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Original Article

Phylogenetic analysis confirms hepatitis C virus transmission among hemodialysis patients in Kosovo

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Abstract

Introduction: It has recently been demonstrated that there is a very high prevalence of hepatitis C virus (HCV) infection among hemodialysis patients in Kosovo with HCV subtype 1 being the most prevalent subtype. In this study, we further detail the molecular epidemiology of HCV outbreaks occurring in seven dialysis centers in Kosovo.

Methodology: In total, 273 samples obtained from HCV RNA positive patients undergoing hemodialysis at one of the seven centers in Kosovo were selected for this study: 171 subtype 1a samples, 91 subtype 4d samples, and 11 subtype 1b samples. A partial HCV NS5B region was amplified and sequenced. Subtype-specific phylogenetic analyses were performed with the inclusion of control sequences and transmission clusters were identified.

Results: NS5B sequences were successfully obtained in 257/273 (94.1%) of samples; 162 subtype 1a, 84 subtype 4d, and 11 subtype 1b sequences. Phylogenetic analyses showed a high degree of phylogenetic clustering of HCV sequences subtyped 1a (99.4%), 1b (63.6%), and 4d (76.2%). Distinct phylogenetic clusters of sequences obtained from hemodialysis patients were observed for all three subtypes studied. In addition, several smaller clusters within the large clusters were identified, mainly from a single dialysis center.

Conclusions: Phylogenetic analyses confirmed nosocomial transmission during dialysis as a major factor in the spread of HCV at the seven dialysis centers in Kosovo.

Key words: HCV; phylogenetic analysis; dialysis; transmission.

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Introduction

Since the identification of hepatitis C virus (HCV) in 1989, patients on hemodialysis have been considered a high-risk group for HCV infection. Transmission of HCV has been relatively frequent in dialysis units, due to breaches in infection control measures [1,2]. Handling blood specimens close to an area allocated for medications and clean supplies, use of mobile carts to deliver injectable medications, use of medications from multi-dose vials, inadequate handling of equipment by staff, such as, improper decontamination of priming receptacles before reuse and inappropriate disinfection and cleaning of surfaces, in conjunction with the high HCV prevalence in the hemodialysis units and lack of human resources have been reported as major factors contributing to HCV transmission in dialysis settings [3,4]. An association between longer duration of dialysis and HCV infection was established over two decades ago [5]. In recent years, HCV transmission in dialysis settings has been greatly reduced in high-income countries with only occasional outbreaks reported; however, in low-resource countries the problem persists [6–9]. Prevention of HCV transmission in dialysis centers is of utmost importance because HCV positivity among patients on maintenance dialysis has been shown to be a significant independent risk factor for death, mainly due to liver and cardiovascular diseases [10].

Kosovo faces a very high prevalence of HCV infection in dialysis units [11,12]. Recently, an average anti-HCV prevalence of 53%, ranging from 22.3 to 91.1%, was reported for the seven dialysis centers in Kosovo [13]. In this study, we further explored the HCV outbreak at the seven dialysis centers in Kosovo by performing phylogenetic analyses. Our aim was to determine whether the high prevalence of HCV

infection among patients on dialysis is a result of nosocomial transmission.

Methodology

Blood samples were collected from all 668 patients receiving dialysis services from January to March 2013 at all seven dialysis centers in Kosovo [13]. Written consents were obtained from all patients, following approval of the study by the Ethics Board of the Medical Faculty, University of Prishtina "Hasan Prishtina" in Kosovo. In total, 354 patients were anti-HCV positive and 323 had detectable HCV RNA; 275 samples were successfully genotyped, as described previously [13]. Samples with the most frequent HCV genotypes were selected for the present study: 171 subtype 1a samples, 91 subtype 4d samples, and 11 subtype 1b samples. A partial NS5B region of HCV was amplified using primers A1b/F1b and B1b/E1b for subtypes 1a and 1b, and primers 4HCV-OS/4HCV-OA and 4HCV-IS/E1B for subtype 4d [14]. The resultant amplicon products were sequenced, as described previously [13]. Separate alignments of the obtained sequences of subtype 1a, 1b and 4d sequences were made using the ClustalW Multiple alignment available through the BioEdit package [15]. A quick neighbor joining tree was created using Mega 5.05 with 100 bootstrap replicates [16]. Major clusters were identified and the BLAST search tool was used to identify HCV control sequences deposited in the GenBank database with the most similarity to the clustered sequences and to the more divergent sequences from Kosovo [17–19]. To serve as local controls, additional sequences were added to the alignment as follows: 39 HCV sequences from injecting drug users (IDUs), nine sequences from chronic HCV patients, and two sequences from blood donors to the subtype 1a analysis; 13 sequences from chronic HCV patients and four sequences from blood donors to the subtype 3a analysis; and four sequences of chronic HCV patients to the subtype 4d analysis. The Find Best DNA/Protein Model tool in Mega 5.05 [16] was employed for the selection of the best-fit evolutionary model. Finally, maximum likelihood phylogenetic trees were constructed using PhyML 3.0 [20] and viewed with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Transmission clusters were identified according to the approximate likelihood ratio test (aLRT) branch support values.

Results

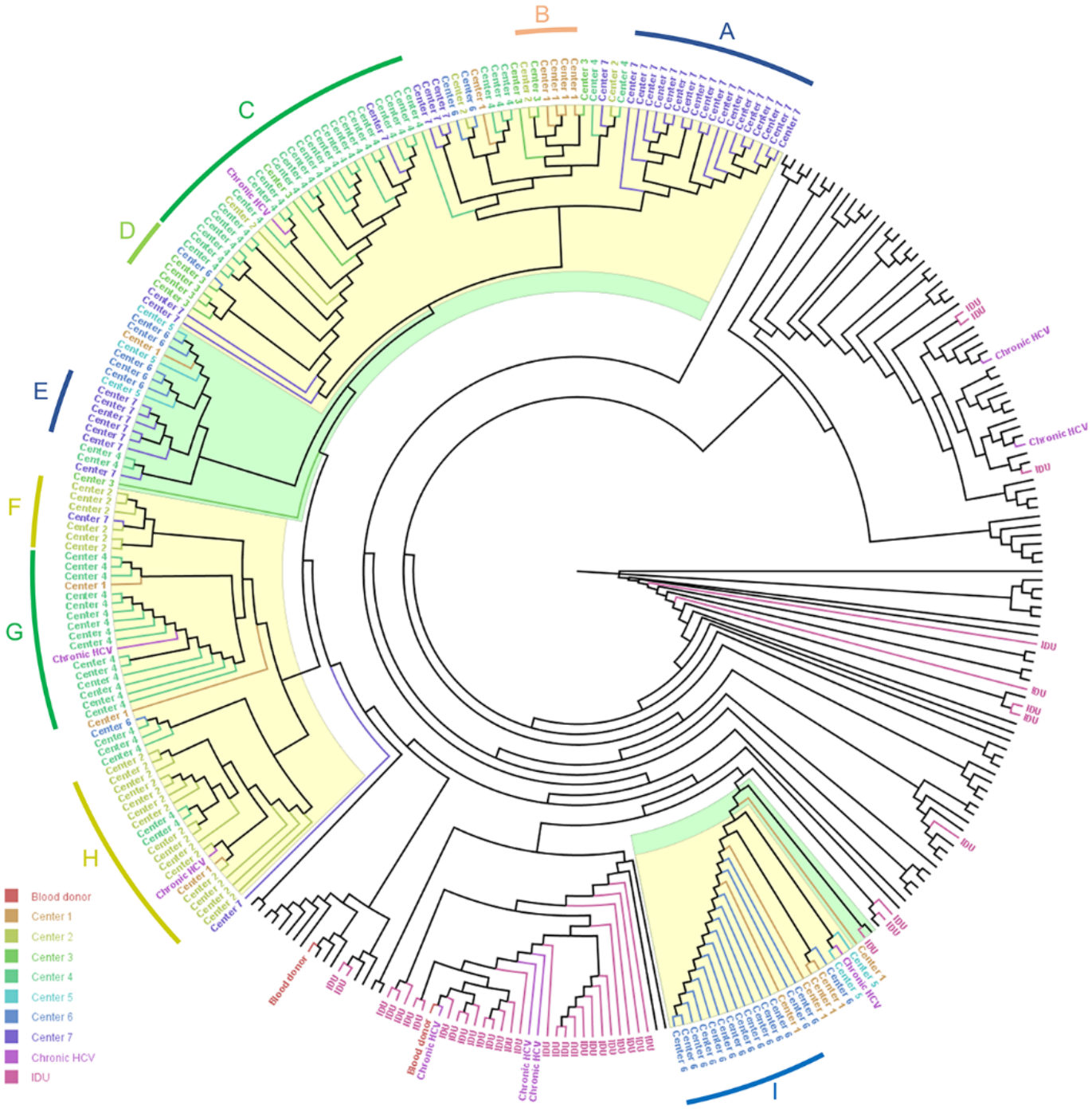
Partial sequences of the HCV NS5B region were generated from 257/273 (94.1%) of samples obtained from dialysis patients: 162 HCV subtype 1a sequences, 84 subtype 4d sequences, and 11 subtype 1b sequences (Table 1).

A phylogenetic tree constructed using HCV subtype 1a sequences showed that the majority of sequences obtained from dialysis patients belonged to distinct phylogenetic clusters (Figure 1). Three large clusters with aLRT > 0.9 were observed, with the largest cluster containing 74 sequences obtained from dialysis patients (74/162; 45.7%), of which 29 were from Center 4, 25 from Center 7, eight from Center 3, five from Center 1, four from Center 2, and three from Center 6. The second cluster included 46 sequences (46/162; 28.4%), of which 21 were from Center 2, 20 from Center 4, three were from Center 1, and one each from Centers 6 and 7. The third cluster consisted of 22 samples (22/162; 13.6%), of which 15 were from Center 6, five from Center 1, and two from Center 5. In total, 87.0% (141/162) of subtype 1a sequences obtained from dialysis patients in Kosovo were part of a transmission cluster with aLRT > 0.9 and 99.4% (161/162) of 1a sequences were found in clusters with aLRT > 0.8. In addition, several smaller clusters with predominantly sequences from patients receiving dialysis at a single Center were observed (Table 2). Notably, sequences obtained from blood donors and IDUs were dispersed on the phylogenetic tree with the exception of one distinct cluster of IDUs (Figure 1).

Table 1. Representation of the obtained partial HCV NS5B sequences among the dialysis centers of Kosovo according to HCV subtypes.

Hemodialysis center	Patients n	HCV genotype 1a/1b/4d n (%)	NS5B sequences n (%)	Subtype 1a n (%)	Subtype 1b n (%)	Subtype 4d n (%)
Center 1	188	32 (17.0)	29 (90.6)	14 (48.3)	3 (10.3)	12 (41.4)
Center 2	68	33 (50.8)	31 (93.9)	25 (80.6)	1 (3.2)	5 (16.1)
Center 3	75	41 (54.7)	40 (97.6)	9 (22.5)	1 (2.5)	30 (75.0)
Center 4	163	86 (52.8)	80 (93.0)	51 (63.8)	1 (1.3)	28 (35.0)
Center 5	39	5 (12.8)	5 (100.0)	5 (100.0)	0	0
Center 6	79	32 (40.5)	30 (93.8)	24 (80.0)	4 (13.3)	2 (6.7)
Center 7	56	44 (78.6)	42 (95.5)	34 (81.0)	1 (2.4)	7 (16.7)
Total	668	273 (40.9)	257 (94.1)	162 (63.0)	11 (4.3)	84 (32.7)

Figure 1. The maximum likelihood phylogenetic tree of HCV subtype 1a.

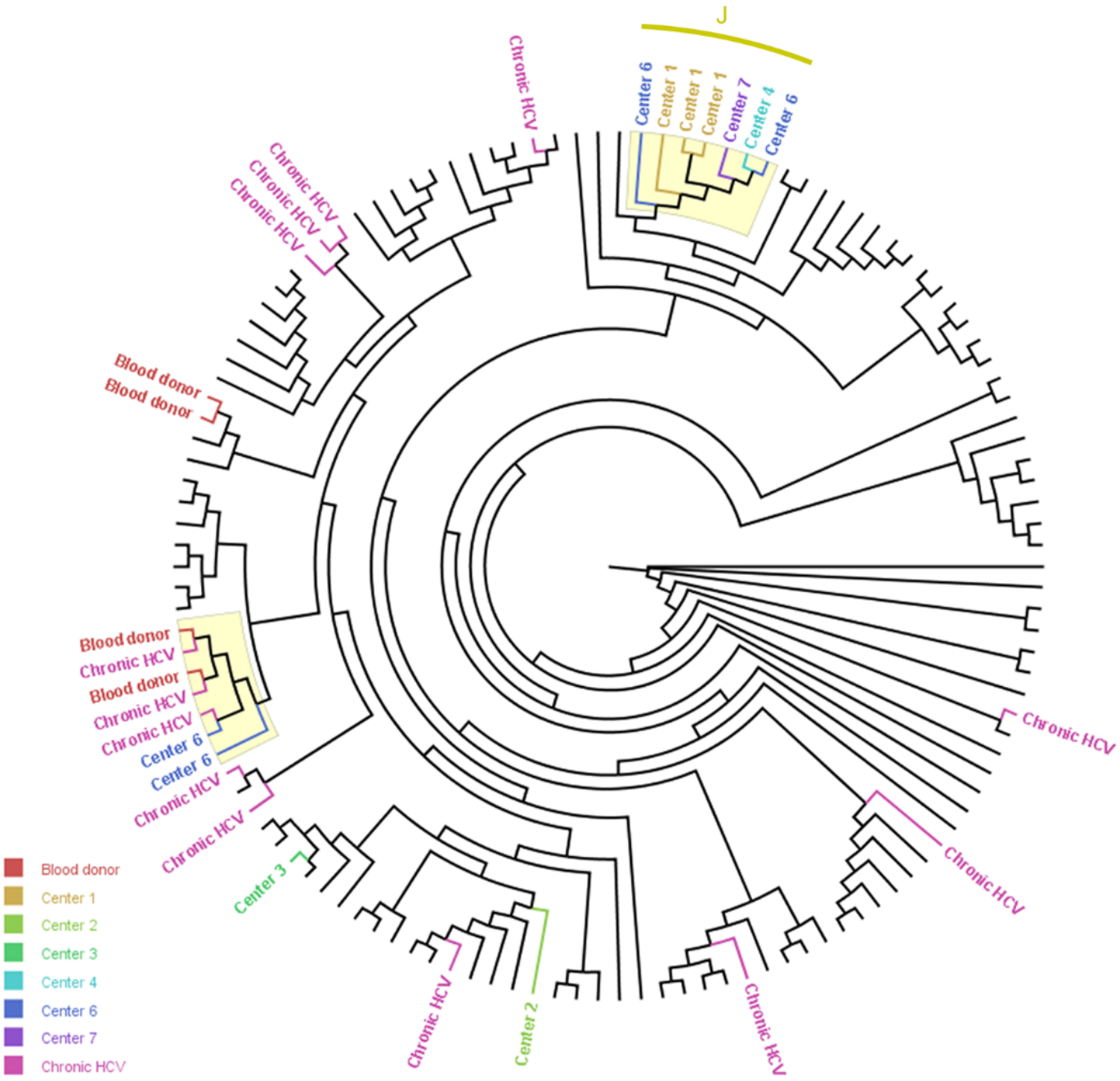


Sequences from different groups (including different dialysis centers) are colored differently. Control sequences obtained by HCV BLAST are shown in black. Clusters with aLRT > 0.9 are highlighted in yellow and clusters with aLRT > 0.8 are highlighted in green. IDU: injecting drug user.

The phylogenetic tree of subtype 1b sequences showed one cluster with aLRT > 0.9 consisting of seven sequences obtained from patients undergoing dialysis, of which three were from Center 1, two from Center 6, and one each from Centers 4 and 7 (Figure 2). In total, 63.6% (7/11) of sequences from dialysis patients with HCV subtype 1b showed phylogenetic clustering with aLRT > 0.9 (Figure 2).

The phylogenetic tree constructed using the NS5B sequences of HCV subtype 4d showed three clusters with aLRT > 0.9, 2 large clusters with aLRT > 0.8, and three clusters with aLRT < 0.8 (Figure 3). The largest cluster with aLRT > 0.9 consisted of 13 sequences

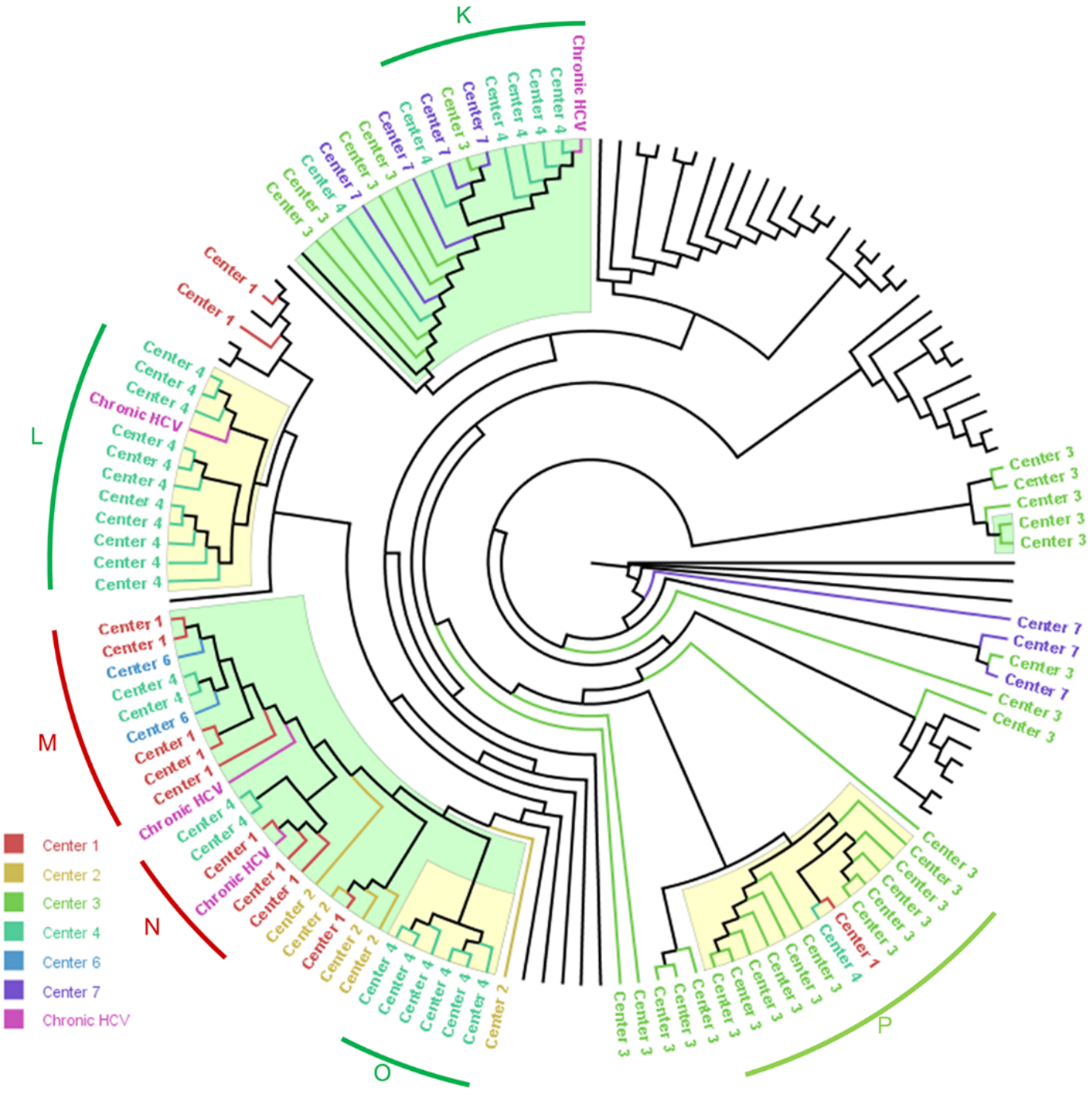
Figure 2. The maximum likelihood phylogenetic tree of HCV subtype 1b.



Sequences from different groups (including different dialysis centers) are colored differently. Control sequences obtained by HCV BLAST are shown in black. Clusters with aLRT > 0.9 are highlighted in yellow and clusters with aLRT > 0.8 are highlighted in green.

(13/84; 15.5%), of which 11 were from patients at Center 3, and one each from Centers 1 and 4. The second cluster with aLRT > 0.9 consisted of 11 sequences from patients at Center 4 (11/84; 13.1%). The third cluster with aLRT > 0.9 consisted of six sequences from patients at Center 4 (6/84; 9.5%). Those sequences were part of a larger cluster with aLRT > 0.8, which additionally included 20 sequences from dialysis patients (26/84; 31.0%). Among them, nine were from the Center 1, six from Center 6, five from Center 2, and four from Center 4. The second cluster with aLRT > 0.8 consisted of 15 sequences from dialysis patients (15/84;

Figure 3. The maximum likelihood phylogenetic tree of HCV subtype 4d.



Sequences from different groups (including different dialysis centers) are colored differently. Control sequences obtained by HCV BLAST are shown in black. Clusters with aLRT > 0.9 are highlighted in yellow and clusters with aLRT > 0.8 are highlighted in green.

17.8%), of which six were from Center 4, five from Center 3, and four from Center 7. In total, 28.6% (24/84) of sequences from dialysis patients with HCV subtype 4d showed phylogenetic clustering with aLRT > 0.9, and 76.2% (64/84) with aLRT > 0.8 (Figure 3).

In total, we detected 16 significant dialysis center-specific clusters with ≥ 5 dialysis patients and aLRT ≥ 0.80 (Table 2). The seven large clusters with n > 10 patients (cluster A, cluster C, cluster G, cluster H, cluster I, cluster L, and cluster P) predominantly consisted of sequences belonging to a single dialysis center with an average of 92% of sequences from the predominant center (range: 83.3–100%), indicating the HCV transmission occurred within the center (Table 2).

The nucleotide sequences of the partial NS5B region obtained from dialysis patients as well as the sequences obtained as local controls were deposited to GenBank (accession numbers MN316701 - MN317028).

Discussion

Phylogenetic analyses of HCV sequences obtained from patients undergoing dialysis at different dialysis centers in Kosovo confirmed nosocomial transmission as the main reason for the spread of HCV in this setting. Phylogenetic analysis was employed to study HCV transmission in several previous studies [21–23]. There are 8 genotypes and 67 subtypes of hepatitis C virus [24–26] and at least ten different viral proteins are synthesized during viral life cycle: the structural proteins include the core, the envelope glycoproteins E1, E2, and the non-structural proteins include the ion channel p7 and NS2, NS3, NS4A, NS4B, NS5A, and NS5B [27]. The NS5B region was shown to be useful for HCV genotyping and subtyping as well as for investigating HCV transmission by performing phylogenetic analysis [28–30].

The previous study among hemodialysis patients in Kosovo indirectly showed underlying conditions that contribute to the spread of HCV infection in dialysis centers in Kosovo. Different anti-HCV prevalence among seven dialysis units in Kosovo was observed, suggesting that specific factors within the dialysis centers could have affected the frequency of infection. Multivariate data analysis showed that the main factors contributing to anti-HCV positivity were duration of dialysis and utilizing dialysis services at more than one dialysis center in Kosovo [13].

The present study demonstrates that HCV sequences obtained from patients undergoing dialysis in Kosovo display a high degree of phylogenetic clustering of HCV subtypes 1a (99.4%), 1b (63.6%), and 4d (76.2%). Distinct phylogenetic clusters of sequences obtained from hemodialysis patients were observed for all three subtypes. In addition, several smaller clusters within the large clusters were detected, where the majority of sequences belonged to single dialysis center, confirming the nosocomial transmission of HCV among the dialysis patients. Center-specific clustering was especially apparent when observing the phylogeny of subtype 1a sequences, probably due to the more comprehensive sampling of this subtype. The clusters with the most individuals belonging to one dialysis center were from Centers 4 and 7 and

Table 2. Center-specific transmission clusters identified from subtype 1a, 1b, and 4d phylogenetic trees.

Subtype	Cluster	Dialysis patients n	Main dialysis center	Sequences from the main center n (%)	aLRT
1a	cluster A	18	Center 7	18 (100.0)	0.84
	cluster B	5	Center 1	3 (60.0)	0.80
	cluster C	26	Center 4	23 (88.5)	0.83
	cluster D	5	Center 3	4 (80.0)	0.86
	cluster E	6	Center 7	6 (100.0)	0.98
	cluster F	7	Center 2	6 (85.7)	0.84
	cluster G	16	Center 4	15 (93.8)	0.85
	cluster H	18	Center 2	15 (83.3)	0.92
	cluster I	14	Center 6	13 (92.9)	0.94
1b	cluster J	7	Center 1	3 (42.9)	0.99
4d	cluster K	8	Center 4	5 (62.5)	0.86
	cluster L	11	Center 4	11 (100.0)	0.97
	cluster M	9	Center 1	5 (55.6)	0.89
	cluster N	5	Center 1	3 (60.0)	0.89
	cluster O	6	Center 4	6 (100.0)	0.95
	cluster P	13	Center 3	11 (84.6)	0.91

aLRT: approximate likelihood ratio test.

interestingly, these two centers were found to have the highest anti-HCV prevalence (68.7% and 91.1%, respectively) among the centers in Kosovo as determined in a previous study [13]. The centers with the lowest anti-HCV prevalence were Center 1 (22.3%) and Center 5 (33.3%) [13]. Indeed, we did not identify any significant center-specific clusters for Center 5, and only a few small clusters for Center 1 with a lower proportion of Center 1 sequences among the clustered sequences (≤60%). This could indicate that the HCV transmission could have occurred outside these two centers or could simply be due to the smaller number of sequences from patients at Centers 1 and 5 that were included in this study. Nevertheless, even though HCV transmission could have occurred in these two centers, significantly less forward transmission was observed. This suggests that either more suitable prevention measures had been in place at these two centers, which was also observed in a previous study, or, the patient population at these centers had different characteristics [13]. Patients at Centers 4 and 7 were at a greater risk of nosocomial transmission because the Centers had reported a shortage of sterile gauze and disinfectant, and furthermore patients did not have anti-HCV tests available to them prior to dialysis [13].

In our phylogenetic analysis we included local control sequences from IDUs in Kosovo. We observed a cluster of HCV subtype 1a sequences obtained from IDUs that was markedly distinct from the majority of the sequences from dialysis patients, suggesting that HCV is not transmitted due to spillover from IDUs. Spillover from IDUs might had occurred only for a separate cluster of 1a sequences, in the most part originating from Center 6. There, the original transmission of HCV could have resulted from a patient with a history of IDU who had undergone dialysis.

The majority of large clusters and most of the smaller, predominantly local clusters, encompassed sequences from more than one dialysis center. This, together with the finding of the previous study demonstrating that HCV infection was more frequently associated with history of receiving dialysis at more than one center, indicates HCV transmission occurred across multiple centers in Kosovo.

Significant efforts to prevent further HCV transmission at dialysis centers in Kosovo are needed. The first step should be to declare the current high rate of HCV transmission in dialysis units a public health emergency. These subsequent steps should follow: (i) development of a sustained strategy to significantly increase human resources and improve dialysis facilities; (ii) provision of sufficient funds to assure a permanent supply of consumables for daily work (including gloves and sterile gauze); (iii) regular serological and molecular screening of all dialysis patients; (iv) strict implementation of infection control practices, followed by routine evaluations and on-going training of staff; and (v) treatment of all HCV-infected dialysis patients with pan-genotypic direct-acting antivirals.

Conclusion

Phylogenetic analysis of HCV subtype 1a, 1b and 4d sequences obtained from patients receiving dialysis at the seven centers in Kosovo revealed significant transmission clusters. The majority of sequences belonged to clusters, confirming that high HCV infection rate among dialysis patients in Kosovo is probably due to nosocomial transmission during dialysis. This finding calls for significant changes in the management of dialysis patients to prevent further HCV transmission at dialysis centers in Kosovo.

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Authors' contributions

XJ designed and coordinated the study and drafted the manuscript. JM and MML participated in study coordination, data collection, data analysis, and drafting the manuscript. IR and LR contributed to the study design, data acquisition, and drafting the manuscript. NPT participated in the study design and manuscript revision. MP designed and supervised the study, data acquisition, data analysis, manuscript preparation, and the final manuscript revision. All authors read and approved the final version of the manuscript.

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