Review Article

Emerging resistance to directly-acting antiviral therapy in treatment of chronic Hepatitis C infection—A brief review of literature

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Abstract

Hepatitis caused by Hepatitis C virus (HCV) is a major cause of chronic liver disease. HCV is transmitted by injection drug use, blood transfusion, hemodialysis, organ transplantation and less frequently sexual intercourse. It has been recognized as a global health problem because of the progression to cirrhosis and hepatocellular carcinoma. Globally, about 170 million people are infected with HCV. Since the discovery of this virus in 1989, the clinical management of chronic hepatitis C infection has undergone a paradigm shift from alpha interferon to direct-acting antiviral (DAA) therapy. However, resistance to many of these antiviral agents has been reported increasingly from all over the globe. This review article focuses on the emerging HCV resistance to DAAs and the relevance of *in vitro* DAA resistance testing in clinical practice.

Keywords: Directly acting antiviral, Hepatitis C virus, resistance

Introduction

Hepatitis C virus (HCV) is a single-stranded RNA virus belonging to Hepacivirus genus of the Flaviviridae family. It causes hepatitis C which is a major chronic liver disease. Hepatitis C is transmitted by injection drug use, blood transfusion, hemodialysis, organ transplantation and, less frequently, sexual intercourse. It has been recognized as a global health problem because of the progression to cirrhosis and hepatocellular carcinoma. Globally, about 170 million people are infected with HCV. Since the discovery of this virus in 1989, the clinical management of chronic hepatitis C infection has undergone a paradigm shift from alpha interferon to directly acting antiviral (DAA) therapy.[1] However, resistance to many of these antiviral agents has been reported increasingly from all over the globe. The aim of this review article is to focus on the emerging HCV resistance to DAAs and the relevance of *in vitro* DAA resistance testing in clinical practice.

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Structural organization of Hepatitis C virus

HCV is an enveloped virus harboring a 9.6 kb positive-sense single-stranded RNA. The genome carries a long open reading frame (ORF) encoding a polyprotein precursor of 3010 amino acids. Translation of the HCV ORF is directed via a 340-nucleotide-long 5' nontranslated region (NTR) functioning as an internal ribosome entry site (IRES) which permits the direct binding of ribosomes in close proximity to the start codon of the ORF. The HCV polyprotein is cleaved co- and posttranslationally by cellular and viral proteases into ten different products which are broadly classified as structural [core (C), E1, and E2)] and nonstructural (NS2-5) replicative proteins.[2] Figure 1 shows the structural organization of the HCV genome and the various proteins encoded by it. A summary of various HCV proteins is been depicted in Table 1.

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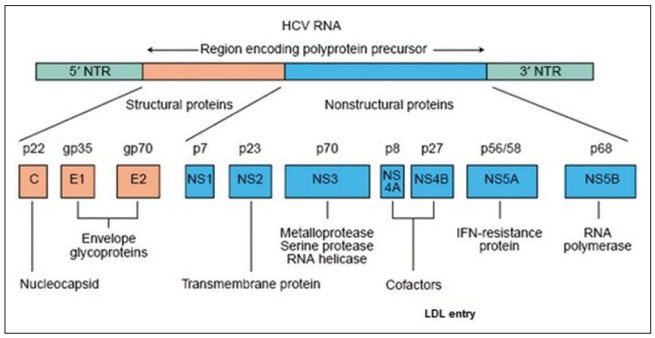

Figure 1: Structural organization of HCV genome[3]

Natural history of HCV infection

Infection with HCV can present either as acute or chronic hepatitis. Acute hepatitis is usually asymptomatic and rarely leads to hepatic failure. Symptomatic acute HCV infection has a mild clinical course with less than one fourth (<25%) of patients presenting with jaundice. About 60–80% patients with acute infection develop chronic infection.[23] The rate of spontaneous viral clearance in patients with chronic HCV is very low. Approximately, one fifth (20–30%) of patients with chronic HCV infection develop cirrhosis over a period of 10–30 years.[24] 1–4% of cirrhotic patients develop hepatocellular carcinoma per year.[25] There are several factors which may determine the rapidity of disease progression which include: HCV acquisition at an advanced age (>40–55 years), male sex, HIV co-infection, high body mass index, presence of hepatic steatosis, and alcohol consumption. Patients with cirrhosis can decompensate with complications like portal hypertension, ascites, spontaneous bacterial peritonitis, esophageal varices, hepatic encephalopathy and hepatic coma, and hepatorenal syndrome leading to renal failure. Chronic infection can also be associated with extra-hepatic manifestations such as cryoglobulinemia, porphyria cutanea tarda, arthralgia, membranoproliferative glomerulonephritis, Sjogren's syndrome, Raynaud's syndrome, idiopathic thrombocytopenic purpura, and non-Hodgkin's lymphoma.[26]

Problem statement

The global prevalence of HCV infection is estimated at 2–3%.[27-30] The prevalence of HCV is highest in Africa and the Middle East. China, India, Egypt, Pakistan, and Indonesia account for approximately half of the global HCV-infected subjects.[31-34] North America, Australia, Japan, and Northern and Western Europe report lower prevalence of HCV infection, with no country showing a rate >2%. Owing to the paucity of large-scale prevalence studies on hepatitis C in the general population of India, the reported prevalence rates are variable ranging from 0.09–2.02%.[35]

The genetic diversity of the virus further complicates the epidemiology of HCV infection. HCV is classified into 7 genotypes (1–7) which differ by more than 30% sequence diversity and at least 67 subtypes, characterized by about 20% sequence divergence.[36,37] Identification of HCV genotypes and subtypes plays a crucial role in defining the epidemiological patterns and effective treatment. Genotype 1 is distributed worldwide and is responsible for majority of the cases reported in the Americas, Europe, Australia, and Japan. Genotype 1a is distributed widely in Northern Europe and United States. Genotype 1b is commonly found in Europe and Asia. Although, genotype 2 is more prevalent in industrialized countries, it is also found in South America and Asia. Genotype 2a has commonly been isolated from Japan and China. Genotype 2b is widespread in Northern Europe and United States. Genotype 2c is the most common subtype found in Western and Southern Europe, Pakistan, and India. Genotype 3a is prevalent in Europe, United States, Australia, and Southern Asia.[38-40] Genotypes 4 and 5 are mainly found in Africa and the Middle East. While genotype 4a is prevalent in Egypt, genotype 4c is highly prevalent in Central Africa. Genotype 5 has mainly been reported from South Africa. Genotype 6 and its numerous subtypes are found mainly in Southeast Asian countries like Thailand, Vietnam, and Myanmar. Genotype 7 has minor clinical relevance and has been recently found in patients from Central Africa and Thailand.[30,40-46]

Treatment of chronic Hepatitis C—An evolutionary perspective

The treatment of chronic hepatitis C virus infection has been rapidly evolving since early 1990s with interferon-alfa. This injectable drug worked by boosting the immune system instead of specifically attacking the virus. However, the response rates of this drug were low, ranging from 10 to 20%. In 1998, the oral drug *ribavirin* was added to interferon. The therapeutic response rates increased dramatically, averaging 37 to 43%. In 2002, pegylated interferon-alfa was approved for treatment of chronic hepatitis C. Combined with ribavirin, permanent response rates of 52% were obtained with this drug. In 2011, two protease inhibitors were introduced namely boceprevir and telaprevir.[47] Treatment of chronic HCV with PEG-IFN and ribavirin and, more recently, with PEG-IFN plus ribavirin and first-generation protease inhibitors (Telaprevir and Boceprevir) for HCV-1, have been the standard of care. However, favorable clinical outcomes could not be achieved in majority of the patients.[48,49] Apart from the several side effects which are associated with interferon therapy, there are various contraindications like pregnancy, breast feeding, and allergic reactions. The recent development of a series of new direct antiviral agents (DAAs) that are directed against HCV target proteins used by the virus for its replication and assembly has ushered a new era in the treatment of hepatitis C.[50] The DAAs are molecules that target specific nonstructural proteins of HCV thereby interfering with viral replication and infection. There are four classes of DAAs, which are defined by their mechanism of action and therapeutic targets [Table 2].[51]

HCV resistance to DAAs

HCV can develop resistance to DAAs due to the lack of proof-reading activity by RNA dependent RNA polymerase coupled with the high replication capacity of the virus. This leads to the generation of large number of genetically distinct

Table 1: Summary of HCV proteins[4-22]

Proten	Type	Functions
HCV core protein	Structural	Makes up the viral nucleocapsid. Affects host cell functions like gene transcription, lipid metabolism, apoptosis, and various signaling pathways Directly or indirectly involved in hepatocarcinogenesis and steatosis hepatitis
Envelope proteins E1 and E2	Structural	Play an important role in cell entry. E1 serves as the fusogenic subunit. E2 acts as the receptor binding subunit of the HCV envelope.
P7 protein	Structural	P7 has characteristics similar to those of a group of proteins called viroporins. Forms ion channels that play an essential role in virus infection. Essential for virus particle assembly and release of infectious virions in a genotype specific manner
NS2 protein	Nonstructural	Essential for completion of the viral replication cycle *in vitro* and *in vivo*.
NS3 protein	Non-structural	It is a multifunctional protein whose N & C terminals have serine protease & NTPase/helicase activities, respectively
NS4A protein	Nonstructural	Acts as a cofactor for NS3 protein. Also required for the phosphorylation of NS5A and can directly interact with NS5A
NS4B protein	Nonstructural	Plays an important role in the recruitment of other viral proteins.
NS5A protein	Nonstructural	Plays an important role in viral replication and modulation of cell signaling pathways and interferon response.
NS5B protein	Nonstructural	Acts as an RNA-dependent RNA polymerase and plays an important role in the synthesis of new RNA genome.

Table 2: Table showing the mechanism of action of DAAs[52-54]

Class of direct antiviral agents	Examples	Mechanism of action	Comments
NS3/4A protease inhibitors	1st generation: Telaprevir, Boceprevir 2nd generation: Simeprevir Next generation drugs: Glecaprevir, Grazoprevir, Paritaprevir, Voxilaprevir	Bind to the active site of the NS3/4A protease	High potency (varies by HCV genotype) Low barrier to resistance (1a<1b) High potential for drug interactions Can cause rash, anemia, and raised bilirubin Later generation drugs like Glecapravir & Grazoprevir are pangenotypic and are expected to have higher barriers to resistance.
NS5A inhibitors	Daclatasvir, Elbasvir, Ledipasvir, Ombitasvir, Pibrentasvir, Velpatasvir	Interact with domain 1 of the NS5A dimer, although the exact mechanism remains to be fully elucidated	Moderate to high potency (consistent across HCV genotypes & subtypes) High barrier to resistance (1a=1b) Low potential for drug interactions; may interact with HIV antiretrovirals (nucleoside reverse transcriptase inhibitors) and ribavirin Can cause mitochondrial toxicity
Nucleos(t) ide analog NS5B polymerase inhibitors	Sofosbuvir	These are incorporated into the nascent RNA chain and result in chain termination by compromising the binding of the next incoming nucleotide	Potency varies by HCV genotype Very low barrier to resistance (1a<1b) Variable potential for drug interactions
Non-nucleoside NS5B polymerase inhibitors	Dasabuvir	Interact with thumb 1, thumb 2, palm 1 or palm 2 domain of NS5B and inhibit polymerase activity by allosteric mechanisms of action	High potency (against multiple HCV genotypes) Low barrier to resistance (1a<1b) Low to moderate potential for drug interactions

viral variants called "quasispecies".[55,56] Some quasispecies bear polymorphisms in drug-targeted genes resulting in reduced susceptibility to DAAs.[57] The prevalence of intrinsically resistant variants within a patient's quasispecies is determined by their replicative fitness. Typically, a dominant variant is detectable within the viral quasispecies along with less-fit variants that are present at lower frequencies. The presence of minor populations of resistance-associated variants (RAVs) at the start of treatment may affect the outcomes of the antiviral therapy. Such variants can become dominant as a result of selective pressure exerted by antiviral drugs, subsequently leading to virological breakthrough during treatment or relapse after treatment cessation.[58] Some RAVs such as those conferring resistance to NS5A inhibitors are very fit and persist as the dominant species for months to years after treatment cessation. Understanding drug resistance is important in clinical settings to optimize treatment regimens, increase success rates, and minimize the impact of treatment failure. Table 3 depicts the most common amino acid substitutions detected in DAA non-responding HCV infected patients.

Drug resistance testing in clinical practice-An overview

The replication cycle of HCV is error prone and many of these errors either do not have any effect on the progeny viruses or result in progeny viruses that are incompetent to replicate. However, for some newly produced viruses, the transcription errors result in changes in critical coding regions that may change the susceptibility of the virus to one or more anti-viral agents. Subtherapeutic levels of antiviral agents often lead to the emergence of drug-resistant viruses by creating selective pressure

Table 3: Most common amino acid substitutions and genotypes/subtypes in DAA nonresponding HCV infected patients[52,59,60]

DAAs approved by FDA	Category of DAAs	Most common amino acid substitutions detected in HCV infected patients who failed to achieve SVR	Most common HCV genotype/ subtype associated with SVR failure
Boceprevir	NS3/4A protease inhibitor	V36M, T54S, R155K	1a
		T54A/S, V55A, A156S, V170A	1b
Telaprevir	NS3/4A protease inhibitor	V36M, R155K	1a
		V36A, T54A, A156S	1b
Simeprevir	NS3/4A protease inhibitor	R155K, D168E/V	1a
		Q80R, D168E/V	1b
Paritaprevir	NS3/4A protease inhibitor	D168A/V/Y	1a
		Y56H, D168V	1b
		D168V	4d
Asunaprevir	NS3/4A protease inhibitor	R155K, D168E	1a
		D168E/V/Y	1b
Vaniprevir	NS3/4A protease inhibitor	R155K, D168T/V/Y	1a
		D168H/T/V	1b
Daclatasvir	NS5A inhibitor	M28T, Q30E/H/R, L31M, H58D, Y93H/N	1a
		L31M/V, Y93H	1b
		Q30H/S	4
Ledipasvir	NS5A inhibitor	Q30E/R, L31M, Y93C/H/N	1a
		Y93H	1b
Ombitasvir	NS5A inhibitor	M28V, Q30R	1a
		Y93H	1b
		L28V	4d
Sofosbuvir	NS5B nucleotide polymerase inhibitor	S282T	2
		L159F, V321A	3
		C316N/H/F	1b
Dasabuvir	NS5B non-nucleoside polymerase inhibitor	M414T, S556G	1a
		S556G	1b
Beclabuvir	NS5B non-nucleoside polymerase inhibitor	A421V, P495L/S	1a

for the resistant viruses to emerge as the dominant species. However, viral variants harbouring substitutions associated with resistance to directing-acting antivirals (DAAs) are detectable prior to antiviral therapy in few chronic HCV infected patients and are referred to as baseline resistance-associated substitutions (RASs). These viruses contain substitutions that are designated as treatment-emergent (or treatment-selected) RASs.[61]

Presently, the most clinically significant RASs are in the NS5A position for genotypes 1a and 3.[61] NS5A and NS3 RASs are frequently selected in patients with failure of NS5A or NS3 inhibitor-containing regimens, respectively. NS5B nucleotide RASs are rarely detected (1% of failures) even after exposure to a failing DAA regimen containing a nucleotide inhibitor.[62,63] In addition, any such substitution would likely render the virus replication incompetent. The clinical impact of NS5A RASs is further compounded by their ability to maintain high replication competence in the absence of continued drug pressure. This allows these viral variants to remain as the dominant viral quasispecies for prolonged periods relative to NS3 protease or NS5B nucleotide polymerase inhibitor RASs.[61]

In general, drug-specific RASs need to be present in at least 15% of the viruses of a given patient to reduce the likelihood of achieving SVR.[64] Drug-specific RASs that are found at a lower frequency may not convey sufficient resistance to reduce SVR with currently available DAA regimens. The magnitude of the negative impact of RASs on treatment outcome varies according to treatment regimen and patient factors such as presence of cirrhosis and fold change decrease in potency conferred by the specific RAS(s).[61]

Testing for RASs in clinical practice becomes important when the results would modify treatment management by impacting the duration of therapy and/or inclusion of ribavirin or result in selection of alternative therapy. However, at present the utility of RAS testing varies by both patient characteristics and DAA regimen. Table 4 depicts the regimen-specific recommendations for use of RAS testing in clinical practice.[61]

Assays for detecting RASs

The methods to detect RASs in NS3, NS5A, and NS5B include population sequencing (Sanger sequencing) and deep sequencing (Next generation sequencing), which differ in their sensitivity for detecting RASs. For the purposes of clinical care and decisions regarding the choice of appropriate DAA regimen, both methods can be considered equivalent if a ≥15% cutoff point is used for the determination of RASs by NGS. However, the presence of RASs with <15% prevalence should not be considered clinically significant. Phenotypic analysis involves laboratory techniques whereby the degree of drug resistance

Table 4: Regimen-specific recommendations for use of RAS testing in clinical practice[61]

Recommended	*Rating
Elbasvir/grazoprevir	I, A
NS5A RAS testing is recommended for genotype 1a-infected, treatment-naive or -experienced patients being considered for elbasvir/grazoprevir. If present, a different regimen should be considered.	
Ledipasvir/sofosbuvir	I, A
NS5A RAS testing can be considered for genotype 1a-infected, treatment-experienced patients without cirrhosis being considered for ledipasvir/sofosbuvir. If clinically importanta resistance is present, a different recommended therapy should be used.	
NS5A RAS testing can be considered for genotype 1a-infected, treatment-experienced patients with cirrhosis being considered for ledipasvir/sofosbuvir. If clinically importanta resistance is present, a different recommended therapy should be used.	
Sofosbuvir/velpatasvir	I, A
NS5A RAS testing is recommended for genotype 3-infected, treatment-naive patients with cirrhosis and treatment-experienced patients (with or without cirrhosis) being considered for 12 weeks of sofosbuvir/velpatasvir. If Y93H is present, weight-based ribavirin should be added or sofosbuvir/velpatasvir/voxilaprevir should be used.	
Daclatasvir plus sofosbuvir	I, B
NS5A RAS testing is recommended for genotype 3-infected, treatment-experienced patients without cirrhosis being considered for 12 weeks of daclatasvir plus sofosbuvir. If Y93H is present, weight-based ribavirin should be added.	
NS5A RAS testing is recommended for genotype 3-infected, treatment-naive patients with cirrhosis being considered for 24 weeks of daclatasvir plus sofosbuvir. If Y93H is present, treatment should include weight-based ribavirin, or a different recommended therapy used	
NOT RECOMMENDED	
Elbasvir/grazoprevir	I, A
RAS testing is not recommended for any genotype 1b-infected patients being considered for elbasvir/grazoprevir therapy.	
Glecaprevir/pibrentasvir	I, A
RAS testing is not recommended for patients with genotype 1, 2, 3, 4, 5, or 6 infection being considered for glecaprevir/pibrentasvir for 8, 12, or 16 weeks	
Ledipasvir/sofosbuvir	I, A
NS5A RAS testing is not recommended for any genotype 1b-infected patients being considered for ledipasvir/sofosbuvir therapy.	
NS5A RAS testing is not recommended for genotype 1a-infected, treatment-naive patients being considered for ledipasvir/sofosbuvir therapy	I, A
NS5A RAS testing is not recommended for genotype 1a- or 1b-infected, treatment-naive patients without cirrhosis and with a viral load <6 million IU/mL being considered for an 8-week course of ledipasvir/sofosbuvir therapy.	I, A
Paritaprevir/ritonavir/ombitasvir with dasabuvir±weight-based ribavirin, or paritaprevir/ritonavir/ombitasvir + weight-based ribavirin	I, A
RAS testing is not recommended for genotype 1- or 4-infected, treatment-naive or -experienced patients being considered for therapy with paritaprevir/ritonavir/ombitasvir with dasabuvir±weight-based ribavirin or paritaprevir/ritonavir/ombitasvir + weight-based ribavirin, respectively.	
Sofosbuvir/velpatasvir	I, A
RAS testing is not recommended for patients with genotype 1, 2, 4, 5, or 6 infection and considered for 12 weeks of sofosbuvir/velpatasvir therapy.	
Sofosbuvir/velpatasvir/voxilaprevir	I, A
RAS testing is not recommended for patients with genotype 1, 2, 3, 4, 5, or 6 infection and considered for 12 weeks of sofosbuvir/velpatasvir/voxilaprevir therapy.	
Clinically important=greater than 100-fold resistance	

*I: Evidence and/or general agreement that a given diagnostic evaluation, procedure, or treatment is beneficial, useful, and effective; A: Data derived from multiple randomized clinical trials, meta-analyses, or equivalent; B: Data derived from a single randomized trial, nonrandomized studies, or equivalent

conferred by an amino acid substitution and the replicative capacity of a particular RAS can be estimated in the presence of a wild-type or consensus strain.[61]

Genotypic analysis

Population-Based Sequencing (Sanger): Population sequencing of the HCV coding region of interest may be performed using reverse transcription polymerase chain reaction (PCR) and standard Sanger sequencing of the bulk PCR product. Although the sensitivity for detection of resistance substitutions is subject to variation, in generally it is 15–25%. As a standard, substitutions are reported as differences compared with a genotype-specific, wild-type strain.

Deep Sequencing Analysis/Next Generation Sequencing (NGS): NGS can increase the sensitivity of detection for minor variants. After sequencing HCV coding regions using PCR, a software algorithm is used to process and align sequencing data via a multistep method to identify the substitutions present at a predetermined level. Although this level, or threshold, can vary, it is often set as low as >1% for research purposes. To approximate results obtained by population sequencing, NGS thresholds are often set to ≥10%.

Phenotypic analysis

This involves laboratory techniques whereby the degree of drug resistance conferred by an amino acid change as well as the replicative capacity (fitness) of a particular RAS can be estimated in the presence of a wild-type or consensus strain. However, these research techniques are not routinely used for clinical practice. To assess the level of resistance, RASs are typically introduced as point mutations into the backbone of an existing standard HCV genome within an existing cell culture/replicon or an enzyme-based assay. Isolates harboring

these RASs are then challenged by appropriate antiviral agents at increasing concentrations and fold changes—based on EC_{50} or $IC_{50}$and EC_{90} or IC_{90} values—are determined for inhibition of replication or enzyme activity, respectively, in comparison to wild-type virus. Comparison of replication levels for variants and wild-type constructs in the absence of drug allows for the estimation of fitness.

Directly acting antiviral therapy and primary healthcare

The treatment and monitoring requirements of regimens containing Direct Acting Antivirals (DAAs) are much lower than those containing interferon and ribavirin. Also, DAAs have a much higher efficacy in treating HCV infections. These characteristics mean that initiating treatment and obtaining SVR on completion of treatment in non-specialist environments should be feasible.[65] To achieve the World Health Organization Hepatitis C virus elimination targets, it is essential to increase access to treatment. Hence, DAA treatment can be provided in primary healthcare services in order to improve accessibility and retention in care.[66] Similar SVR rates were demonstrated when services cited in community settings where compared to published studies and real-world clinics in secondary care. However, stronger study designs are needed to confirm the precision of effect size seen in most of these studies.[65]

Currently in India, few states are providing treatment and care for HCV infected patients with DAAs and some healthcare facilities are also providing antiviral treatment for chronic Hepatitis B infection/disease. The National Viral Hepatitis Control Programme (NVHCP) under National Health Mission (NHM), Government of India, aims at smooth transitioning of all state-level programmes to a country-level programme in order to align with the national protocols and guidance on testing and management of different types of viral hepatitis (namely A, B, C, D, and E viruses, respectively). The objectives of NVHCP are as follows[67]:

1. Enhance community awareness on hepatitis and lay stress on preventive measures among general population, especially high-risk groups and in hotspots.
2. Provide early diagnosis and management of viral hepatitis at all levels of healthcare.
3. Develop standard diagnostic and treatment protocols for management of viral hepatitis and its complications.
4. Strengthen the existing infrastructure facilities, build capacities of existing human resource and raise additional human resources, where required, for providing comprehensive services for management of viral hepatitis and its complications in all districts of the country.
5. Develop linkages with the existing National programmes towards awareness, prevention, diagnosis, and treatment for viral hepatitis.
6. Develop a web-based "Viral Hepatitis Information and Management System" to maintain a registry of persons affected with viral hepatitis and its sequelae.

Owing to the recent developments pertaining to the management of chronic HCV infection with DAA therapy at primary healthcare level, it is imperative for physicians to be aware of the emerging resistance to these drugs in order to avoid their misuse.

Conclusion

Management of HCV infection is a challenge for both hepatologists and virologists alike. Emergence of resistance to many currently available anti-HCV drugs is alarming and, therefore, it is imperative to perform *in vitro* RAS testing especially in patients on DAA therapy who fail to attain a sustained viral response. Keeping in mind the emerging concept of management of chronic HCV infections at the primary healthcare level, physicians should be made aware of the importance of emerging resistance to directly acting antiviral drugs to avoid their misuse.

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Conflicts of interest

There are no conflicts of interest.

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