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Thalassemia Patients from Baluchistan in Pakistan Are Infected with Multiple Hepatitis B or C Virus Strains

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Abstract. There are an estimated 2,000 children with β-thalassemia in the province Baluchistan of Pakistan. These children are at high risk of acquiring transfusion-transmitted infections (TTIs) due to their need of regular blood transfusions for survival. Therefore, we investigated the frequencies of TTIs among these multi-transfused patients in a region where the WHO guidelines for blood safety are not always followed. Sera from 400 children (mean age 7.7 ± 4.70 years) treated at two thalassemia centers in Baluchistan were investigated for TTIs. Eleven (2.8%) were hepatitis B surface antigen positive, and 72 (18.3%) had anti-hepatitis C virus (HCV), two of which were infected with both viruses. Only 22% of the children had been reached by the program for universal hepatitis B virus (HBV) vaccination which started in 2004. Half (51%) of the HCV infected had also been HBV infected. The HBV- and HCV-infected patients were older and had received more blood transfusions than the uninfected patients ($P < 0.001$). Molecular characterization of the viral strains revealed the presence of several genetically different strains in at least three HBV- and seven HCV-infected children. This is the first study to demonstrate infections with multiple HBV or HCV strains simultaneously infecting thalassemia patients. These may become the source for new emerging recombinant viruses of unknown virulence. The high prevalence of anti–HCV-positive children, and the presence of HBV infections among children who should have been vaccinated, highlights an urgent need for improvements of blood safety in this region of Pakistan.

INTRODUCTION

There are five provinces in Pakistan, of which Baluchistan is the largest, but only 13 million of the country's total 208 million inhabitants live in this province. The capital Quetta in the northern part has one million inhabitants (Figure 1). Baluchistan is a poor province and has the lowest human development index in the country[1]; in 2018, it was 0.477, whereas it was 0.678 for Islamabad. Vaccination against hepatitis B virus (HBV) as part of the Expanded Program on Immunization (EPI) was launched nationwide in 2004. This was financed by a grant received from the Global Alliance for Vaccines and Immunization in 2001–2002.[2] There have been failures in the EPI for preventing the spread of infections of public concern in Baluchistan, and poliomyelitis is still endemic in the area with 12 cases reported in 2019.[3]

Thalassemia is one of the most common genetic disorders and affects 1.5% of the world population.[4] β-thalassemia is caused by mutations in the hemoglobulin subunit beta gene on chromosome 11[5] and is inherited in an autosomal, recessive trait.[5] The disorder is associated with populations originating from the Mediterranean, Middle East, and Indian subcontinent, but is now widespread also in other areas.[6] The severity of the disease depends on the nature of the mutation and on the presence of mutations in both alleles (thalassemia major as β-thalassemia) or in one allele (thalassemia minor). β-thalassemia is common in Pakistan and affects 5–7% of its population.[7] Regular blood transfusion is required to reduce anemia in these patients.[8] Because this remains the only treatment, these patients are at high risk for developing liver cirrhosis or cardiac complications and often die because of iron overload at the age of 30 years or earlier.[9]

Viral hepatitis infections are considered among the top eight causes of deaths, with approximately 1.34 million yearly deaths globally.[10] It has been estimated that 257 million persons are chronically infected with HBV and 71 million with hepatitis C virus (HCV).[10] The diseases are called silent killer because many patients remain undiagnosed and untreated for many years before their health complications arise. To reduce the negative impact of hepatitis on global health, the World Health Assembly adopted the Global Health Sector Strategy on viral hepatitis to eliminate hepatitis by 2030.[11] In Pakistan, almost 12 million people are suffering from HBV or HCV, with a yearly incidence of about 200,000 new cases.[12] In the general population, the prevalence of HCV is almost 5% and of HBV is 3–5%.[13–16] There are probably more infected persons because these infections are blood transmitted, and blood safety is not well handled in all regions of Pakistan. In addition, many of healthcare workers may be infected without being aware of it, and there is no general testing of health personnel.[12,17,18]

Safe blood is not always readily available, and consequently, β-thalassemia patients often acquire transfusion-transmitted infections (TTIs), and many Pakistani patients have been infected.[19,20] The reasons for the high number of TTIs include practices and measures of blood supply below the standard recommended by the WHO[21] and other nosocomial transmissions due to lack of resources and poorly trained staff.[22] In 2005, the prevalence of hepatitis B was 4% in the general population and less than 2% in children in Pakistan,[23] whereas it was twice as high in β-thalassemia patients.[24] For HCV infections, the prevalence is around 6% in the general population,[25] but 20–58% in β-thalassemia patients. Transfusion-transmitted infections are also common among thalassemia patients in India, Iran, Iraq, and Pakistan.[26–28]

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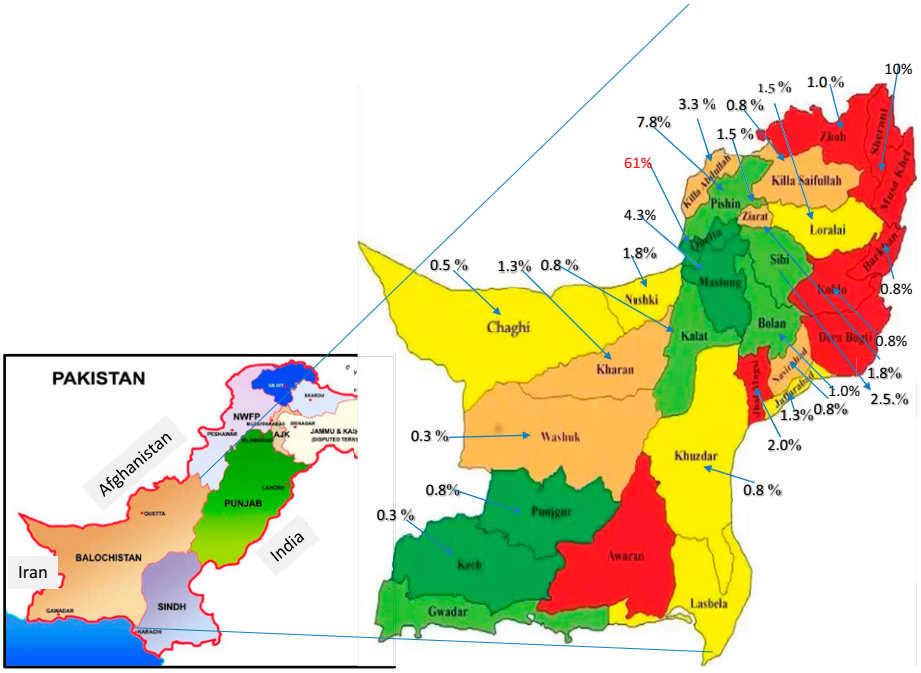

FIGURE 1. Map of Pakistan with the Baluchistan Province enlarged. The figures indicate the origin of the 400 children with β-thalassemia included in the study. This figure appears in color at www.ajtmh.org.

An earlier study of TTIs in multiply transfused patients with thalassemia majorly revealed high incidences of HCV and HBV in Rawalpindi, Islamabad, and Karachi,[29] but studies have so far not been conducted on these patients in Baluchistan. Therefore, the aim of this study was to investigate the prevalence of TTIs in multi-transfused β-thalassemia patients treated in this province.

MATERIALS AND METHODS

Patients. The study was conducted at the two thalassemia centers in Baluchistan, the Bolan medical college hospital (BMCH) and the Civil Hospital Quetta (CHQ), which provide screened blood and medical care for β-thalassemia patients. In total, 400 of 2,000 patients with confirmed β-thalassemia were investigated: 239 patients were from BMCH and 161 from CHQ. Blood samples were collected from March 2017 until June 2018 at the Department of Pathology at the BMCH.

β-thalassemia was diagnosed by hemoglobin (Hb) electrophoresis. A questionnaire was used to record clinical data and family history of the patients. The records included age, gender, ethnic origin, and family history (parent consanguinity and number of individuals with β-thalassemia in the household family). The total number of transfusions was estimated by the parents, who also informed about the age of the child at diagnosis. Information was also obtained regarding previous or present jaundice, and splenectomy or hepatomegaly, and previous vaccination against HBV (Supplemental Table S1).

Ethical approval. Ethical approval was obtained from the research committee of the University of Baluchistan in Quetta (Reg No UOB/06/2020). Informed consent was obtained from all patients and/or their guardians/parents before sampling.

Serological analyses. Hemoglobin was analyzed for in an XS-500i Sysmex analyzer (GmbH, Norderstedt, Germany). Serum ferritin was quantified by Cobas Integra 400 plus (Roche, Basel, Switzerland), and alanine amionotransferase test (ALT) by Selecta Pro XL (ELITech Group, Puteaux, France).

All sera were tested for HBV surface antigen (HBsAg), antibodies against HCV (anti-HCV), antibodies against hepatitis delta virus (anti-HDV), antibodies against HIV (anti-HIV), and antibodies against cytomegalovirus (anti-CMV) by ELISA (Dia 710 Diamate; Bio-technologies, Hertfordshire, England) at the BMCH. Antibodies against HBV core antigen (anti-HBc), against hepatitis B surface antigen (anti-HBs), hepatitis B e antigen (HBeAg), antibodies against HBeAg (anti-HBe), and HCV core antigen were analyzed at the Clinical Microbiology Laboratory (CML) at Sahlgrenska University Hospital in Gothenburg, Sweden. The analyses were performed by chemiluminescent microparticle immunoassay from Abbott Laboratories (Abbott Park, IL) on ARCHITECT i2000SR platform. For HCV core antigen, samples with < 3 fmol/L were considered negative, those with 3–10 fmol/L boarder line, and those with > 10 fmol/L were considered positive.

PCR amplification and sequencing. Nucleic acids (NA) in 200 μL of serum reactive for either anti-HCV or HBsAg were extracted on NucliSENS EasyMAG automated system (BioMerieux, Marcy l'Etoile, France) at CML. The extracted NAs were stored at –80°C until further analyzed. Hepatitis C virus RNA detection, cDNA synthesis, PCR, purification of amplified PCR products and cycle sequencing were performed as described previously.[30] The sequences obtained are deposited in GenBank with accession numbers MW234350–MW234406.

Phylogenetic analysis. The HBV and HCV sequences were analyzed in the SeqMan Pro 13 program in the DNAStar Program package version 10.1.2 (DNAStar Inc, Madison, WI). The HBV S-gene, the HCV core, and the NS5B sequences were aligned with corresponding sequences from GenBank. Evolutionary distances were calculated using the Hasegawa–Kishino–Yano algorithm with gamma correction in the

DNADIST program in the PHYLIP package version 3.65 (University of Washington, Seattle, WA).[31]

Statistical analysis. For means and corresponding SDs (mean ± SD), range is presented when proportions were expressed by percentages. Prevalence of HBV and HCV infections were compared with regard to age, number of transfusions received, and serum ferritin levels between HBsAg and anti-HCV reactive and nonreactive participants using Fisher's exact test. All statistical analyses were carried out using SPSS software version 24.0 (IBM, Chicago, IL), and *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Demographical data of the patients. The mean age of the patients was 7.70 ± 4.70 years (range 2–27 years). One patient was older than 25 years. There were slightly more boys than girls (228 versus 172; Table 1). Most patients (244; 61%) were from Quetta, since many families had moved there due to poor blood facilities in other districts of the province. Because of poor availability of blood, families pay relatives or students to donate blood at a nearby pharmacy. This blood, which is often unscreened for TTIs, is then transfused to the children at their homes by the parents. Consanguineous marriages are common in Baluchistan. In this study, there were 300 (75%) interfamily marriages, and 71% of the families had several members with thalassemia.

Clinical data. The average age at diagnosis of β-thalassemia was 5 months, and for the first transfusion it was 6 months. To reach Hb levels of ≥ 9 g/100 mL, 37% of the patients had to receive two to four blood units monthly, the others received one blood unit every month (Table 1).

ALT and serum ferritin values were related to the number of blood units the patients had received during their lifetime (Table 2). For all children the serum ferritin levels were more than four times the upper normal limit, and 70% had elevated ALT levels (Supplemental Table S2).

Transfusion transmitted infections. At inclusion, 11 patients were HBsAg positive and 73 had anti-HCV (Table 1). Two patients, aged 10 and 18 years, were infected with both viruses. One 22-year-old patient was infected with HDV, and another 16-year-old with HIV. The number of TTIs increased with the number of blood transfusions received (Table 2). Children with TTIs had more often splenectomy than noninfected children ($P < 0.0001$; Supplemental Table S2).

Despite the EPI, in which 87% of the children should have been included, only 22% had been vaccinated against hepatitis B according to their parents. To confirm this, anti-HBc and anti-HBs were analyzed in available sera from 64 of the HCV infected patients used for HCV typing. Four of seven children lacking these markers were born after 2004. Anti-HBs without anti-HBc, indicating vaccination, were found in 12 of 38 children born after 2004, and in five of 26 older patients. Evidence of past HBV infection with high levels of both anti-HBc and anti-HBs was found for 23 patients; six of them were vaccinated according to their parents. Reactivity was found only for anti-HBc in 11 patients.

Hepatitis B. The HBV-infected patients were older than uninfected, 13.2 (range 6–22 years) versus 6 years (range 1–17 years; $P = 0.001$), and had received more transfusions (mean 264 versus 96; Supplemental Table S2). They had higher levels

TABLE 1
Demographic data and prevalence of transfusion-transmitted infections in the 400 children with β-thalassemia

Variable	*n* = 400 (%)	Hepatitis B virus positive, *n* = 11 (%)	*P*-value	Hepatitis C virus positive, *n* = 72 (%)	*P*-value	CMV positive, *n* = 349 (%)	*P*-value
Age-group (years)			**< 0.001**		**< 0.001**		**< 0.001**
0–5	124 (31)	0 (0.0)		1 (0.8)		93 (75)	
5–14	236 (59)	6 (2.4)		45 (19)		217 (92)	
≥ 15	40 (10)	5 (12)		27 (68)		39 (98)	
Gender			0.541		0.873		0.983
Male	228 (57)	5 (2.2)		41 (18)		199 (87)	
Female	172 (43)	6 (3.5)		32 (19)		150 (87)	
Number of transfusions			**< 0.01**		**< 0.001**		**< 0.001**
< 50	128 (32.0)	0		1		97	
50–199	161 (40.3)	4		25		145	
200–349	78 (19.3)	4		23		74	
≥ 350	33 (8.3)	3		24		30	
Transfusion frequency every 4 weeks			–				–
Once	252 (63)	4		18		205	
Twice	114 (28)	4		37		11	
Three times	33 (8.3)	3		18		33	
Four times	1 (0.3)	0		0		1	
Splenectomy	91 (22.8)	5 (5.5)		37 (41.7)	**< 0.001**		
Hepatitis B vaccination			–				–
According to parents							
Vaccinated	97 (24)	4 (36)		19 (26)		87 (25)	
Not vaccinated	213 (53)	6 (54)		47 (64)		182 (52)	
Unknown	90 (22)	1 (10)		7 (10)		80 (23)	
Patients born after 2004	350	4	–	43	–	301	–
Vaccinated	79 (22)	2		9		70	
Not vaccinated	188 (54)	2		30		158	
Unknown	83 (24)	0		4		73	
Treatment center			0.032				0.653
Civil hospital	239 (59.8)	3 (5)		35 (22)	0.138	139 (86)	
Bolan medical complex	161 (40.2)	8 (1.3)		38 (16)		210 (88)	

Bold values denote statistical significance values.

TABLE 2
Number of transfusions in relation to age and hepatitis B virus and HCV infections at the end of the study period among the 400 children with β-thalassemia

Number of transfusions	Number of patients	Age-group (years)	Number HBV surface antigen positive	Number anti-HCV positive	Serum ferritin (ng/mL) (normal range 12–300)	ALT (IU/L) (normal range 7–45)
< 50	128	< 5	0	1	1,190 ± 868	–
		5–14	0	–	–	36.1 ± 12.3
		≥ 15	0	–	–	–
Subtotal			*0*	1 (0.8%)	–	–
50–199	161	< 5	0	0	3,381 ± 1,807	–
		5–14	4	24	–	53.8 ± 18.5
		≥ 15	0	1	–	–
Subtotal			4 (2.5%)	25 (15.5%)	–	–
200–349	78	< 5	0	–	–	–
		5–14	2	16	5,278 ± 2,338	73.8 ± 19.1
		≥ 15	2	7	–	–
Subtotal			4 (5.1%)	23 (29.5%)	–	–
> 350	33	≥ 15	3 (9%)	24 (72.7%)	6,284 ± 2,293	92.7 ± 23.7
Total	400		11 (2.8%)	73 (18.2%)	–	–

HCV = hepatitis C virus.

of serum ferritin (4,559 versus 2,769 mg/mL) and ALT (85 versus 47 IU/mL) than the noninfected ($P < 0.001$). Three of the HBsAg-positive children had HBeAg, and the other eight had anti-HBe. Hepatitis B virus DNA could be amplified by PCR in sera from eight of the 11 HBsAg-positive patients. The S-gene could be sequenced in six samples, three each from patients with HBeAg and anti-HBe. The sequences were ambiguous for the other two samples, probably because of the presence of several strains. The three children with anti-HBe were infected with at least two different HBV strains, whereas those with HBeAg were infected with one genotype D strain each (Supplemental Table S3). Two of the multiply infected had different genotype D strains, one had both a genotype D and a genotype A strain (Table 3, Figure 2). The multiplicity of strains could be revealed because of varying reactivity by the sequencing primers. All children were infected with unique strains, a repeatedly consistent finding. Five strains were divergent from reported genotype D strains and were not similar to the HBV A/D and C/D recombinants described from India.[32,33]

Hepatitis C. The risk of HCV infection was higher than that of HBV when considering the number of transfusions received, with a risk ratio of 34 ($P < 0.0001$; Table 1). Patients with anti-HCV were 13.2 years (range 4–27 years) older than the noninfected patients ($P < 0.001$). They also had higher levels of serum ferritin (5,233 versus 2,769 mg/mL) and ALT (86 versus 47 IU/mL) than the noninfected ($P < 0.001$).

Sera were available for HCV core antigen analysis for 44 anti–HCV-positive patients. Hepatitis C virus RNA was detected in 19 of 33 patients with high or intermediate HCV core antigen and in one of the 11 patients negative for this antigen. The core and/or NS5B regions could be amplified in 46 of the 73 anti–HCV-positive patients. The strains in 16 patients were difficult to type because of the presence of multiple strains. Genotype 3a strains were identified in 14 children, 3b in three, 2b in five, and 1b in one child. Some sequencing primers amplified only one of several strains in a sample. Multiple strains of subtypes 3a, 3b, and 2b could thereby be identified in at least seven children (Table 3, Supplemental Table S4). The samples with multiple strains were genetically divergent from each other as is shown for the 3a strains based on the core region in Figure 3, and for the NS5B region in Supplemental Figure S1.

DISCUSSION

A high prevalence of TTIs was found in children with β-thalassemia in Baluchistan. The number of HBV- or HCV-infected patients increased with age and number of blood transfusions received. High incidence of HBV or HCV infections in children with β-thalassemia has also been described from other regions of Pakistan.[29,34–36] Many of the children in this study were transfused with unscreened blood because several blood banks and pharmacies in Baluchistan have not implemented infection control measures and lack methods for blood screening, and there are many paid donors in Pakistan.

Several children were infected with more than one HBV or HCV strain. The number of infecting strains and multiple

TABLE 3
Genotypes and subtypes of sequenced HBV and HCV strains

			HBV genotype		HCV subtype				
Infecting virus	*N* infected	*N* sequenced	A	D	1b	2b	3a	3b	Multiply infected
HBV	11	6	0	3					3*
HCV region sequenced	73								
NS5B		14			0	5	6	2	0
Core		9			1	0	6	1	0
Both regions		7			0	0	1	0	7†
Subtotal		30			1	5	13	3	8
Total	82‡	36	0	3	1	5	13	3	11

HBV = hepatitis B virus; HCV = hepatitis C virus.
* Two children had two different genotype D strains, one child had genotype A and genotype D strains.
† Two children were infected with two 3a strains, three children were infected with one 3a and one 3b strain, and two children were infected with one 2b and one 3a strain.
‡ Two children were infected with both HBV and HCV.

FIGURE 2. Hepatitis B virus (HBV) strains in a phylogenetic tree based on the complete S gene. The strains from this study are marked with red. The strains from children infected with multiple strains are indicated with arrows. The strain indicated with arrow and box is from a child infected with HBV genotype A and D strains. This figure appears in color at www.ajtmh.org.

infected patients may be even higher because some of the strains were only identified because of variations in sensitivity of the sequencing primers. Next-generation sequencing would be needed to determine the exact number of strains in the sera. Multiple infections may be the source for recombinants as shown for HBV in Northern India.[32,33] The prevalence of hepatitis C in the children with β-thalassemia was nearly as high as in first-time blood donors in Quetta,[37] and is the second highest reported prevalence worldwide.[38] The prevalence of HBV and HCV among the patients in this study was equal or somewhat lower than that described from other patients with β-thalassemia in Pakistan.[34,39] In the current study, only one patient was positive for HIV. This is in accordance with other studies on HIV in this patient group in Pakistan[40] and indicates that luckily, for the time being, that HIV infection is not a health burden for thalassemia patients in Pakistan. This may be due to somewhat low prevalence rate of HIV infections in Pakistan.[41]

Because the efficient direct-acting antiviral drugs against HCV now are available in Pakistan,[42] β-thalassemia patients need to be treated. However, possible new recombinants may influence on this treatment and need to be monitored. For both HBV and HCV, the phylogenetic analyses of the sequences showed the presence of several genetically divergent strains. This may either be due to sequencing bias, if multiple strains were present in the samples, or that new divergent strains

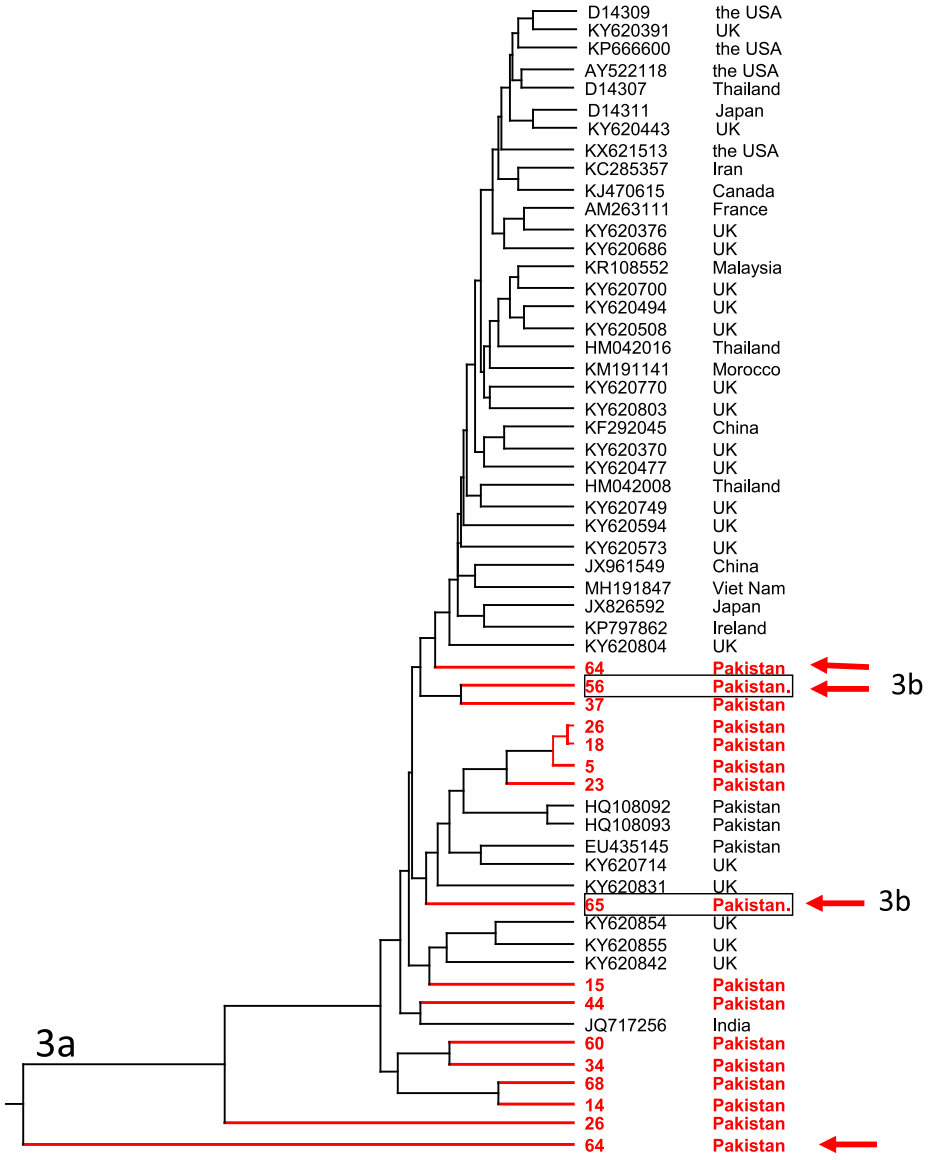

FIGURE 3. Branch of hepatitis C virus (HCV) subtype 3a strains based on partial core region. The strains from this study are marked with red. The strains from children infected with multiple strains are indicated with arrows. The strains indicated with arrows and boxed are from children also infected with subtype 3b strains. This figure appears in color at www.ajtmh.org.

have evolved. The virulence and spread of these viral strains is unknown, and needs further investigation.

Thalassemia is presumably the most common fatal genetic disorder in Pakistan with 5,000–9,000 children born with this blood disorder yearly.[7] Despite this high prevalence, there is a low awareness regarding β-thalassemia in the general population. In a study from Karachi, persons were interviewed regarding their knowledge on thalassemia.[43] Most did not know that it is a genetic disorder which causes a lifelong burden for the affected individuals[43] and low quality of life.[44,45] With modern treatments, as iron chelation therapy, and advances in the knowledge of the disorder, the mortality related to thalassemia has been reduced significantly worldwide. These treatments are, however, not affordable for many families in Baluchistan. The number of newborns with β-thalassemia is declining in Western countries because of the introduction of screening programs. Such programs and education regarding the cause of the disorder is needed also for Pakistan as has been proposed for India.[46] There is also an urgent need for improvement of blood safety in all regions of Pakistan, both with regard to donor selection and screening methods for TTIs. Improvement of blood and injection safety and interventions for well-functioning immunization are strongly needed, especially in Baluchistan.

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