP1501.05	3	3	20/23 (87%)	15/15 (100%)	12/12 (100%)	2/3 (67%)	6/8 (75%)	6/8 (75%)
HCV-GTP1501.06	1a	1a	13/23 (56%)	9/15 (60%)	7/12 (58%)	2/3 (67%)	4/8 (50%)	4/8 (50%)

Table 2. HCV Genotype Results - 2016

Code No	TRUE GENOTYPE RESULTS		ALL PARTICIPANTS SUCCESS RATES	COMMERCIAL METHODS SUCCESS RATES	REAL TIME PCR METHODS SUCCESS RATES	STRİP TEST METHODS SUCCESS RATES	IN HOUSE METHODS SUCCESS RATES	PCR DNA SEQUENCING METHODS SUCCESS RATES
	Genotype	Subtype						
HCV-GTP1601.01	4	4	17/20 (85%)	13/14 (93%)	11/12 (92%)	2/2 (100%)	4/6 (67%)	4/6 (67%)
HCV-GTP1601.02	-	1a	17/20 (85%)	12/14 (86%)	10/12 (83%)	2/2 (100%)	5/6 (83%)	5/6 (83%)
HCV-GTP1601.03	-	1b	18/20 (90%)	12/14 (86%)	12/12 (100%)	0/2 (0%)	6/6 (100%)	6/6 (100%)
HCV-GTP1601.04	2	2/1b	6/20 (30%)	5/14 (36%)	3/12 (25%)	2/2 (100%)	1/6 (17%)	1/6 (17%)
HCV-GTP1601.05	-	1a	19/20 (95%)	13/14 (93%)	11/12 (92%)	2/2 (100%)	6/6 (100%)	6/6 (100%)
HCV-GTP1601.06	-	1b	19/20 (95%)	13/14 (93%)	11/12 (92%)	2/2 (100%)	6/6 (100%)	6/6 (100%)

Although the HDV RNA results were evaluated qualitatively and quantitatively, the scores of quality control reports were given according to the qualitative results. HDV RNA results were evaluated qualitatively. Accordingly, the HDV RNA detection rates of participant laboratories were between 71-100% in 2015 and 50-100% in 2016. The detailed findings of the HDV RNA tests are given in Tables 5 and 6. In addition, false negativity rates were 6.6% (1/15) for Tubes 1, 2, and 4, and 26.6% (4/15) for Tube 3 in 2015. In 2016, the false negativity rates were 25% (4/16), 50% (8/16), 18.75% (3/16), and 37.5% (6/16) for Tubes 1, 3, 4, and 5, respectively. The quantitative results of each participant laboratory were given in Tables 7 and 8.

DISCUSSION

We reported the results of the first national quality control study on HCV RNA genotypes and qualitative results of the test on HDV RNA in Turkey. In total, our study involved 23 different laboratories that were distributed nationwide. In this manner, we were able to compare the relative performances of all the assays that are routinely used in Turkey.

Table 3. Frequency of HCV Genotypes - 2015

CODE NO	TRUE GENOTYPE RESULTS	RATES OF REPORTED GENOTYPES n=23											
		1. FREQUENCY[a]			2. FREQUENCY[b]			3. FREQUENCY[c]			4. FREQUENCY[d]		
		Genotype	n	%	Genotype	n	%	Genotype	n	%	Genotype	n	%
HCV-GTP1501.01	1a	1a	17	74	1	5	22	1b	1	4			
HCV-GTP1501.02	4	4	16	70	Negative	4	17	4+1b	1	4	1b	1	4
HCV-GTP1501.03	2	2	22	96	3	1	4						
HCV-GTP1501.04	1b	1b	21	91	1	1	4						
HCV-GTP1501.05	3	3	19	83	1a	1	4	1b	1	4			
HCV-GTP1501.06	1a	1a	13	57	1	5	22	1a+1b	1	4	3	1	4

Table 4. Frequency of HCV Genotypes - 2016

CODE NO	TRUE GENOTYPE RESULTS	RATES OF REPORTED GENOTYPES n=23											
		1. FREQUENCY[a]			2. FREQUENCY[b]			3. FREQUENCY[c]			4. FREQUENCY[d]		
		Genotype	n	%	Genotype	n	%	Genotype	n	%	Genotype	n	%
HCV-GTP1601.01	4	4	17	85	1a	2	10	5a	1	5			
HCV-GTP1601.02	1a	1a	17	85	1b	2	10	Unknown	1	5			
HCV-GTP1601.03	1b	1b	18	90	1a	2	10						
HCV-GTP1601.04	2/1b	1b	11	55	2	6	30	Unknown	1	5	6a	1	5
HCV-GTP1601.05	1a	1a	19	95	Unknown	1	5						
HCV-GTP1601.06	1b	1b	19	95	4	1	5						

Table 5. Qualitative Success Rates of Participating Laboratories - 2015

	HDV-K1501.01 n=14	HDV-K1501.02 n=14	HDV-K1501.03 n=14	HDV-K1501.04 n=14	HDV-K1501.05 n=14
Expected Viral Load	10.000 IU/mL	4.000 IU/mL	1.000 IU/mL	4.000 IU/mL	Negative
Rates of True Results	13/14 %93	13/14 %93	10/14 %71	13/14 %93	14/14 100%

Table 6. Qualitative success rates of participating laboratories - 2016

	HDV-K1601.01 n=16	HDV-K1601.02 n=16	HDV-K1601.03 n=16	HDV-K1601.04 n=16	HDV-K1601.05 n=16
Expected Viral Load	70.000 IU/mL	Negative	7.000 IU/mL	40.000 IU/mL	4.000 IU/mL
Rates of True Results	12/16 %75	16/16 100%	8/16 %50	13/16 %81	10/16 %63

Due to the potential clinical implications of different HCV genotypes, reliable methods are required to classify and characterize viral genomes from patient specimens (27). However, the availability of commercial kits for HCV genotyping is scarce (8). Although these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating between the different subtypes (9). In our study, largely com-

Table 7. Quantitative HDV RNA Results of each participant laboratory - 2015

					HDV-K1501.01	HDV-K1501.02	HDV-K1501.03	HDV-K1501.04	HDV-K1501.05
Commercial/ In House	Kit Name	Method	Quantitative/ Qualitative	Expected Qualitative Results	Positive	Positive	Positive	Positive	Negative
				Expected Quantitative Results	10.000 IU/mL	4.000 IU/mL	1.000 IU/mL	4.000 IU/mL	Negative
				Participating Laboratories	Copy/mL	Copy/mL	Copy/mL	Copy/mL	Copy/mL
Commercial N=14	Kit C	Real Time PCR N=14	Quantitative /Qualitative	TR015-35	<410	<410	Negative	<410	Negative
	Kit D			TR015-28	9.807	4.200	2.459	4.974	Negative
	Kit A			TR015-02	44	122	Negative	118	Negative
	Kit D			TR015-38	5.320	6.267	731	5.693	Negative
	Kit E			TR015-34	399.000	215.200	35.400	232.400	Negative
	Kit E			TR015-70	704.000	146.000	32.000	176.000	Negative
	Kit A			TR015-19	36.000	19.600	4.210	29.600	Negative
	Kit C			TR015-69	15.200	580	<400	<400	Negative
	Kit A			TR015-87	391	805	8	1.149	Negative
	Kit C			TR015-29	122.080	30.800	6.986	34.440	Negative
	Kit A			TR015-91	45	122	Negative	118	Negative
	Kit B			TR015-48	Positive	Positive	Positive	Positive	Negative
	Kit B			TR015-79	Negative	Negative	Negative	Negative	Negative
	Kit B			TR015-44*	4	1	1	2	Negative
In House n=1		Nested-PCR n=1	Qualitative	TR015-09	Positive	Positive	Positive	Positive	Negative

Table 8. Quantitative HDV RNA Results of each participant laboratory - 2016

					HDV-K1601.01	HDV-K1601.02	HDV-K1601.03	HDV-K1601.04	HDV-K1601.05
Commercial/ In House	Kit Name	Method	Quantitative /Qualitative	Expected Qualitative Results	Positive	Negative	Positive	Positive	Negative
				Expected Quantitative Results	70.000 IU/mL	Negative	7.000 IU/mL	40.000 IU/mL	Negative
				Participating Laboratories	Copy/mL	Copy/mL	Copy/mL	Copy/mL	Copy/mL
Commercial N=15	Kit A	Real Time PCR N=15	Quantitative Qualitative	TR016-91	1.582	Negative	108	3.061	1.444
	Kit A			TR016-87	16.353	Negative	1.348	1.105	75
	Kit B			TR016-44	11	Negative	Negative	Positive	Negative
	Kit B			TR016-79	130	Negative	Negative	79	Negative
	Kit C			TR016-69	820	Negative	400	538	400
	Kit A			TR016-68	<38	Negative	<38	>38	<38
	Kit B			TR016-48	Negative	Negative	Negative	Negative	Negative
	Kit D			TR016-38	16.571	Negative	1.536	9.973	1.276
	Kit A			TR016-35	Negative	Negative	Negative	Negative	Negative
	Kit E			TR016-34	13.100	Negative	1.125	803	90
	Kit C			TR016-29	1.400	Negative	Negative	Negative	Negative
	Kit D			TR016-28	72.400	Negative	5.840	5.540	521
	Kit A			TR016-19	Negative	Negative	Negative	642	281
	Kit A			TR016-17	49	Negative	Negative	1.180	105
	Kit A			TR016-02	Negative	Negative	Negative	1.400	Negative
In House n=1		Nested-PCR n=1	Qualitative	TR016-103	Positive	Negative	Positive	Positive	Positive

mercial real time PCR kits were used, followed by laboratory-designed methods, and the least-used method was nucleotide sequencing. In 2015, the lowest success rate belonged to the HCV genotype 1a (56%), while the other tube with genotype 1a was detected higher with a rate of 74% accuracy. Viral RNA may be lost in the serum through storage or improper laboratory procedures (9). In 2016, the tube with 30% success rate had recombinant HCV genotype, therefore, it could not be detected in most of the laboratories. Only 17% of laboratories that used DNA sequencing for HCV genotyping could detect the recombinant genotype. The ideal candidate method to standardize HCV sequencing should cover all three protein coding regions of interest (NS3, NS5A, and NS5B) (28).

The quantification of HDV RNA has become a major diagnostic issue, for both the management of patients and the evaluation of new specific anti-HDV therapies, which are currently in progress. There are several challenges in developing accurate, sensitive, and consensus tests for HDV RNA viral load quantification in blood samples, in particular the tests for HDV genetic diversity. The most conserved regions of the genome are located in genomic and antigenomic ribozymes (21).

We performed an unprecedented comprehensive national quality-control study to evaluate almost all the commercially available and in-house assays for plasma HDV-RNA quantification. In the present study, the success rates of qualitative HDV RNA assessments in the participating laboratories were higher in 2015 and control serum number 3, which had the lowest viral load (1000 IU/mL), had the highest false negativity rate. In 2016, the serum with 7000 IU/mL viral load had the highest false negativity. The high genetic diversity of HDV has been problematic for the standardization of in-house real-time PCR assays, especially when using probe-based techniques, because HDV has small genomes combined with its high genetic diversity hamper design of HDV hydrolysis probes that bind efficiently to all viral genotypes (29). In addition, to avoid false negative results, it is especially important to make PCR primers for highly conserved sequence regions, because RNA viruses, including HDV, are prone to mutations that can cause primer mismatch (30). Therefore, the development of international quality controls for the various in-house or commercial assays should be organized and implemented, to assess the specificity and sensitivity of the techniques and to judge their ability to quantify all circulating strains (21). The high genetic variability of HDV is a key factor in the discrepancies observed in these tests, which also suffer from a lack of standardization as mentioned above. The recent WHO-developed HDV international standard (WHO-HDV-IS) will greatly contribute to providing a solution to this drawback (13).

Consequently, all participant laboratories compared their own results with other laboratories and re-evaluated the tests they used. This study was the first national external quality control program regarding HCV RNA genotyping and HDV RNA in the field of molecular microbiology that was implemented successfully in Turkey.

MOTAKK HCV Genotype and HDV Study Group
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