Tracing the epidemic history of hepatitis C virus genotypes in Saudi Arabia

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Abstract

HCV genotype 4 is highly prevalent in many Middle Eastern countries, yet little is known about the genotype's epidemic history at the subtype-level in this region. To address the dearth of data from Saudi Arabia (SA), we genotyped 230 HCV isolates in the core/E- and NS5B-region and analyzed using Bayesian phylogenetic approaches. HCV genotype 4 (HCV/4) was positive in 61.7% (142/230) of isolates belonging to 7 different subtypes with the predominance of type 4 (HCV/4) was positive in 61.7% (142/230) of isolates belonging to 7 different subtypes with the predominance of genotype 4d (73/142; 51.4%) followed by 4a (51/142; 35.9%). Phylogenetic analysis also revealed a distinct epidemiological cluster of HCV/4d for Saudi Arabia. HCV/1 appeared as the second most prevalent genotype positive in 31.3% (72/230) of isolates with the predominance of 1b (53/72; 73.6%) followed by 1a (16/72; 22.2%), and 1g (3/72; 4.1%). A small proportion of isolates belonged to HCV/3a (12/230; 5.2%), and HCV/2a (4/230; 1.7%). We estimate that the genotype 4 common ancestor existed around 1935 (1850–1985). Genotype 4 originated plausibly in Central Africa and multiple subtypes disseminated across African borders since ~1970, including subtype 4d which dominates current HCV infections in Saudi Arabia. The Bayesian skyline plot (BSP) analysis showed that genotype 4d entered the Saudi population in 1900. The effective number of HCV infections grew gradually until the second half of the 1950s and more rapidly until the early-80s through the use of imported blood units and blood products. Subsequently, the rate of HCV infection in the Saudi Arabian population was stabilized through effective screening of blood and infection control measures.

1. Introduction

An estimated 170 million people worldwide possess hepatitis C virus (HCV) infection. Carrying infection over decades can lead to liver cirrhosis and hepatocellular carcinoma. Hepatitis C virus belongs to the Flaviviridae family of RNA viruses and is considered to have a high spontaneous mutation rate (Khan et al., 2009; Pybus et al., 2009; Tanaka et al., 2002). This is because of the lack of a proofreading capacity of the viral non-structural gene 5B (NS5B) encoded viral RNA-dependent RNA polymerase. HCV has been classified into seven major genotypes and numerous subtypes on the basis of approximately 70% nucleotide sequence homology (Nakano et al., 2012). It has been found that the mode of transmission, population movements, and as well as the geographic position of an area determine the distribution of a particular HCV genotype. HCV genotypes or subtypes distribution, therefore, can change over time and it alleviates the importance of genotyping as the gold standard to investigate the spread of HCV within a community (Pybus et al., 2001).

The uniqueness of HCV genotypes and/or subtypes with respect to their response to therapy and chronic outcome of HCV infection is now well established (Foster et al., 2015; Cane et al., 2015; Yoshida et al., 2015). In North America, Europe, and Australia, genotype 1, 2, and 3 are common (>80%) among HCV-infected patients (Kamal and Nasser, 2008). Similarly, HCV genotype 4 (HCV/4) is responsible for >80% of HCV infections in the Middle East and Africa (Khattab et al., 2011). The prevalence of HCV in Saudi Arabia is considered from 1 to 3% with predominant HCV/4 infections (Shobokshi et al., 1999). In Egypt, the country with highest HCV prevalence (>15%) in the world, HCV/4 is responsible for 90% of all hepatitis C infections (Al Ashgar et al., 2013). However, unlike a good therapeutic response rate observed in Egyptian patients (Khattab et al., 2011), the therapeutic response rate among Saudi patients is not as promising (Al Ashgar et al., 2009; Alfafeh et al., 2004). It has been
reported that HCC is the 2nd most cause of cancer among the Saudi population (Pouсти et al., 2010) and it is yet to define the causes for such a high rate of HCC.

Despite the recent development of greatly effective, interferon-free anti-hepatitis C virus (HCV) drugs, the global burden of this pathogen remains enormous. Control or eradication of HCV still requires the effective and comprehensive measures to prevent virus transmission, which could be facilitated by precise molecular epidemiological tools. Acute HCV infection is difficult to detect in many cases due to unspecific symptoms and as a result HCV transmission in real time cannot be established. As a consequence, contemporary viral sequences have been utilized to estimate viral divergence and dissemination in different parts of the world. HCV phylogenetics using phylogenetic and coalescent-based methods made possible to estimate the viral effective population size through time for a variety of HCV subtypes that caused twentieth-century epidemics (Iles et al., 2014; Jackowiak et al., 2014; Khan et al., 2011; Magiorkinis et al., 2009). Although Central Africa and the Middle East hold a high burden of HCV/4 infections caused by multiple of its subtypes, the current distribution and the geographic source of these lineages is unknown. HCV/4a spread in Egypt has been described as ‘local epidemic’ (Iles et al., 2014) but apart from that, there is no study addressing HCV spread in the rest of Middle Eastern countries.

The present study was aimed to evaluate the distribution of HCV genotypes/subtypes among Saudi chronic liver disease patients and increase our understanding of the spread of different HCV subtypes in Saudi Arabia by using a molecular epidemiological approach. To our knowledge, this is the first study to have used molecular evolutionary analysis to describe the spread of HCV in Saudi Arabia.

2. Materials and Methods

2.1. Study population

The consecutive Saudi patients (n = 230), positive for hepatitis C virus antibodies (anti-HCV), attending the outpatient and inpatient clinics of King Fahad Hospital, Riyadh from January 2011 to April 2011 were enrolled in this study. Serum samples collected were tested for anti-HCV, attending the outpatient and inpatient clinics of King Fahad Hospital, Riyadh from January 2011 to April 2011 were enrolled in this study. Serum samples collected were tested for anti-HCV, attending the outpatient and inpatient clinics of King Fahad Hospital, Riyadh from January 2011 to April 2011 were enrolled in this study. Serum samples collected were tested for anti-HCV, attending the outpatient and inpatient clinics of King Fahad Hospital, Riyadh from January 2011 to April 2011 were enrolled in this study. Serum samples collected were tested for anti-HCV, attending the outpatient and inpatient clinics of King Fahad Hospital, Riyadh from January 2011 to April 2011 were enrolled in this study.

2.2. Reverse transcription-PCR, detection of HCV-RNA and sequencing

Total RNA was extracted from the serum samples using the Qiagen Nucleic acid extraction kit (Qiagen) in accordance with the manufacturer’s protocol. Viral RNA was reverse transcribed into complementary DNA using SuperScript II RNase HReverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random Hexamer primer as described previously [Ohno et al., 1997]. Confirmation of the presence of HCV RNA in the samples was carried out by amplifying the highly conserved 5′ UTR region with primers, KK30 (5′-CTGTCTTCAC GCACAAAGGC-3′ and KM3 (313-294): 5′-CCTGCAGACCGCCCTTATCA-3′. Samples positive for HCV RNA were subsequently amplified in the partial core/E1 and NS5B regions of the partial NS5B region of our sequences. The primers used to amplify samples in the NS5B region were: NS5B_8278S (8258-8278): 5′-GTACCTGATTCCCTG-3′. The primers used to amplify samples in core/E1 region were: HVE1-s1 (834-859): 5′-GCAACAGGAAAT TTCYGCTTGCCTC-3′ and HVE2-as1 (1299-1321): 5′-GACATCTTCT CATCAIATCCA-3′ (Tanaka et al., 2002). Amplicons obtained were sequenced directly with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer.

2.3. Sequence assembly

Sequences obtained in this study were aligned with a set of reference sequences downloaded from Los Alamos sequence database representing each known genotype and subtype. Based on this primary result, homologous blast search was carried out for each genotype/subtype to determine the most closely related sequences. To have a composite phylogenetic tree we randomly removed sequences to create an alignment having a representation of each subtype and closely matched to study sequences. We developed this dataset proportional to these newly isolated sequences, keeping in view that homologous sequences (high base identities and score) stay in the final alignment.

2.4. Phylogenetic analysis

Phylogenies were estimated for the partial core/E1 and NS5B genome alignments using maximum likelihood (ML) as implemented in Mega v.6. Our analysis used a Kimura 2 nucleotide substitution model, uniform rates, and a gamma distribution model of among-site rate variation. This model was used after model comparison test implemented in Mega v.6. Models with the lowest BIC scores (Bayesian Information Criterion) was considered to describe the substitution pattern the best (Tamura et al., 2013). Phylogenetic clustering was statistically calculated using an ML bootstrap approach with 1000 replicates. Phylogenies were visualized and annotated using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree).

2.5. Bayesian evolutionary analysis

To reconstruct the evolutionary history of HCV/4 in Saudi Arabia we built an alignment of 338 nucleotides of the partial NS5B region of HCV genome using Saudi HCV-4 isolates and selected HCV/4 reference sequences with good temporal information (e.g. the year of sampling and the country of origin) by surveying online databases (Los Alamos HCV sequence database, GenBank, and DDBJ), associated primary literature and/or published elsewhere. We used uniform prior distribution between 0 and 100 for our analysis. To measure the temporal evolutionary signal in the dataset prior to molecular clock analysis, we employed program Path-O-Gen v1.4 (available from http://tree.bio.ed.ac.uk) to estimate evolutionary rates specific to the NS5B genome region of our sequences. Evolutionary rates were then estimated using Bayesian Markov Chain Monte Carlo (MCMC) inference framework implemented in BEAST v1.8.0 (Drummond et al., 2012). Our analyses employed an SDR06 nucleotide substitution model, an uncorrelated lognormal relaxed molecular clock model, and a Bayesian skyline plot models. In Bayesian model selection tests, SDR06 clearly outperformed GTR + F model. Bayes factors were compared and model comparison test was run in Tracer v1.6 [Suchard et al., 2001]. To measure the evolutionary change over time in our samples, we used both strict and relaxed (lognormal) molecular clock models and all included gamma-distributed rate.

We used uniform prior distribution between 0 and 100 for our analysis. Since we were unable to precisely estimate the evolutionary rates from the alignments due to narrow sampling times and minimum sequence length. As reported earlier by [Iles et al., 2014], normal prior distribution was used because the substitution rate matched the analyzed sequence estimates. Each MCMC run contained 100 million samples, sampled every 10,000 states. At least two MCMC runs were performed to ensure convergence and combined using log-combiner for better accuracy of parametric estimates. The obtained trees were summarized using TreeAnnotator v1.8.0 (Drummond et al., 2012) and Maximum clade credibility tree was visualized and annotated in FigTree v1.4.2.

2.6. HCV/4d transmission history

Maximum likelihood phylogenetic analysis was performed solely on the HCV/4d isolates in order to determine the epidemiology of this lineage geographically. Furthermore, Bayesian MCMC analysis was performed on
distinct Saudi HCV/4d cluster to estimate the epidemic history and basic reproductive number since its introduction in Saudi Arabia. BEAST model settings were the same as above except the use of the Bayesian skyline plot coalescent model (Shapiro et al., 2006).

2.7. Statistical analysis

Statistical differences were evaluated using one-way analysis of variance. A p-value of < 0.05 was considered statistically significant. The analysis was carried out using the Graph Pad Software (Graph Pad Prism5).

3. Results

3.1. Patients

Out of 230 patients, 46.1% (106/230) were male and 53.9% (124/230) were female. The median age of the patients was 59 years with ages ranging from 4 to 84 years having no significant difference between male and female. ALT and AST levels were relatively high in male patients but didn’t reach statistical significance (Table 1).

3.2. Phylogenetic analysis

Maximum-likelihood phylogenies estimated from the core/E1 and NS5B alignments. Genotype and sub-genotype reference sequences were chosen by blast search and it was ensured that closely matched, formally defined subtype sequences stay in final alignment. Fig. 1 shows the ML phylogeny in the core/E1 region. HCV positive samples sequenced in this study were corresponding to four different HCV genotypes, that is, genotype 1 (72/230; 31.3%), genotype 2 (2c) (4/230; 1.7%), genotype 3 (3a) (12/230; 5.2%), and genotype 4 (142/230; 61.7%). Table 1 summarizes the distribution of HCV genotypes and subtypes determined in this cohort. Genetically diverse HCV genotype 4 consisted of six different subtypes, that is, 4d (73/142; 51.4%), 4a (51/142; 35.9%), 4r (11/142; 7.7%), 4l (1/142; 0.7%) and two cases (2/142; 1.4%) each of 4n, 4o and an unassigned HCV/4 subtype. The subtype distribution within HCV/1 cases was 1a (16/72; 22.2%), 1b (53/72; 73.6%), and 1g (3/72; 4.1%). HCV/3 was significantly higher in male as compared to female (p = 0.01). A relatively high percent of male cases was infected with HCV/4d and 4a. Strains of HCV/4o and unassigned HCV/4 subtype were exclusively isolated from male patients while HCV/4l and 4n from female patients. Phylogenetic analysis revealed a distinct epidemiological cluster of HCV/4d for Saudi Arabia. The closest matched strains of HCV/4d were from France in NS5B region and the UK in core/E1. HCV/4a, 1a, and 1b strains were more closely related to the strains from Egypt, the United States, and Japan, respectively. The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers LC109103-LC109261 and LC109975-LC11078.

Table 1

Demographic and clinical features of Saudi chronic liver disease patients infected with a variety of HCV genotypes (subtypes).

<table>
<thead>
<tr>
<th>Features</th>
<th>Total (n = 230)</th>
<th>Male (n = 106)</th>
<th>Female (n = 124)</th>
<th>p</th>
<th>&lt;0.05</th>
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<tr>
<td>Age (years)</td>
<td>59 (8–84)</td>
<td>60 (8–84)</td>
<td>58 (19–79)</td>
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<tr>
<td>ALT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.82 ± 65.9</td>
<td>68.81 ± 71.76</td>
<td>50.12 ± 59.74</td>
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<tr>
<td>AST&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.19 ± 187.2</td>
<td>96.24 ± 233.2</td>
<td>64.97 ± 137</td>
<td>NS</td>
<td></td>
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<tr>
<td>ALP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120.3 ± 83.19</td>
<td>124.4 ± 78.54</td>
<td>117.2 ± 87.07</td>
<td>NS</td>
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<tr>
<td>T. Bil.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.46 ± 67.03</td>
<td>34.99 ± 68.84</td>
<td>24.85 ± 65.40</td>
<td>NS</td>
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</table>

HCV Genotypes (subtypes)<sup>c</sup>

| 1 | 16 (6.9) | 11 (10.4) | 5 (4) | NS |
| 1a | 53 (23) | 25 (23.6) | 28 (22.6) | NS |
| 1b | 3 (1.3) | 1 (0.9) | 2 (1.6) | NS |
| 2 | 2 (0.9) | 0 | 2 (1.6) | ND |
| 2a | 0 | 2 (1.6) | ND |
| 2c | 0 | 2 (1.6) | ND |
| 3 | 12 (5.2) | 10 (9.4) | 2 (1.6) | 0.01 |
| 3a | 21 (9.8) | 30 (24.2) | NS |
| 4 | 73 (31.7) | 45 (36.3) | NS |
| 4a | 1 (0.4) | 1 (0.8) | ND |
| 4n | 2 (0.9) | 2 (1.9) | ND |
| 4o | 1 (0.4) | 5 (4) | NS |
| Unassigned subtype | 2 (0.9) | 2 (1.9) | 0 |


<sup>a</sup> Median (interquartile range).

<sup>b</sup> Mean ± SD.

<sup>c</sup> n (%).

3.3. Maximum clade credibility phylogeny

We employed a range of coalescent models with SDR06 substitution model and an uncorrelated lognormal relaxed molecular clock. The constant size coalescent model outperformed other models. The mean nucleotide substitution rate (subs/site/year) was 5.12 × 10<sup>−3</sup> (95% CI: 3.89 × 10<sup>−3</sup>, 6.39 × 10<sup>−3</sup>). Fig. 2 is the maximum clade credibility (MCC) phylogeny obtained by Bayesian molecular clock analysis of HCV-4 sequences in the partial NS5B region. The date of the genotype 4 common ancestor was 1935 with a wide 95% HPD credible region (1850, 1985) likely reflecting the reduced phylogenetic information. MCC phylogeny was also used to estimate the date of origin of clearly discernable 5 clusters (indicated as C1, C2, C3, C4, and C5) that enclosed multiple sequences from this study. The distinct Saudi cluster of HCV/4d was separable from its ancestor French cluster by a posterior probability value of >0.70 and its sub-clusters C1 and C2 also diverged with a posterior probability value of >1. Cluster C1 enclosed 36 isolates from this study. The estimated time of MRCA of this cluster was 1997 (95% HPD: 1978, 2007) while MRCA for its sub-clusters (C1a and C1b) was dated to 2000 (1986, 2008). Cluster C2 contained 5 sequences from this study and

<table>
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<td>VN</td>
<td>Vietnam</td>
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</table>

Table 2

Country codes following ISO 3166 used in this study to refer samples.
MRCA was dated to 2000 (95% HPD: 1984, 2006). Cluster C2 appeared as a trajectory of a previously isolated Saudi strain whose MRCA was dated to 1993 (95% HPD: 1971, 2009). Clusters C3 and C4 were present in subtype 4a; each contained 12 new Saudi isolates, however, did not appear with high posterior values. The estimated MRCA was dated to 1999 (95% HPD: 1984, 2008) and 2000 (95% HPD: 1985, 2007), respectively. Finally, cluster C5 was present in the subtype 4r and comprised 6 isolates from this study. The estimated time of MRCA for this cluster was 2002 (95% HPD: 1989, 2007).

Along with many Saudi isolates that appeared outside of these five clusters, two isolates may be grouped under a new subtype (provisionally named as 4s) supported by high posterior probability N0.9 in MCC phylogeny and clearly discerned in the ML phylogeny of Core/E1 region. These isolated were closely related to some of the sequences from Tunisia and Europe. The estimated time of MRCA of this subtype was 1989 (95% HPD: 1958, 2004) while MRCA of Saudi isolates was somewhat more recent dated to 2001 (95% HPD: 1983, 2011).

3.4. HCV/4d spread analysis

Maximum likelihood phylogeny of HCV/4d (Fig. 1s) shows this lineage has been endemic in France and Netherlands. It seems that HCV/4d spread in Saudi Arabia earlier than Turkey. The Bayesian skyline plot summarizes the spread and epidemic growth of HCV/4d in Saudi Arabia.
(Fig. 3). This plot describes the effective number of HCV infections through time, back to the estimated time of MRCA of this subtype. It shows that genotype 4d entered the Saudi population in 1900 and the effective number of infections grew gradually until the second half of the 1950s and more rapidly until the early-1980s when it reached a plateau that still persists.

4. Discussion

Viral gene sequences constitute a potentially significant source of information about epidemiological processes. HCV genotypes and even subtypes show different distribution patterns and phylogenetic analysis has been used as a successful tool to describe the historical movement of viral lineages worldwide (Magiorkinis et al., 2009; Pybus et al., 2009; Tanaka et al., 2006). The Middle East is geographically vast and out, HCV spread in this region is largely unknown. The only country in this region, which has been surveyed for HCV spread is Egypt, where it appeared to be a local epidemic as an aftermath of the antischistosomal campaign (Frank et al., 2000). In this study, we report HCV genetic diversity in Saudi Arabia based on phylogenetic analysis of NS5B and core/E1 region, which are considered consensus regions to genotype HCV with confidence in the absence of complete genome sequences (Smith et al., 2014). HCV genotypes that are, HCV/1 and HCV/4 stood out as the predominant infectious HCV genotypes, causing a combined ~92% of all infections in this study. HCV/4 showed a large phylogeographic diversity, with a total of seven subtypes (subtype 4d, 4a, 4r, 4o, 4l, 4n and an unassigned subtype 4). The strains of this unassigned subtype may have diverged from its ancestral subtype 4o in 1989 (95% HPD: 1958, 2004) with a high posterior probability value >1 in MCMC phylogeny. However, full genome sequencing of these 4s isolates would confirm their subtype status, since subtype assignments can only be made (as confirmed assignments) when sequence data from three or more isolates including at least one complete or nearly complete coding region is performed (Smith et al., 2014).

Our estimates about HCV/4 emergence and dissemination differed from previous report rates of (Iles et al., 2014) largely because their estimates were based on concatenated sequence alignment generated by combining NS5B and Core sequences while our study results were based solely on the alignment of a partial NS5B region. However, the molecular clock and phylogeographic estimates well coincided with the key finding of HCV/4 emergence in Central Africa and subsequent dissemination in North Africa, Europe, and the Middle East (Iles et al., 2014). In the wake of the catastrophic events like World War II (1943–45) and Congo crisis (1960–65) which brought humanitarian crises in the form of vast population movements within and outside of Africa, troops close association with local populations with a high likelihood of parenteral medical treatments and surgical procedures paved the way for the spread of HCV across African borders (Iles et al., 2014; Mays, 2010; Nigel, 1991).

The ML phylogenies in core/E1 region and the MCMC phylogeny in the NS5B region revealed not only two sub-clusters of HCV/4a but a distinct phylogenetic cluster of HCV/4d in Saudi Arabia. The closest matches of HCV/4a strains were from Egypt point to labor dependency and strong socio-religious ties with Egypt. The estimated MRCA analysis of these cluster points to previous findings where the spread of HCV/4a has been shown during the 1980s in Egypt as part of the anti-schistosomiasis vaccination campaign. The closest matches of Saudi HCV/4d isolate in the NS5B region were strains reported from France, and in the core/E1 region, they resembled more closely to strains reported from the UK. This is to note that HCV/4d has been associated in explosive HCV outbreak in high-risk groups (de Bruijn et al., 2009). In order to investigate the origin and spread of HCV genotype 4d in KSA, we estimated divergence dates and demographic parameters in a Bayesian coalescent framework (Duchene et al., 2015). The Bayesian skyline plot population dynamics analysis showed that genotype 4d spread explosively in Saudi population during the 1950s and grew rapidly until the early-1980s when it reached a plateau that still persists. These time frames lead to the finding that the epidemic history of 4d in Saudi Arabia may be dated back to the history of medical services and blood banking in Saudi Arabia which started in the 1950s (http://www.slideshare.net/SUSRIS/saudi-health-and-medical-services). Because of the un-flourished culture of blood donation in Saudi Arabia, blood units were imported fortnightly, mainly from France and the United States of America. As these units were received as finished products, no tests were carried out again for transfusion transmitted infection on arrival in Saudi Arabia. The blood units were issued for...
transfusion after the necessary cross-match with the recipient. We spec-
ulate that HIV/4d epidemic spread in Saudi Arabia is transfusion-mediat-
ed through these imported blood products. A break in explosive nature of
HCV transmission during the 90s has been shown in Fig. 3, mainly corre-
ponding to the period in which the source of blood shifted dramatically
from imported blood to locally recruited blood donors (Abdel Gader et al.,
2011). A steady HCV spread afterward may be attributed to other modes of
transmission like intravenous drug use, sexual and/or the use of unsteri-
lized surgical and dental equipment.

Consistent with our finding for HCV/4d, the HCV/1 transmission may also
be linked with the blood import from the US. Our results also support the
findings of other HIV-related studies, which show that imported blood from
Europe and the US lead to the transmission of HIV in Saudi Arabia and other Gulf states (Alrajhi, A.A., 2004. Human immuno-
Alfa l e h, F.Z., Hadad, Q., Khuroo, M.S., Aljumah, A., Algamdi, A., Alashgar, H., Al-Ahdal, M.N.,

	ransitivity analysis. The authors also would like to commend Ms. Zoe P.

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Of particular concern as they may be refractory to currently

available therapeutic regimens and could potentially spread epidemically

if introduced into high-risk groups.

In conclusions, transmission of HCV in Saudi Arabia may be linked to

the history of blood transfusion in this country and other socioeconomic

groups of population movements. Divergent yet uncommon

strains is of particular concern as they may be refractory to currently

available therapeutic regimens and could potentially spread epidemically

if introduced into high-risk groups.

Supplementary data to this article can be found online at http://dx.

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