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DISTRIBUTION OF HEPATITIS C VIRUS GENOTYPES AND SUBTYPES IN A GROUP OF PATIENTS WITH CHRONIC HEPATITIS C FROM CANTON SARAJEVO, 2012-2018

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ABSTRACT

Background: Hepatitis C virus (HCV) genotypes and subtypes exhibit significant geographic variations.

Aim: To analyse the distribution of genotypes/subtypes of HCV in a group of patients with chronic hepatitis C from Canton Sarajevo during 2012-2018.

Material and methods: The study enrolled 247 human plasma samples of HCV-RNA positive patients with available results of HCV genotyping test.

Results: During 2012-2018, the domination of subtypes 1a (34.01%), 1b (28.34%) and genotype 3 (23.89%) was registered. In 2012 and 2013, HCV subtype 1a was the most common (27/63; 42.86% and 17/40; 42.50%, respectively). In 2014, the leading HCV genotype/subtype were 3 and 1b (17/57; 29.82%). In 2015, the dominance of HCV genotype 3 (14/39; 35.90%) continued, while in 2016, the same number of HCV subtypes 1a and 1b (11/30; 36.67%) was recorded. Although in a small number of tested, during 2017, HCV subtype 1b was the most prevalent (7/14; 50.00%), and in 2018, it was replaced by a HCV subtype 1a (3/4; 75.00%). Distribution of HCV genotypes/subtypes by age group of patients varied significantly (p=0.000). The largest number of patients (71/247; 28.74%) belonged to the age category 30-39 years and HCV genotypes/subtypes 1, 3, 4, 1a and 1b were identified. Except in 2017, male gender significantly dominated (p=0.000). In males, HCV subtype 1a (68/170; 40.00%) was the most common, while in women it was HCV subtype 1b (44/77; 57.14%).

Conclusion: This six-year retrospective study showed the time variations of the circulating HCV genotypes/subtypes among patients with chronic hepatitis C in Canton Sarajevo. Genotyping of the HCV has an important implications for diagnosis and treatment of the patients.

Key words: hepatitis C, genotype, prevalence

INTRODUCTION

Based on the WHO estimation from 2015, 71 million persons were living with chronic hepatitis C virus (HCV) infection worldwide and 399 000 died from cirrhosis or hepatocellular carcinoma caused by HCV infection. In the same year, 1.75 million new HCV infections occurred, mostly among people who inject drug (PWID) and with unsafe health care [1]. According to the phylogenetic and sequence analysis of whole viral genomes worldwide, HCV infection may be caused by one of seven recognized genotypes (1-7) [2]. Within genotypes, HCV is then classified into 67 confirmed and 20 provisional subtypes. The differences between HCV genotypes vary in 30-35% of the nucleotides, while the strains within the same subtype differ at <15 % of nucleotide sites [3].

Distribution of HCV genotypes and subtypes exhibits significant geographic variations. In meta-analysis involving 1.217 studies, representing 117 countries and 90% of the global population, HCV genotype 1 was the most prevalent worldwide (83.4 million cases; 46.2% of all HCV cases), with approximately one-third recorded in East Asia. Genotype 3 was the next most prevalent globally (54.3 million, 30.1%); genotypes 2, 4, and 6 were responsible for a total 22.8% of all cases; genotype 5 comprises the remaining <1%. Genotypes 1 and 3 dominated in most countries regardless of their economic

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status, while genotypes 4 and 5 were mostly present in low-income countries [4].

With a more detailed insight into the prevalence of HCV genotypes, it was seen that the south and eastern European countries follow the dominance of subtypes 1b, 3 and 1a. Genotype 1 is distributed worldwide and is responsible for most cases in America, Europe, Australia and Japan; subtype 1b is most common in Europe and Asia, while subtype 1a is widespread in northern Europe and the United States. Genotype 2 is more common in industrialized countries, as well as in South America and Asia, especially in Japan and China, where subtype 2a is usually isolated; subtype 2b is widespread in Northern Europe and the United States; genotype 2c is the most common subtype in Western and Southern Europe, Pakistan and India. Genotype 3, and especially subtype 3a, predominates in Europe, the US, Australia and South Asia [5-7].

Genotypes 4 and 5 are mainly identified in Africa and the Middle East: the subtype 4a is prevalent in Egypt, while the subtype 4c is widespread in Central Africa; genotype 5 is largely isolated in South Africa. Genotype 6 and its many subtypes are found predominantly in Southeast Asia, and in some countries such as Thailand, Vietnam and Myanmar, genotype 6 is responsible for most new HCV cases. It is believed that genotypes 4, 5 and 6 are limited to North Africa, Central Africa and Southeast Asia, but rising migration and globalization processes cause the spread of these genotypes beyond those areas, in the nearby regions of Asia, and far to Western countries such as the United States, Canada and Northern Europe. Genotype 7 has little clinical relevance and has recently been found in patients from Central Africa and Thailand [8-14]. In developing countries, an age-related distribution of cases and a virus transmission linked to unsafe medical procedures and blood transfusions have been observed; however, recent data indicated an increasing role in the spread of infection played by injection drug use [15].

In Bosnia and Herzegovina (B&H), a limited range of data based on molecular epidemiology of HCV is available so far, showing the major prevalence of subtypes 1a and/or 1b and genotype 3, respectively, depending on age, gender and risk category of study population [16-17]. Because of its high genetic diversity, HCV has been considered a major challenge for the development of HCV vaccines and pan-genotypic therapies [18]. Currently, the duration of treatment, cure rates, and the need for adjuvant interferon and ribavirin with the new direct-acting antiviral (DAA) therapies remain dependent in part on HCV genotype and subtype.

An initial therapy that has been used in B&H consisted of pegylated interferon plus ribavirin combination therapy administered for 48 weeks in patients with genotype 1, while treatment with lower doses of ribavirin has been equally effective in patients with genotype 2 and 3 for 24 weeks. Because of the lack of data on the nature of the disease caused by the HCV genotypes 4, 5 and 6, a rigorous regimen of treatment as for genotype 1 was in place.

Treatment of patients with chronic hepatitis C using interferon free new DAA therapy (e.g. sofosbuvir/ledipasvir) has been available in B&H since 2016. The therapy is administered as a simple one-tablet regimen, once a day for 8-12 weeks for most patients, alone or with ribavirin, and has shown exceptional efficiency (94-100%) for HCV genotypes 1, 4, 5 and 6 [19-21].

However, the development of national treatment strategies using DAA therapies requires better knowledge of the epidemiology of HCV and of the distribution of its genotypes. Therefore, the aim of this study was to analyse the distribution of genotypes/subtypes of HCV in a group of patients with chronic hepatitis C from Canton Sarajevo during 2012-2018, as a contribution to the planning of the preventive and therapeutic actions, allowing determination of the need for treatment of infected persons.

SUBJECTS AND METHODS

The research was carried out at the Clinical Center of the University of Sarajevo, Unit for Clinical Microbiology. Study enrolled 247 HCV RNA-positive patients suffering from chronic hepatitis C from Canton Sarajevo (one of the ten cantons of the entity Federation of B&H within the country B&H) during the period of 2012-2018. Participants were divided into eight age groups: I (0-9 years), II (10-19 years), III (20-29 years), IV (30-39 years), V (40-49 years), VI (50-59 years old), VII (60-69 years) and VIII (>70 years) and considered by gender (male, female). For the patients' identity protection, all samples were tested anonymously and coded. Blood samples were collected by standard vacutainer system method and plasma fraction was used for molecular diagnostics.

HCV genotyping was performed by automated system m2000 (Abbott Molecular Inc., IL, SAD) at Clinical Center of the University of Sarajevo, Unit for Clinical Microbiology. The system consisted from m2000sp instrument for extraction and purification of RNA from human plasma samples and preparation of multiplex real-time reverse-transcription polymerase chain reactions (rtRT-PCR), and instrument m2000rt for running of rtRT-PCR.

The Abbott m2000sp provides automated sample preparation using a magnetic microparticle-based protocol (Abbott mSample Preparation System, Promega Corporation, Madison, WI, SAD) to process 0,5 mL of samples. During the sample preparation protocol, HCV virions were disrupted by guanidine isothiocyanate, RNA was captured on the magnetic microparticles, inhibitors were removed by washing steps, and RNA was eluted off the microparticles. The

bound nucleic acids were eluted and transferred to a 96 deep-well plate for amplification. The Internal Control (IC) was introduced into each specimen at the beginning of the sample preparation process to demonstrate that the process was completed correctly for each specimen and control.

The Abbott m2000sp instrument automated the assembly of three amplification master mixes (A, B, and C) by combining the respective Abbott RealTime HCV Genotype II Oligonucleotide Reagent (A, B, or C) with thermostable rTth DNA polymerase enzyme and Activation Reagent. The instrument dispensed the resulting master mixes into the 96-well optical reaction plate along with aliquots of the nucleic acid samples prepared by the Abbott m2000sp. Each processed sample was added to one well containing Master Mix A, one well containing Master Mix B, and one well containing Master Mix C.

The Abbott RealTime HCV Genotype II assay used four sets of PCR primers. One set of primers targeted a sequence within the 5' untranslated region (UTR) of the HCV genome. This primer set was designed to amplify all HCV isolates. The second primer set was designed to amplify the non-structural 5b (NS5b) region of genotype 1a. The third HCV primer set amplified the NS5b region of genotype 1b. The IC primer set targeted a portion of the hydroxypyruvate reductase gene of the pumpkin plant, Cucurbita pepo, and was delivered in an Armored RNA® particle that was diluted in negative human plasma.

The assay required three separate reactions to detect genotypes 1, 1a, 1b and 2-5: Reaction A detected all HCV isolates, type 3 isolates, and subtype 1a isolates. Reaction B was designed to detect type 1 isolates, type 2 isolates, and subtype 1b isolates, and reaction C was used to detect type 4 and type 5 isolates.

Statistics

Descriptive statistics were expressed by frequency, arithmetic mean, standard deviation (SD), minimum and maximum values, and percentages. As statistical procedures, Kolmogorov-Smirnov test, Kruskal-Wallis test and Chi-square test were applied. Statistical analysis was performed using Excel (Microsoft Office) and IBM SPSS Statistics 23.0 (IBM, New York, USA). A p-value of<0.05 was considered statistically significant.

RESULTS

The results were analysed in the total population tested for HCV genotyping in the period of 2012-2018, and annually. The most prevalent HCV genotype/subtype in the population during 2012-2018 was 1a (84/247; 34.01%), followed by subtype 1b (70/247; 28.34%), and genotype 3 (59/247; 23.89%) (Table 1).

Table 1. Distribution of HCV genotypes/subtypes by age groups, 2012-2018.n- number of participants

HCV genotype/ subtype	Age group (years)																*Total*	
	I (0-9)		II (10-19)		III (20-29)		IV (30-39)		V (40-49)		VI (50-59)		VII (60-69)		VIII (>70)			
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1	/	/	/	/	3	12.50	5	7.04	2	3.33	3	5.45	2	6.90	/	/	**15**	***6.07***
2	/	/	/	/	/	/	/	/	2	3.33	1	1.82	/	/	/	/	**3**	***1.21***
3	/	/	1	25.00	12	50.00	21	29.58	18	30.00	5	9.09	1	3.45	1	33.33	**59**	***23.89***
4	1	100.00	/	/	3	12.50	6	8.45	2	3.33	2	3.64	2	6.90	/	/	**16**	***6.48***
1a	/	/	1	25.00	5	20.83	36	50.70	29	48.33	12	21.82	1	3.45	/	/	**84**	***34.01***
1b	/	/	2	50.00	1	4.17	3	4.23	7	11.67	32	58.18	23	79.31	2	66.67	**70**	***28.34***
Total	**1**	0.40	**4**	1.62	**24**	9.72	**71**	28.74	**60**	24.29	**55**	22.27	**29**	11.74	**3**	1.22	**247**	***100.00***

The largest number of HCV genotyping tests performed was recorded in 2012 (63/247; 25.51%), followed by 2014 (57/247; 23.08%) and 2013 (40/247; 16.19%). In the period of 2015-2018, there was a continuous decline in the number of tested individuals (Figure 1).

In 2012 and 2013, HCV subtype 1a was the most common (27/63; 42.86% and 17/40; 42.50%, respectively). In 2014, the leading HCV genotype/subtype were 3 and 1b (17/57; 29.82%). In 2015, the dominance of HCV genotype 3 (14/39; 35.90%) continued, while in 2016, the same number of HCV subtypes 1a and 1b (11/30; 36.67%) was recorded. Although in a small number of tested, during 2017, HCV subtype 1b was the most prevalent (7/14; 50.00%), and in 2018, it was replaced by a HCV subtype 1a (3/4; 75.00%) (Figure 1).

The genotype distribution varied across age categories. An average age of the participants was 44.11 years (STDEV±12.44). The youngest among them was 9, and

the oldest was 79 years old. The 50% of patients had ≤43 years. According to the age group, the majority of patients (71/247; 28.74%) belonged to the group IV (30-39 years), followed by the group V (40-49 years) with 60/247 (24.29%) patients, while the smallest number of tested (1/247; 0.40%) was recorded in the group I (0-9 years) (Table 1). In the youngest age group (I, 0-9 years), only one patient was present with HCV genotype 4 identified. The widest spectrum of genotypes/subtypes (1, 2, 3, 4, 1a and 1b) was identified in the age groups V (40-49 years) and VI (50-59 years), while in the group of oldest patients VIII (>70 years), HCV genotype 3 and subtype 1b were recorded (Table 1).

The highest frequency among age groups belonged to HCV subtype 1a (36/71; 50.70%) found within the age group IV (30-39 years) and then by HCV subtype 1b (32/55; 58.18%), which was the most frequently identified in age group VI (50-59 years). The dominance of individual genotypes/subtypes among age groups of the patients is given in Table 1.

The difference in the prevalence of HCV genotypes/subtypes according to the age of participants was statistically significant (p=0.000). Actually, the age variable for HCV genotype/subtype 1 (p=0.543), 1a (p=0.487), 2 (p=0.960), 3 (p=0.384), and 4 (p=0.884) followed the normal distribution (p≥0.05; a nonparametric Kolmogorov-Smirnov test was used) while for subtype 1b, the normal distribution of the values was not followed (p<0.05; p=0.038) and a nonparametric test for k independent samples was applied (Kruskal-Wallis test).

In the period 2012-2018, the testing of the male population significantly dominated (170/247; 68.83%; p=0.000) and the most common HCV genotype/subtype was 1a (68/170; 40.00%), followed by the genotype 3 (50/170; 29.41%), while in the female population the subtype 1b was the most common (44/77; 57.14%), followed by subtype 1a (16/77; 20.78%) (Table 2).

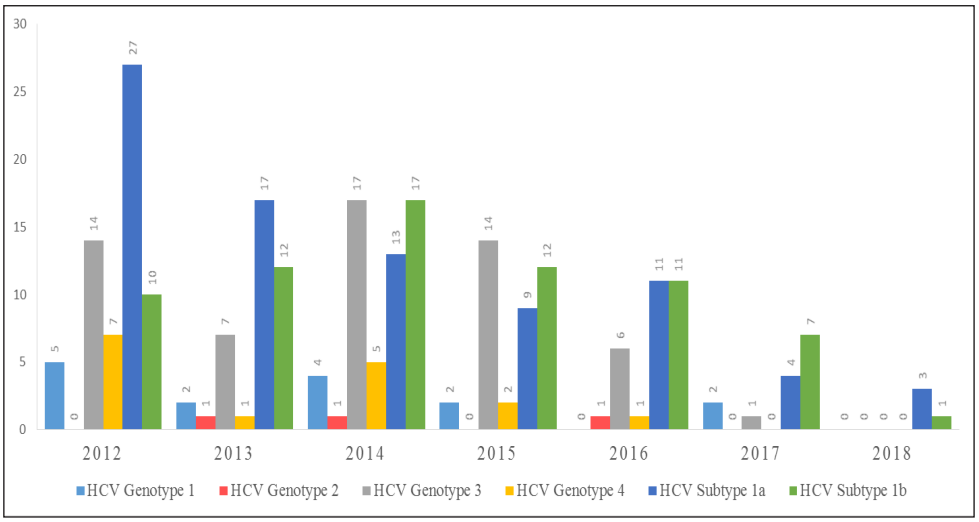

Figure 1. Annual distribution of HCV genotypes/subtypes, 2012-2018.

Table 2. Distribution of HCV genotypes/subtypes by gender, 2012-2018.

HCV genotype/subtype	Male		Female		Total	
	n	%	n	%	n	%
1	10	5.88	5	6.49	15	6.07
2	2	1.18	1	1.30	3	1.21
3	50	29.41	9	11.69	59	23.89
4	14	8.24	2	2.60	16	6.48
1a	68	40.00	16	20.78	84	34.01
1b	26	15.29	44	57.14	70	28.34
Total	**170**	68.83	**77**	31.17	**247**	**100.00**

DISCUSSION

Persistent HCV infection is associated with the development of liver cirrhosis, hepatocellular cancer, liver failure, and death [22].Since epidemiological data are the basis for the development of preventive strategies necessary for eradication of HCV infection, the aim of this study was to update and review the molecular epidemiology of HCV in a part of B&H (capital region) as a contribution to the development of national-specific screening programs and the international HCV surveillance program. Several studies in B&H were conducted on the prevalence of HCV genotypes/subtypes. In the study published in 2007 [16], with the group of treated chronic hepatitis C patients, subtype 1a was the most prevalent (46%), followed by subtype 1b and genotype 3 in the same percentage (22%).

In the later study from 2009 [17], which enrolled the samples collected during 2006-2008, the prevalence of HCV genotypes in patients with chronic hepatitis C and first time blood donors from northeastern B&H region, according to gender and age group, was assessed. In both analysed populations, subtype 1b was the most frequent, followed by genotype 3 and subtype 1a. Subtype 1a was more prevalent in the first time blood donors but with limited statistical significance. The largest age group among both populations assessed was 30-50 years, and male gender dominated among all respondents of the study.

The most prevalent HCV infection revealed in the patients with chronic hepatitis C during 2012-2018 in Canton Sarajevo was caused by subtype 1a (34.01%), followed by subtype 1b (28.34%), and genotype 3 (23.89%). Our results showed a continuing trend of dominance of subtypes 1a and 1b over the time in this part of B&H, reffering the higher similarity to study from 2007 [16]. Comparing with the available data from the neighbouring countries, the results of eight-year (2008-2015) retrospective study on the distribution of HCV genotypes and subtypes in 3.655 HCV-infected individuals from Croatia showed that the majority of infections were also attributed to genotypes 1 and 3, without major changes in the molecular epidemiology in Croatia in the past 20 years [23].

In Europe, subtype 1a and genotype 3 (including subtype 3a) are mostly found in PWID, whereas subtype 1b and genotypes 2 are usually associated with blood transfusion and unsafe medical procedures [24]. The most common genotype found in Central European countries is genotype 1 (70.0%), followed by genotype 3 (21.0%), 4 (4.9%) and 2 (3.2%). Only a small percentage of mixed genotypes and genotype 6 has been found, whereas genotype 5 cases were not reported according to the study from 2015 [25]. Actually, in Romania, Hungary and Slovakia, genotype 1 is almost the only genotype found (98.0; 94,1 and 89,9%, respectively). A significant percentage of genotype 3 was described in Macedonia (44,6%), Slovenia (37.8%) and Croatia (35.6%), while a considerable prevalence of genotype 2 was revealed only in Albania (20.0%) and of genotype 4 in Montenegro (19.6%) and Albania (16.0%) [25].

It is necessary to emphasize that the range of detected genotypes largely depends on the method used to determine the HCV genotype. In our study, we were limited to the detection of HCV genotypes/subtypes 1, 1a, 1b, 2-5 by the method used, not excluding the possibility of the presence of other genotypes/subtypes circulated in the region. The gold standard of HCV genotyping is the sequencing of NS5B region, capable to accurately assign the genotype, and the resulting sequence can be used for phylogenetic analysis to epidemiological purposes [26-29]. HCV prevalence in the majority of developed countries is classified as low, but marked differences in the epidemiological picture exist among countries, principally related to temporal and transmission factors and resulted in diverse age-specific distribution of HCV cases [30].

In our study, the prevalence of HCV genotypes/subtypes according to the age group of patients was significantly different. Thus, the majority of patients (28.74%) belonged to the group of 30-39 years, followed by the group of 40-49 years with 24.29% of patients. Looking for the similar results among other countries, we found that also in Australia and in the United States, the peak prevalence was recorded in people aged 30-39 years, probably related to an increase of parenteral drug use throughout the 1980s and 1990s [31-32]. Among developed countries with a low prevalence of HCV infection, Japan shows the most of HCV cases recorded in people aged 40-69 years, while HCV prevalence in younger people was very low, suggesting the occurrence of infection in the distant past, linked to the possible improper sterilization procedures and unsafe medical practice [33-35].

In the period 2012-2018, testing of the male population significantly dominated (68.83%) with the most common HCV subtype 1a and genotype 3 detected, while in the female population, subtype 1b was the most common, followed by the subtype 1a. The male population was also more prevalent in the study from 2009 [17], but the distribution was somewhat different, with the dominance of subtypes 1b and 3 in both, males and females. Among the study group, during the 2015-2018, a continuous decline in the number of tested people was observed, indicating the possible benefits of the use of effective therapy on the population. Currently, the development of highly effective therapy against HCV infection could substantially contribute to better control and the treatment of the patients.

Although HCV genetic diversity on a national and regional level indicates the need for continuous surveillance of HCV molecular epidemiology, according to the updated WHO Guidelines recommendation, the use of pan-genotypic regimens will reduce the need for genotyping before initiation of the treatment [36]. The potential limitation of the study was an incomplete total number of patients from Canton Sarajevo, representing the group of those tested at the Clinical

Center of the University of Sarajevo during the study period.

In conclusion, consideration of the trend in the prevalence of HCV genotypes in a particular region is a challenge for the improved development of vaccines and pan-genotypic treatments. Our results confirmed the domination of genotypes 1a, 1b and 3 during 2012-2018 in a group of patient with chronic hepatitis C in Canton Sarajevo, which is accordance to the similar results from 2007 (B&H) and countries from the region. Distribution of genotypes indicates the possible trnsmission routes by PWID, blood transfusion and unsafe medical procedures.

SAŽETAK

Uvod: Genotipovi i podtipovi hepatitis C virusa (HCV) pokazuju značajne geografske varijacije.
Cilj: Analizirati distribuciju genotipova i podtipova HCV-a kod grupe bolesnika sa hroničnim hepatitisom C iz Kantona Sarajevo, u periodu 2012-2018.
Materijal i metode: U istraživanje je uključeno 247 uzoraka humane plazme HCV-RNK pozitivnih ispitanika sa raspoloživim rezultatima testa genotipizacije HCV-a.
Rezultati: Tokom 2012.-2018. godine, zabilježena je dominacija HCV podtipova 1a (34,01%), 1b (28,34%) i genotipa 3 (23,89%). U 2012. i 2013. godini, HCV podtip 1a bio je najčešći (27/63; 42,86% i 17/40; 42,50%). U 2014. godini, preovladavali su HCV genotip 3 i podtip 1b (17/57; 29,82%). U 2015. godini nastavljena je dominacija HCV genotipa 3 (14/39; 35,90%), dok je u 2016. godini zabilježen isti broj HCV podtipova 1a i 1b (11/30; 36,67%). Iako u malom broju testiranih, tokom 2017. godine, HCV podtip 1b je bio najraširenij (7/14; 50,00%), a 2018. godine je bio zamijenjen HCV podtipom 1a (3/4; 75,00%). Distribucija HCV genotipova/podtipova prema starosnoj kategoriji bolesnika značajno je varirala (p=0,000). Najveći broj ispitanika (71/247; 28,74%) je pripadao starosnoj kategoriji od 30-39 godina, u kojoj su identifikovani HCV genotipovi/podtipovi 1, 3, 4, 1a i 1b. Osim u 2017. godini, značajno je dominirao muški spol (p=0,000) među testiranim. Kod muškaraca je najčešći bio HCV podtip 1a (68/170; 40,00%), dok je kod žena bio HCV podtip 1b (44/77; 57,14%).
Zaključak: Ova šestogodišnja retrospektivna studija pokazala je vremenske varijacije cirkulirajućih HCV genotipova/podtipova među ispitanicima sa hroničnim hepatitisom C u Kantonu Sarajevo. Genotipizacija HCV-a ima važne implikacije za dijagnozu i liječenje pacijenata.

Ključne riječi: hepatitis C, genotip, prevalenca

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