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Emergence of genetically variant Hepatitis C virus population in response to increased antiviral drug pressure, Pakistan

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Abstract Mutations in NS5B gene of Hepatitis C virus (HCV) have been reported in patients undergoing antiviral therapy. In the present study, we report emerging clade of HCV-3a in patients administered with IFN plus ribavirin therapy for 24 weeks and having low viral loads (<250 IU/mL). Mutations D/N244E, K304R, N/K307G, Q/T329V, and *A338V* were found associated with these emerging strains. This distinct HCV could be associated with the increased antiviral drug pressure.

Keywords Hepatitis C virus · Antiviral therapy · Interferon resistance · Ribavirin

Introduction

Hepatitis C virus (HCV) infection is estimated to affect 170–200 million persons globally Alter et al. [1]. Based on genetic analysis of the complete genomes and partial sequences of HCV, six genotypes (genotype 1–6) with 66–69 % sequence similarities, have been described, which are further classified into multiple subtypes with sequence similarity of >75 % [2, 3]. Such classification is important for clinical management of chronic HCV infection, vaccine development, and epidemiological analysis [4].

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The most frequently prevalent HCV genotype in Pakistan is 3a, and SVR rates to standard IFN-α plus ribavirin in Pakistan have been reported to be 27.8–51.26 % [5, 6]. Our recent studies show high prevalence of untypable (UT) HCV isolates in the patients administered with interferon plus ribavirin standard therapy, particularly relapsers or non-responders [7, 8]. However, very little is known about genetic diversity of HCV isolates prevalent in such patients. The present study investigates the genetic variability of HCV in patients administered with IFN plus ribavirin standard therapy and remained non-responders with particular focus on isolates with low viral loads of <250 IU/mL at the end of treatment.

Results and discussion

Patients

Figure 1 shows the patients disposition. Out of total 32 patients included in the present study, 22 were males and 10 were females with mean age 37.68 ± 12.05 years (18–56 years). All the patients were from the Punjab province, which is the most populated province of Pakistan. Most of the samples were obtained from Lahore, the second most populated city in Pakistan with a population of 7.2 million and is reported to have high rate of HCV infection [11].

Phylogenetic analysis shows emerging clade associated to IFN plus RBV combination therapy

In the present study, we specifically focused on UT genotypes which are non-responders and have low viral loads <250 IU/mL. Here, we report a new emerging HCV-3a

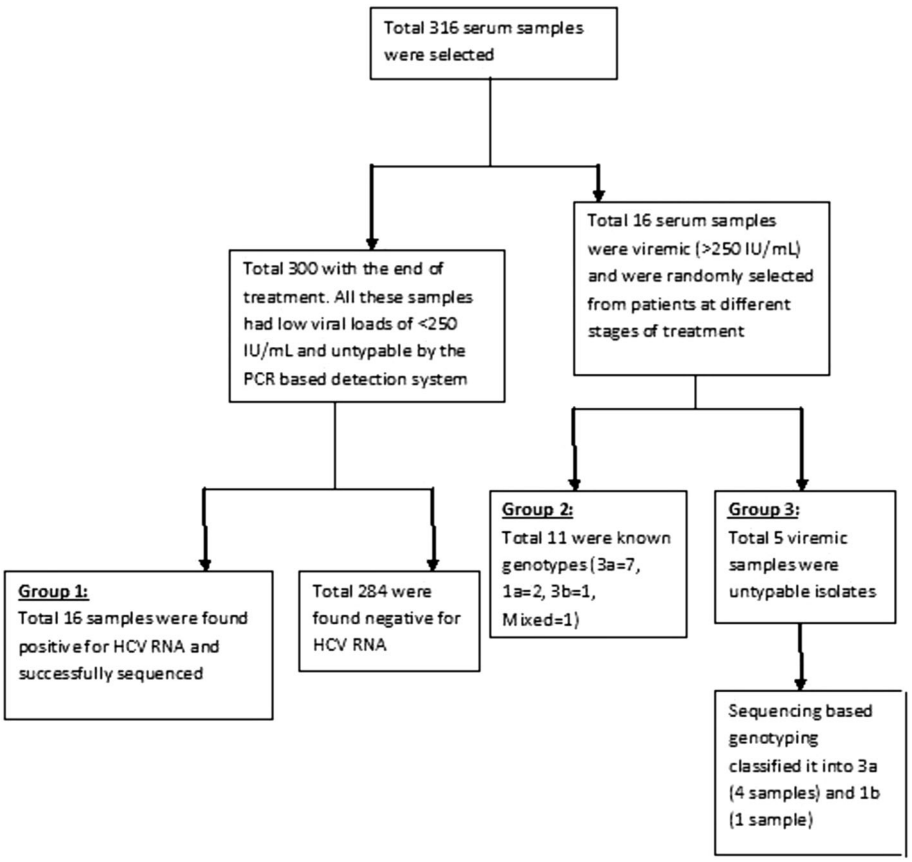

Fig. 1 Patients disposition

strain in Pakistan that is associated with IFN plus RBV combination therapy. In a study, Khan et al. [12] investigated molecular evolution of HCV in Pakistan and reported an increased number of HCV infection in Pakistan that appeared in 1920s with rapid spread in 1950s, and it related the latter exponential spread to poor healthcare system and population movement due to the partition of Indian subcontinent. In another study based on NS5B genome, HCV-3a Pakistani isolates were reported more evolutionary related to sequences from Japan and UK [13]. However, the present study suggests that HCV-3a prevalent in Pakistan seems to be more related to that prevalent in Tajikistan and China (Fig. 2).

Mutation D/N244E associated to the emerging clade

We found a novel mutation D/N244E associated with the emerging HCV clade (patients with low viral loads at the end of treatment) (Fig. 3). Amino acid residue D244 is located at flexible loop connecting two helixes in the NS5B protein (Fig. 4) and could interfere with protein–protein interaction of NS5B protein. Previously, D244N substitution was found extensively in HCV-3a associated with RBV therapy [14–16], also found in drug naive HCV patients [15]. As both aspartic acid (D) and glutamic acid (E) have the same side chains, we propose that this substitution could be associated with resistance to IFN plus RBV therapy in the studied population.

D310N is associated with HCV-3a

D310N mutation (functional domain D) of NS5B polymerase in patients administered with RBV monotherapy has been reported to be associated with increased sensitivity to IFN therapy and is suggested to be critical region in positive viral selection [14], especially in genotype 3a [16]. In the present study, all HCV 3a genotypes were found to be D310N region signifying high susceptibility to IFN therapy. A potentially resistant novel mutation D310E was found in one Pakistani isolate (GenBank accession No. Gu294484) encouraging further epidemiological studies based on NS5B sequences.

Q/L309R and A333R mutations associated with HCV-3a and its emerging isolates in Pakistan

Mutations at amino acid residues Q309R and A333R located at the surface of the catalytic domain of the enzyme

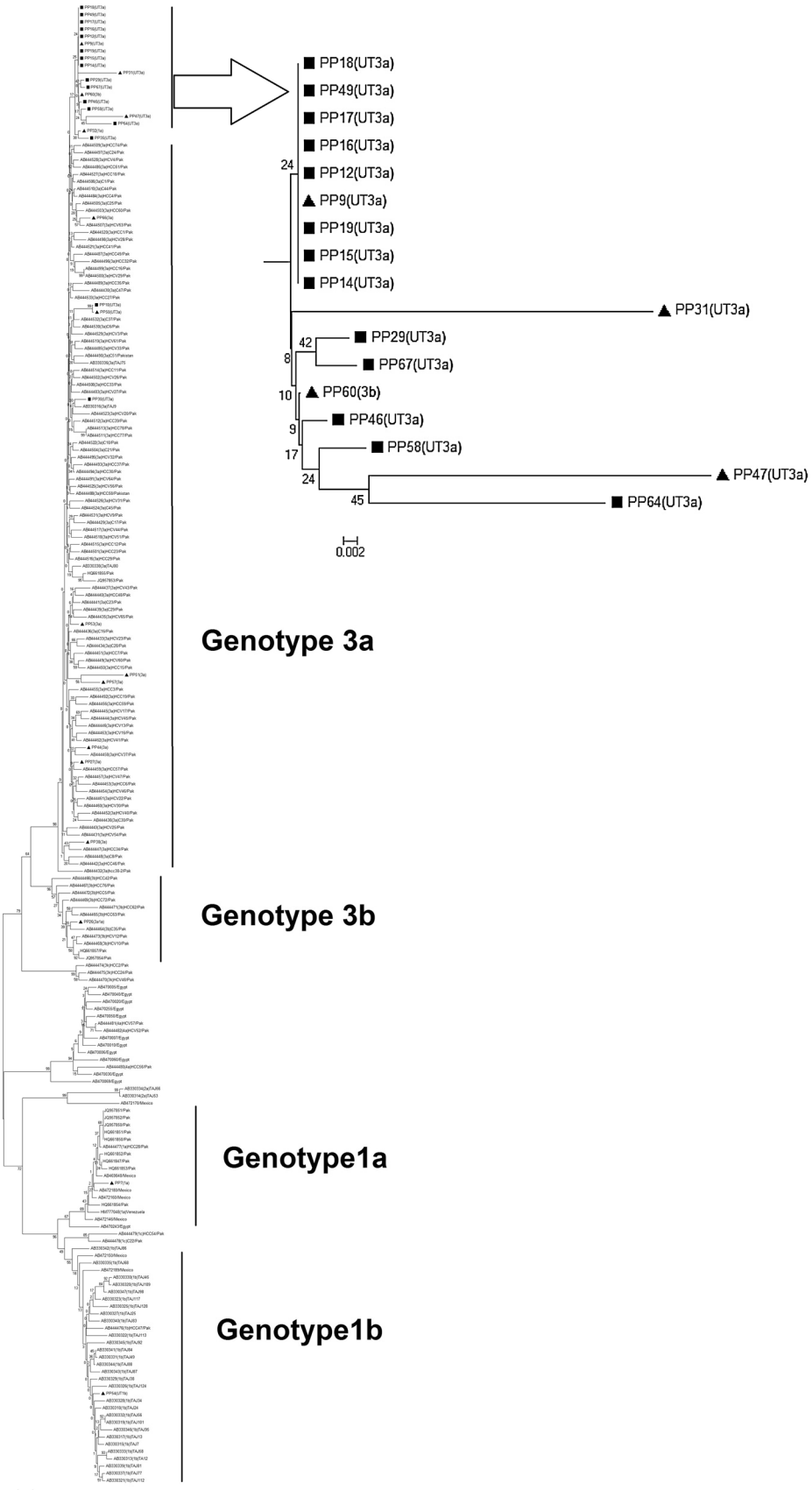

Fig. 2 Phylogenetic analysis shows emergence of HCV-3a isolates in patients undergoing IFN plus RBV therapy. Sequences from the present study are represented by their isolate names. The emerging clade is shown in bold and was found to be associated with M/N244E mutation. The bar at the base of each figure shows the scale for nucleotide substitution per site. Reference sequences obtained from GenBank/DDBJ/IMBL are represented by their accession numbers

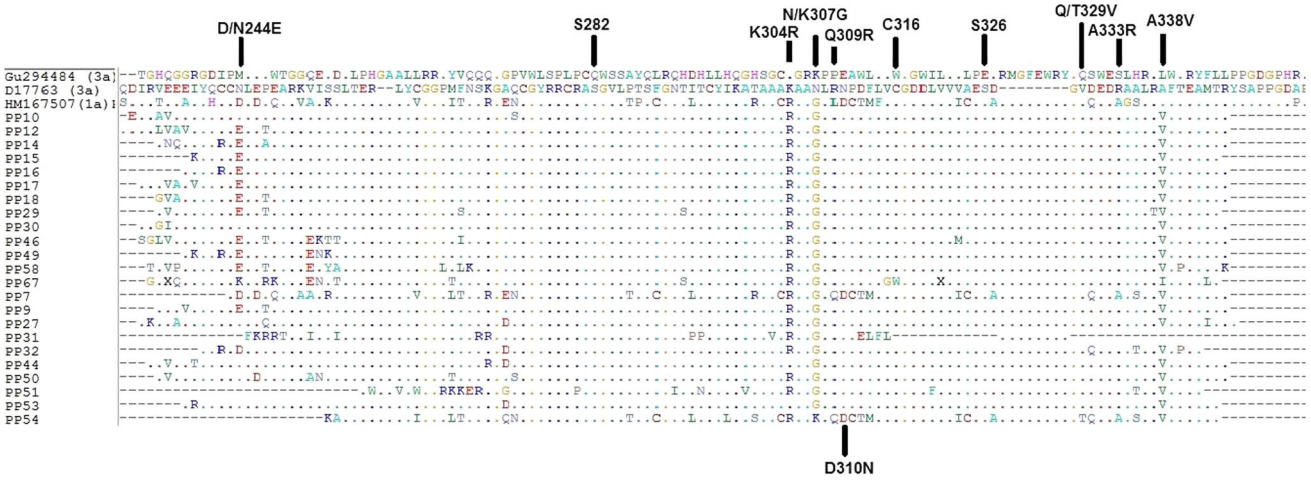

Fig. 3 Alignment of the amino acid sequences of the HCV strains as compared to standard HCV-3a sequences (BAA04609 and ADD69958) and genotype 1a (ADI99198). Mutation D/N244E was found in all the emerging strains

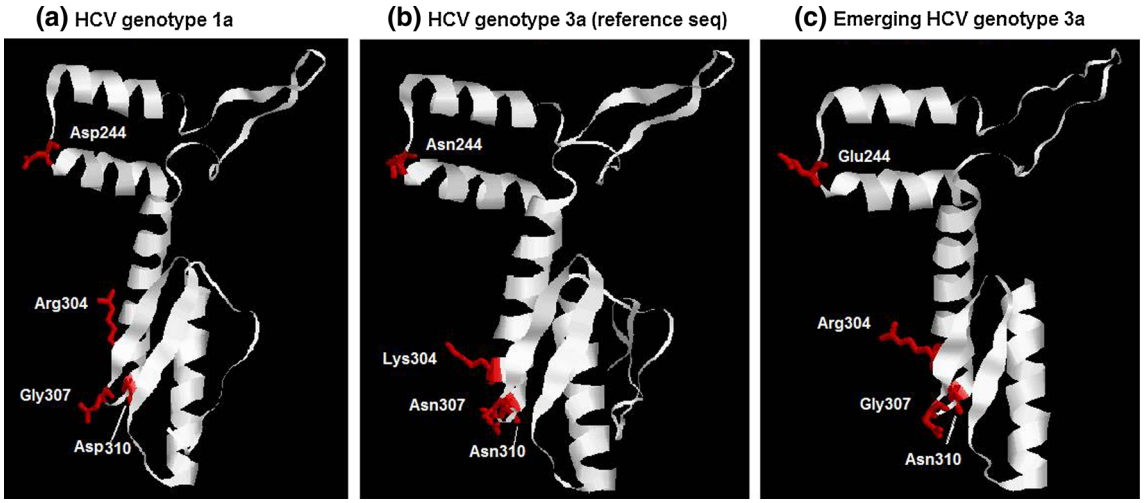

Fig. 4 Structural comparison of NS5B segment of HCV genotype 1a (**a**), genotype 3a (**b**), and emerging HCV-3a strain (**c**). Mutation D/N244E in the emerging strain causes slight extension in the flexible loop. Mutations K304R and Q309R are common in HCV-3a in Pakistan (**c**) that cause conformational change making it similar to the loop similar found in the genotype 1a (**a**)

and have been found to be associated to ETR and SVR [17]. In another study, Castilho et al. [15] observed A333E in one patient infected with genotype 1a administered with RBV monotherapy. However, we observed that Q/L309R and A333R are positively selected in the emerging HCV-3a isolates. These results are in agreement with the results found in HCV infected patients from Venezuela [16].

S326 and T329V are associated with HCV genotype 3a and 1a

S326G and T329I mutations (located in the loop connecting two helixes) have been reported to be induced by RBV monotherapy [14]. None of these mutations were found associated with the emerging HCV clade. However, a variant T329V was observed in all the isolates except genotype 1b, which shows that RBV could also result in substitution of threonine with valine at this position.

Mutations S282T and C316Y/N resistant to NS5B inhibitors were not found in our studied population

S282T has been found resistant to 2′-C-methyl cytosine [18]. In our study, wild-type S282 was found in all the isolates. C316Y/N polymorphism has been reported to be associated with resistance to NS5B inhibitor (HCV-796) [19, 20]. Jaspe et al. [16] reported a high prevalence of C326N substitution in HCV genotype 1b population from East Asian countries (Japan, China, Taiwan, and Hong Kong) resistant to NS5B inhibitors. An unexpected

Table 1 Hepatitis C virus in patients with end of IFN plus RBV therapy

S. no.	HCV isolate	Genotype by conventional method[a]	Genotype by sequencing	Sex	Age (years)	City	Viral load
1	PP10	UT	3a	Male	24	Lahore	<250
2	PP12	UT	3a	Female	30	Lahore	<250
3	PP14	UT	3a	Male	60	Lahore	<250
4	PP15	UT	3a	Male	44	Lahore	<250
5	PP16	UT	3a	Male	18	Qusur	<250
6	PP17	UT	3a	Female	35	Qusur	<250
7	PP18	UT	3a	Female	40	Lahore	<250
8	PP19	UT	3a	Female	50	Lahore	<250
9	PP29	UT	3a	Female	27	Lahore	<250
10	PP30	UT	3a	Male	51	Lahore	<250
11	PP35	UT	3a	Male	39	Lahore	<250
12	PP46	UT	3a	Male	36	Tobatek Singh	<250
13	PP49	UT	3a	Male	21	Lahore	<250
14	PP58	UT	3a	Male	22	Lahore	<250
15	PP64	UT	3a	Male	27	Lahore	<250
16	PP67	UT	3a	Female		Lahore	<250

UT untypable

[a] The isolates that failed to classify within the established subtypes by nested PCR-based method reported by Idrees [9]

mutation C326W was found in one isolate PP67 (genotype 3a; viral load <250 IU/mL; classified in the emerging clade in Fig. 2) which needs further studies for its association with the resistance to anti-viral therapies.

Contradictory results have been reported in the past about specific nucleotide/amino acid substitutions in NS5B associated with antiviral therapy

About threefold increase in mutation rate and considerable shift in mutation spectrum have been observed in HCV isolates from patients administered with interferon (IFN) plus ribavirin (RBV) therapy for 6 months [21]. The most important substitutions in response to antiviral therapy in HCV are D244N, S282T, Q309R, D310N, C316N, S326G, T329I, and A333E [14–16], with contradictory results [14, 17, 22–25]. Sugihara et al. [23] has reported that no specific HCV NS5B nucleotide/amino acid sequence variations were found associated with clinical treatment response or selected by the combination therapy in Japanese patients with HCV/1b infection. More recently, Dietz et al. [26] reported increased C-to-U and G-to-A nucleotide transitions in HCV genome of genotype 1 infected the patients treated with RBV monotherapy. Cuevas et al. [21] suggested that several changes along the HCV genome enable virus to overcome strong selective pressure of immune system and antiviral therapy. Such variations could be mediated by mutagenesis in NS5B, an RNA dependent RNA polymerase [22]. Based on our observation of emerging HCV clade, we suggest that IFN plus RBV combination therapy could result in both specific and non-specific mutations in NS5B gene, followed by emergence of potentially resistant isolates under the strong selective pressure from host immune system and antiviral therapy.

Methods

A total of 32 HCV isolates were included in the present study (Tables 1, 2). The criteria for selection of the patients are illustrated in Fig. 1. Patients were classified into three groups: (1) HCV non-responders patients with low viral loads of <250 IU/mL. These patients were selected among 300 patients with continuously raised liver function tests (LFTs) and had shown decompensate liver findings even after the completion of apparently successful antiviral therapy courses. Group (2) Patients with relatively high viral loads (>250 IU/mL) and known genotypes; Group (3) patients with high viral loads (>250 IU/mL) but unknown UT genotype. Group 2 and 3 isolates were obtained from viremic patients in different stages of IFN plus ribavirin treatment and were randomly selected with mean viral load of $2.02 \times 10^6 \pm 3.73 \times 10^6 \pm 3.73\ 445 \times 10^6$ IU/mL (range, 4.012×10^3–1.243×10^7 IU/mL) (Table 2). Before sample collection, a written informed consent was obtained from each patient. A printed data sheet was also

Table 2 Hepatitis C virus in viremic patients

S. no.	HCV isolate	Genotype by conventional method	Genotype by sequencing	Sex	Age (years)	City	Viral load (IU/mL)
1	PP7	1a	1a	Female	23	Lahore	1947063
2	PP9	UT	3a	Female	18	Rawalpindi	55802
3	PP26	3a/1a	Cluster with mixed genotypes	Female	40	Lahore	90232
4	PP27	3a	3a	Male	35	Sargodha	101818
5	PP31	UT	3a	Male	51	Lahore	8134143
6	PP32	1a	1a	Male	40	Lahore	12849
7	PP38	3a	3a	Female	33	Lahore	12430805
8	PP44	3a	3a	Male	24	Lahore	6941925
9	PP47	UT	3a	Male	45	Lahore	42039
10	PP50	UT	3a	Male	56	Lahore	572337
11	PP51	3a	3a	Male	35	Lahore	12691
12	PP53	3a	3a	Male	32	Lahore	924264
13	PP54	UT	1b	Male	52	Lahore	247890
14	PP57	3a	3a	Male	38	Gujranwala	4012
15	PP60	3b	3b	Male	40	Lahore	13310
16	PP66	3a	3a	Male	30	Lahore	822481

The isolates that failed to classify within the established subtypes by nested PCR-based method reported by Idrees et al. [11]

UT untypable

filled from all patients contained demographic, biochemical, and biological information.

Genotyping was performed following the previously described protocols [9]. The reported sensitivity of this assay is 10 IU/mL. HCV isolates that remained undetermined by this method were declared as UT. For sequence analysis, NS5B sequence (~340-nt) was amplified with gene specific primers (Forward set: 5′-tggggatcccgtatgatacccgctgctttga-3′ and 5′-cggaattcctggtcatagcctccgtgaa-3′; Reversed: 5′-gacacccgctgctttgactc-3′ and 5′-cggaattcctggtcatagcctccgtgaa-3′) and used as templates for sequencing in the Big-Dye Terminator cycle sequencing reaction kit (Applied Biosystems). Sequencing was performed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., 850 Lincoln Center Drive, Foster City, CA94404). Products were sequenced from both strands to get consensus sequences. Evolutionary relationship among the isolates was determined by phylogenetic analysis using MEGA 5 software system [10].

Conclusions

(1) We successfully amplified and sequenced HCV isolates from patients with low viral loads (<250 IU/mL) at the end of treatment and report emerging HCV clade in such patients

(2) Mutations K304R, Q309R, and A3338V are common in HCV-3a prevalent in Pakistan

(3) Studies involving low viral loads as a result of antiviral therapy in HCV infected patients could be useful for investigating potentially emergent strains

(4) As all the non-responders were of UT/unclassified genotype, we suggest more sensitive methods for genotyping of such clinical samples must be used

Accession numbers of the sequences

The nucleotide sequences reported in this study are published in GenBank/DDBJ/EMBL database under the accession numbers JQ038432, JQ038434, JQ038435, JQ038437, JQ038439, JQ038440–JQ038444, JQ038451, JQ038452, JQ038454–JQ038457, JQ038460, JQ038463, JQ038469, JQ038471, JQ038472, JQ038474–JQ038476, JQ038478, JQ038479, JQ038482, JQ038483, JQ038485, JQ038489, JQ038491, and JQ038492.

List of reference sequences from Pakistan

Reference sequences reported in the previous studies from Pakistan (2009–2012) were obtained from GenBank/DDBJ/EMBL database and used for the comparison: AB444429–AB444582, HQ661847, HQ661850, HQ661851, HQ661852, HQ661853, HQ661854, HQ661857, HQ661855,

JQ957850, JQ957851, JQ957852, JQ957853, JQ957854, HM167507, and Gu294484.

Reference from the rest of the world obtained from GenBank/DDBJ/EMBL database

D17763, AB330346, AB330344, AB330342, AB330340, AB330338, AB330336, AB330334, AB330332, AB330330, AB330328, AB330322, AB330316, AB330326, AB330320, AB330318, AB330314, AB330347, AB330345, AB330343, AB330341, AB330339, AB330337, AB330335, AB330333, AB330331, AB330329, AB330327, AB330325, AB330323, AB330321, AB330319, AB330317, AB330315, AB330313, AB470005, AB470006, AB470007, AB470010t, AB470020, AB470030, AB470040, AB470050, AB470060, AB470069, AB470243, AB470255, AB469848, AB472146, AB472150, AB472160, AB472170, AB472180, AB472189, and HM777048.

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