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ONE HEALTH EPIDEMIOLOGY OF HEPATITIS C VIRUS IN PAKISTAN: INTEGRATING PREVALENCE, GENOTYPE DIVERSITY, MOLECULAR EVOLUTION, AND TRANSMISSION RISK

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Abstract

Pakistan has one of the world's highest burdens of blood borne infections of the hepatitis C virus (HCV) and has a major public health problem. In this review, we summarize the current state of epidemiological evidence on HCV in Pakistan, including the ecological niche and molecular evolution of HCV in Pakistan, from a One Health perspective. Genotype 3a is the predominant genotype, representing around 55-65% of infections, and with notable regional variations, while there is an alarming rate of untypeable infections (21.3%) and mixed infections (3.5%). Epidemic expansion of HCV 3a in South Asia dates back to the twentieth century and has reached saturation in diversity, with >100 years of circulation in the region. Various factors such as HACE, community practices like barbering and personal service rituals, occupational exposures and intrafamilial spread contribute to the dynamics of transmission. HCV is an indicator of a number of systemic weaknesses in infection control, informal healthcare, and socioeconomic factors. Combined with genomic surveillance, spatial epidemiology and risk mapping could provide a roadmap for precision elimination policy, however the coverage of such datasets is fragmented and the available longitudinal genomic data are limited.

1. INTRODUCTION

Hepatitis C virus infection is one of the urgent public health problems in Pakistan. Currently the country has the second largest prevalence of HCV infection in the world and it is estimated that 10 million people are infected with the HCV. The natural history of chronic HCV infection after persistent hepatitis to cirrhosis, hepatocellular carcinoma and hepatic decompensation is a significant burden on the

individual, family and health care system. Liver disease due to HCV is now the most common cause of liver transplantation in the country and is a major cause of liver-related death[1]. The importance to public health goes beyond individual morbidity. The incidence of new cases is around 240,000 a year, and it is steadily increasing. The burden of HCV is expected to increase in coming decades owing mainly to widespread use of unsafe medical procedures. Strategies to address HCV infection disproportionately affect working-age adults, resulting in reduced work productivity, and increasing social and economic vulnerability[2]. The first attempts to describe the HCV epidemic in Pakistan mainly included seroprevalence figures based on blood donors screening and hospital-based surveys. Although these studies documented the magnitude of the infection, they failed to provide much information regarding the viral factors responsible for viral transmission and progression of disease. With the advent of molecular epidemiological methods, the analytical paradigm has shifted from the descriptive mapping of prevalence to a more complex picture of the distribution of genotypes, dynamics of evolution and networks of transmission[3].

Genotyping studies showed that genotype 3 is the predominant genotype and 3a is the predominant sub-genotype in Pakistan. But the finding of mixed infections as well as untypeable variants have pointed to a more complicated viral landscape than first thought, and regionally distinct subtype distributions. Phylogenetic analysis has started to shed light on the historical geographic dispersal of HCV lineages to inform when and how the virus was introduced and spread in the area[4]. The transmission ecology of HCV in Pakistan can't be understood simply in a clinical and virological perspective. The virus exists in the interconnected human and environmental environment, including formal and informal health care, community-based personal-service practices, working environment and home environment. Traditionally used in zoonotic pathogens at the human-animal-environment interface, a One Health approach can be modified and applied here to focus on systemic and ecological aspects of HCV persistence[5]. This framework assumes that HCV transmission is the result of structural factors, and highlights the lack of infection control infrastructure, unregulated informal healthcare providers, socioeconomic factors that lead to unsafe practices and environmental routes of HCV exposure. The combination of molecular, spatial and social epidemiological data, therefore, is needed to develop interventions that try to target the root causes of transmission rather than just individual risk factors[6].

This review will attempt to collate the existing evidence on the epidemiology of HCV in Pakistan using an integrated One Health approach. Specific objectives include: (1) characterizing the epidemiology landscape, including prevalence trends and high-risk populations; (2) examining the transmission ecology and risk determinants across interfaces of healthcare, community and environment; (3) analysing genotype diversity and molecular evolution of circulating HCV strains; (4) exploring phylogeographic and phylogenetic patterns that reveal the course of viral dissemination; (5) evaluating the findings for their implications in diagnosis, treatment and elimination strategies; and (6) developing a research agenda and identifying key research gaps for a national genomic surveillance framework.

2. Epidemiological Landscape of HCV in Pakistan

2.1 National prevalence and temporal trends

In the past few decades, the HCV transmission has changed from hypo-endemic to hyper-endemic in Pakistan. Different population and methodology result in different national prevalence estimates. A detailed survey of published data from 2010 to 2015 revealed that the seroprevalence of HCV in the general adult Pakistani population was ~6.8% and the prevalence of HCV active infection was ~6% in the population[7]. Based on temporal trend analysis it was found that the epidemic is not yet at saturation. There is still a significant burden of new infections with an estimated 240,000 new infections being reported each year. This sustained increase in the number of infected

people indicates ongoing infection despite the fact that there are now cure-oriented direct-acting antiviral drugs – and it is because the number of people diagnosed and the barriers to access to effective treatment are still small, and there is continued exposure to transmission risk. There is strong, positive evidence for the prevalence in liver-related disease groups and evidence of prevalence increase in Pakistan with no evidence of prevalence decline[8].

2.2 Provincial and geographic heterogeneity

There is significant provincial variation in HCV prevalence. The following provincial prevalence estimates were available from a systematic review of data collected in the period 2000 – 2013: Punjab 5.46%, Sindh 2.55%, Khyber Pakhtunkhwa 6.07%, Balochistan 25.77% and federally administered tribal areas 3.37%. This analysis indicated a prevalence of 8.64% overall, across provinces[9]. Geographic variation is not only in terms of prevalence, but also in genotype distribution. While genotype 3a is dominant nationwide, a recent phylogenetic analysis of isolates from Sindh province showed that all sequences clustered with genotypes 3a and 3b, except for one isolate clustering with genotype 1a . This finding indicates that genotype 3a is indigenous to this area. A study conducted in the North West Frontier Province (NWFP) now known as Khyber Pakhtunkhwa (KPK) has reported genotype 3 (65.17%), genotype 1 (21.42%) and genotype 2 (11.60%) with huge variation in genotype distribution in different districts[10].

2.3 Urban–rural and socioeconomic disparities

Spatial variations in HCV infections in urban and rural areas are linked to differences in access to health care, infection-control measures, and socioeconomic status. HCV prevalence has been reported as high as 25% in rural and underdeveloped areas, and suggests a greater attention is required in this hitherto neglected socioeconomic group[7]. A consistent correlate of HCV infection identified in studies is socioeconomic status. Pakistan's place in the list of 134th of 174 countries on United Nations Human Development Index (UNDP) highlights the status of low standards of education and health care which are important factors in the transmission of HCV. Lower educational levels and earnings are associated with higher seroprevalence, which may be due to greater exposure risk and lower access to prevention health care[11].

2.4 High-risk and underserved populations

There are several populations in Pakistan which are disproportionately burdened with HCV. The pooled HCV prevalence rate is 32.33% among the hemodialysis patients, and provincial subt group analysis reveals that the prevalence is significantly higher in Punjab hemodialysis patients (37.52%) than that of Balochistan (34.42%) and Sindh (27.11%) and Khyber Pakhtunkhwa (22.61%). This is a high prevalence, which is reflective of the cumulative risk from frequent use of vascular access and the possible nosocomial transmission in dialysis units[12]. Patients with beta-thalassemia major are most frequent recipients of blood transfusion in Pakistan and the HCV prevalence is 29.79% in these patients. Another well-defined high-risk population is injecting drug users, who were estimated in two systematic reviews at 51.0% and 57% prevalence, respectively. The prevalence of the multi-transfused populations is 48.67%[13].

According to a systematic review, the prevalence of HCV in Middle East and North Africa in populations with liver-related diseases was 55.8% in Pakistan compared to only 15.6% in other countries of the Middle East. Patients with hepatocellular carcinoma (HCC) had the highest mean prevalence of 56.9%, followed by cirrhosis of 50.4%[14].

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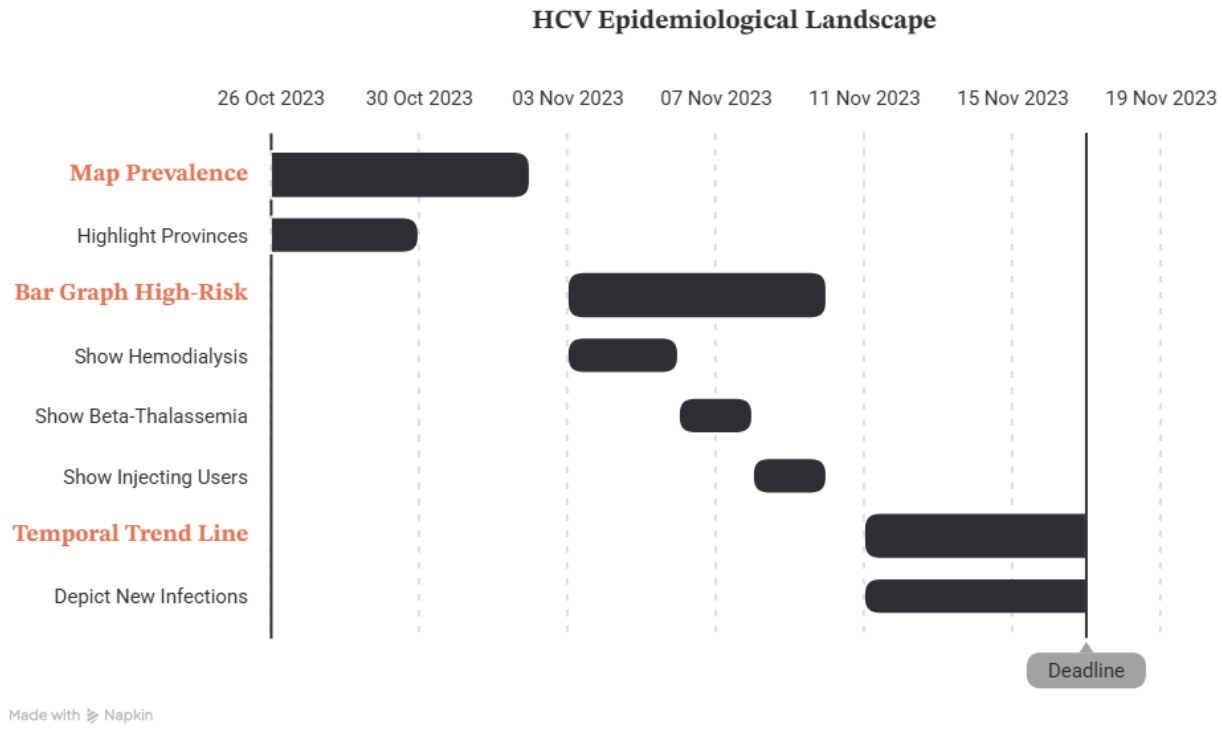


Figure 1.0. Epidemiological landscape of hepatitis C virus (HCV) infection in Pakistan.

3. Transmission Ecology and Risk Determinants

3.1 Healthcare-associated transmission

HAT is considered one of the big links of HCV transmission in Pakistan. Unsafe medical care was the primary contributor to the expected growth in HCV morbidity in the next decades, according to a comprehensive review. Reuse of syringes and needles, failure to adequately sterilize surgical instruments, and poor infection control practices can result in the efficient nosocomial transmission[15]. Nosocomial infection has been especially identified as a frequent risk factor in the region. If the nosocomial transmission rate is suspected to be high, urgent measures should be implemented for the fostering of safe medical practices. History of hospitalization has been consistently found to be an important risk factor for HCV infection. Another risk factor that has come to the fore is dental procedure; a study has found it to be the most common conventional risk factor (32.55% of patients)[16].

3.2 Blood transfusion and invasive procedures

Although, blood screening policy has been introduced in Pakistan, there remains a residual risk of transmission of HCV through blood transfusion, especially in areas with limited quality assurance capacity. Blood donors have been reported to be seropositive at 2.45% and it is urgent to reduce the risk of transfusion-transmitted HCV. The group with the highest prevalence of exposure to blood products is the population of thalassemia which needs transfusion support throughout their lives, where the prevalence of HCV is nearly 30%[17]. In addition to transfusion, invasive medical or dental procedures such as surgery, dialysis and other invasive procedures can cause them to be spread if the equipment is not reprocessed well. In several studies History of surgery and Blood transfusion is important risk factors. The recognition of HCV detection in healthcare facilities is complicated by the frequently non-symptomatic nature of acute HCV infection, and the identification of transmission events is not readily possible without the use of molecular epidemiology[18], [19].

3.3 Community and occupational exposures

Community-based transmission pathways contribute significantly to the HCV epidemic in Pakistan and may be currently underappreciated. Unsterilized needles/mounted equipment used for giving medicines and barber activities have been considered as a major risk factor for HCV infection. Community barber shops are especially very efficient ways to transmit HCV, and more than two-thirds of HCV patients are in the 40–50-year-old age group[20].

Street barbers are enumerated in this regard as one of the major transmission routes in Pakistan besides intravenous drug abuse, unsafe blood transfusion, recycled syringes and use of unsterilized surgical tools. Such practices are entrenched in cultural norms, and often carried out informally, with low levels of instrument sterilization. The community aspect of transmission places emphasis on the inadequacy of interventions in clinical practice and the importance for public health to engage with informal service providers[21].

3.4 Injecting drug use and sexual transmission

Injection drug use is a widely documented transmission route in Pakistan; sharing of injection equipment is a highly effective means of virus transmission. Systematic reviews have reported prevalence of HCV of 51.0% and 57% amongst IDU. Combined with other factors, such as socioeconomic marginalization, limited access to health services and incarceration, the combination of drug use and these risk factors generates an added vulnerability[22]. Sexual transmission is involved in transmission dynamics (possibly for household members and sexual partners). Sexual history was found to be an independent risk factor for HCV transmission in familial clustering studies, in which 17% of HCV-positive patients were found to have sexually active HCV positive spouses, whereas only 4% of those in the control group did. This discovery highlights the need for partner notification and screening as a part of a comprehensive prevention strategy[23].

3.5 Mother-to-child and household transmission

Unlike hepatitis B, maternal HCV transmission does not occur at a high rate, but it is still a problem with the high rate of HCV infection in women of reproductive age. In familial clustering studies, only 3.7% of HCV-positive individuals had HCV-positive mothers, and there was no significant difference in the risk of transmission between patients with and without HCV-positive mothers.

Infection in the household has been reported, including intrafamilial spread (household transmission). Thirty-one percent of patients (30.1%) had familial clustering of HCV versus 17.4% of controls. It is also a true possibility that non-conventional (person-to-person) transmission occurs in households, which is currently underestimated. In one study, the total of all non-conventional risk factors was found to be similar to and even greater than the predominant hematogenous risk factors, and household contact was the most important non-conventional risk factor. No group contained a risk factor alone and there were patients who were not known to have a risk factor (39.69%), meaning that HCV infection was apparently acquired by non-conventional modes in these patients, most likely by person-to-person interaction[24].

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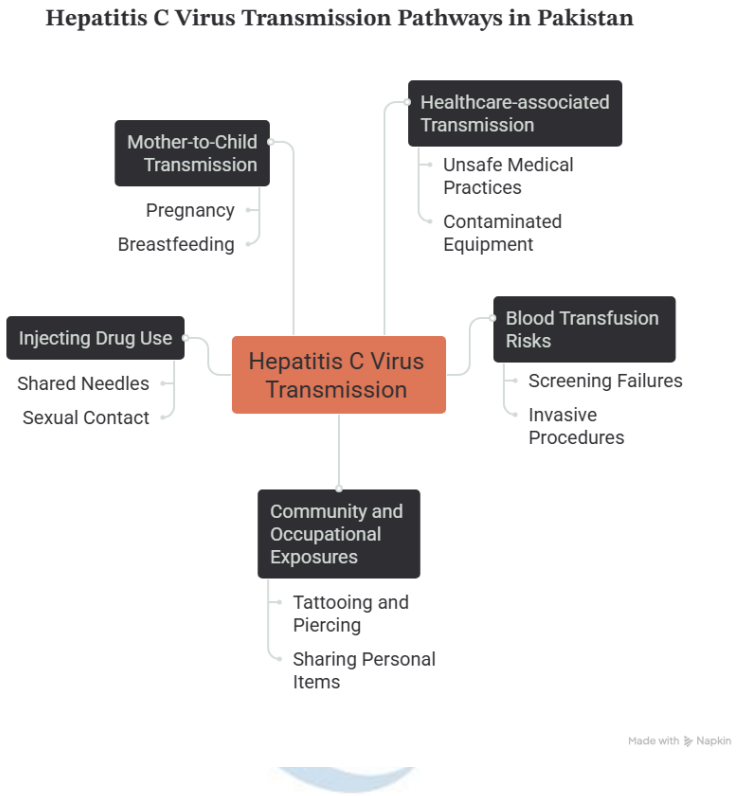


Figure 2.0. Transmission pathways and risk determinants of hepatitis C virus (HCV) infection in Pakistan. The schematic illustrates major routes contributing to HCV transmission, including healthcare-associated exposure, blood transfusion risks, injecting drug use, mother-to-child transmission, and community and occupational exposures. Key associated practices include unsafe medical procedures, contaminated equipment, shared needles, invasive procedures, tattooing and piercing, and sharing of personal items

4. Genotype and Subtype Diversity of HCV in Pakistan

4.1 Geographic distribution of major genotypes

Genotype 3 is the predominant genotype in Pakistan and the genotype landscape is strongly dominated by genotype 3. A systematic review of various studies published from 1996-2020 revealed that Genotype 3 was the most prevalent (65.35%) in which subtypes 3a (55.15%) and 3b (7.18%) were the most common. 5.1% of patients were of genotype 1, of which 4.38% were 1a. The prevalence of genotype 2 was of 2.66%, mainly distributed by subtype 2a (2.11%). Genotypes 4, 5, and 6 were scarce, with frequencies of 0.97%, 0.08%, and 0.32% respectively. This distribution is different from the global distribution where genotype 1 is the most prevalent worldwide. The high prevalence of genotype 3 in Pakistan and neighboring countries indicates Pakistan-specific transmission patterns. A systematic review of HCV epidemiology in Asia, Australia, and Egypt gave same results that genotype 3 is predominant in India and Pakistan[7].

4.2 Dominance and diversification of genotype 3

Since the first-ever HCV genotyping done in Pakistan, genotype 3a has been found to be the predominant HCV strain in Pakistan. The prevalence rate of genotype 3a was 63.45%, the largest of all types of genotypes in a systematic review from 2000 to 2013. The recent data not only corroborated this but also revealed that genotype 3a was the most prevalent (61.3%) in a recent comprehensive review. Overall, the genetic heterogeneity of genotype 3a is significant in Pakistan. Phylogenetic analysis of

NS3 and NS5b have previously demonstrated that the Pakistani 3a variants are genetically as diverse as those found around the world and intermingled extensively. This diversity suggests a long history of evolution in the region, as opposed to a recent introduction. The variant of genotype 3a found in the HCV sequence data obtained from a patient in Pakistan is quite different to the other genotype 3a variants and its origin is different as was confirmed by full genome sequencing of this HCV isolate[25].

4.3 Regional subtype variation

The genotype 3a is largely predominant throughout the country but the distribution of this genotype is variable throughout regions. The genotype 3 was reported in 65.17% of the cases, genotype 1 in 21.42% and genotype 2 in 11.60% of samples in Khyber Pakhtunkhwa where genotype 3a accounted for 42.85% and 3b genotype accounted for 22.32% of the samples. The HCV genotype distribution was found to be different in the various districts (Mardan, Charsadda, Peshawar, Sawabi, Nowshehra) of Khyber Pakhtunkhwa. A remarkable regional difference is the rise of the frequency of subtype 2a in geographic sub-regions. Subtype 2a was the second most common genotype in Khyber Pakhtunkhwa (17.3%) and Sindh (11.3%) provinces, which changed from patterns in the past. Phylogenetic analysis in Sindh province has indicated that most (all) of the isolates are of the genotype 3a and 3b with one sequence belonging to genotype 1a[25].

4.4 Mixed and undetermined infections

The epidemiology of HCV infection in Pakistan is characterised by many unique attributes, such as a higher number of untypeable and mixed-genotype HCV infections. Prevalence of untypeable genotype (21.34%) and mixed infection (3.53%) were estimated through meta-analysis of the studies performed from 1996 to 2020. Sequence variability of the most commonly used genomic region for genotyping, the 5' untranslated region (5'UTR), has been cited as the reason for the high rate of untypable infections. Using RFLP, a study with HCV samples from Pakistan (n=33) yielded genotype 3 in 25 and an undetermined result in 8. Genetic distance analysis showed significant differences between the isolates which were present in different clusters when compared with the reference sequences, and when pairwise comparison was done, it revealed more restriction sites which were not resolved. These mutations account, in part, for the lack of ability of conventional RFLP to type some isolates of HCV[26], [27].

4.5 Emerging genotype patterns

The whole genotype distribution has been fairly constant, but there are some signs of trends that should be monitored. The shifting distribution of different types of genotypes calls for more research to find new therapeutic regimes. Higher prevalence of mixed infections and unique sequence variation in the 5'UTR of genotype 3 isolates from Pakistan may suggest the possibility of new sublineages with changed biological features. The use of next-generation sequencing has now started to uncover the smaller-scale genetic heterogeneity of HCV in Pakistan. In the non-high-risk group of HCV patients, the predominant genotypes were 3a (86.6%), 1a (6.6%), 1b (3.3%) and 3b (3.3%). Within each patient, NGS showed high genetic diversity of HCV populations, with an average of 0.055 in the nucleotide sequence of HVR1. These data indicate that there might be more genetic diversity of HCV in Pakistan than was thought before[28].

5. Molecular Evolution of HCV Circulating in Pakistan

5.1 Genetic variability and evolutionary dynamics

Our results indicate that the HCV genotype 3a evolutionary dynamics in Pakistan have been reconstructed using Bayesian coalescent analysis. The most recent common ancestor of Pakistani genotype 3a variants was estimated to have happened during the late 19th century or early 20th century (around 1896-1914 CE), indicating a century-long circulation of these variants in the region. The size of the virus population increased from around 1905 and reached a plateau of about 1970. Demographic modelling

suggests that HCV 3a has reached saturating genetic diversity with a decreasing turnover rate and positive extinction. This is typical of an established epidemic and indicates that the virus has become well established within the host population and the virus transmission has stabilized, but at a high endemic level. Subtypes 1a, 1b and 3a are now established as epidemic strains and are found worldwide, with HCV genotype 3a being found to originate in South Asia, where it has become stably adapted to the host environment[29].

5.2 Mutation and selection pressure

The interplay of viral replication dynamics, host immune responses and transmission bottlenecks gave rise to the mutation rate and selection pressures acting on HCV in Pakistan. High genetic diversity was found within genotype 3a, suggesting a significant amount of genetic change over the course of 100 years circulating. But the nature of this diversity (whether it is neutral evolution or adaptive selection) is not well studied. A 5'UTR, which is usually very conserved, has shown some sequence variability in Pakistani isolates. Variants in this region have functional consequences for the virus replication and translation, and it is possible that some variants might be able to replicate with different fitness. It is not known whether these mutations are incidental to the rapid replication of HCV or adaptations to the host population[30].

5.3 Conserved versus rapidly evolving genomic regions

Across the HCV genome there are heterogeneous rates of evolution. The 5'UTR and core regions are relatively conserved and suitable targets for diagnostic assays and genotyping. However, the variation in sequence of the 5'UTR of Pakistani isolates introduces additional restriction sites that affect the ability of conventional RFLP methods. The NS3 and NS5b regions are more variable with higher resolution for phylogenetic analysis. Sequencing of these subgenomic segments of genotype 3a variants isolated in Pakistan has played a significant role in the genetic history reconstruction of HCV in Pakistan. The envelope regions (E1 and E2), especially the hypervariable region 1 (HVR1) of E2, are the areas with the highest mutation rates. Analysis of the E1-HVR1 regions by NGS has demonstrated a high level of intra-host genetic diversity with a maximum sequence diversity of 0.14 and an average of 0.055[31].

5.4 Antiviral resistance-associated substitutions

New selection pressures on HCV populations have been the introduction of direct-acting antiviral (DAA) therapy. Resistance-associated substitutions (RASs) may develop during therapy and affect therapeutic efficacy in the NS3, NS5A, and NS5B regions. Although there currently are few comprehensive RAS surveillance data from Pakistan, genotype 3a, which has historically had varying responses to some DAA combinations, should be a concern. A useful technique to help determine the length and choice of antiviral therapy is to estimate the genotype. Different HCV genotypes may have varying responses to certain antiviral treatments. The natural history of different genotypes may vary; some may produce more rapid liver damage than others, and differences may occur in the severity and progression of disease. The rapid turnover of polymorphisms that confer reduced susceptibility to DAA in naturally occurring infections may thwart scale-up of treatment, and the fact that mixed and untypable infections are common will add to the difficulty of monitoring resistance[32].

5.5 Evolutionary implications for treatment and control

The evolutionary history of HCV in Pakistan will have implications for the control strategy. Overall, positive extinction signals and saturating diversity of the demographic models indicate that there has been high transmission over decades with little evidence of recent transmission growth. This suggests that the risk of transmission is sustained in the current environment and not due to a high rate of epidemic transmission. The stable endemic state indicates that elimination will not happen as a result of short-term campaigns but will need a sustained intervention. This genetic diversity of circulating variants also implies that vaccine

development (if possible) would have to consider the predominant lineage of genotype 3a and allow for the possibility of immune escape variants. Evidence of high nosocomial transmission warrants urgent actions to improve safe medical practices, and the shifting frequency distribution of genotype warrants greater focus on research for new therapeutic approaches[33].

6. Phylogenetics and Phylogeography of Pakistani HCV

6.1 Phylogenetic clustering of Pakistani isolates

Phylogenetic studies of HCV sequences from Pakistan invariably show them to group into genotype 3, and subtype 3a is the largest subgroup. In Sindh province, a recent phylogenetic analysis revealed that all the isolates were grouped with genotype 3a/3b reference sequences, with one isolate being grouped with genotype 1a. The analysed sequences had 98% similarity with the reference and isolated sequences. Punjab studies have also reported the prevalence of 3a with 1a and 1b sequences in some cases. The clustering pattern indicates that the HCV epidemic in Pakistan is more likely to be due to a high number of endemic transmissions of 3a lineages than the high number of introductions of different genotypes from outside sources. High within-genotype diversity, however, suggests that these have been evolving within the region for a longer time[34].

6.2 Geographic lineage structure

Phylogenetic analysis of the Pakistani genotype 3a sequences with sequences from around the world has shown that these Pakistani variants are no less genetically diverse than those in other parts of the world, with extensive intermixing. This pattern is not expected to exhibit geographic clustering and indicates a lot of historical viral migration. The earliest molecular characterization of HCV from Pakistan showed it had a high similarity to a Japanese but low similarity to other reference strains, and thus a South Asian specific genotype was assigned. Sequence analysis of the entire genome of an HCV 3a strain from Pakistan (PK-1) and six other HCV genotype 3a complete genome sequences of different countries in the world revealed that the HCV genotype 3a of Pakistan (PK-1) is of a different origin than the other sequences, suggesting the circulation of HCV genotype 3a of different origin in Pakistan[35].

6.3 Viral migration and population mobility

The phylogeographic analysis of HCV in Pakistan is similar with the migration of humans and population mobility. HCV 3a has been traced back to South Asia and the region has been considered the source of global dissemination. Finally, there is extensive mixing between the Pakistani variants and those from elsewhere in the world, indicative of historical viral movement along trade routes and mixing through population movement. Geographic distribution of HCV lineages within the country is indicative of internal migration. Genotype 1a, for instance, may be present due to networks of transmission to regions where it is more common, like Sindh. The population increase beginning around 1905 and continuing until 1970 corresponds well with times of major population shifts and health care advances in the area[36].

6.4 Introduction and dissemination of viral lineages

Molecular clock analyses suggest that HCV was introduced in Pakistan in the 1980s. The most recent common ancestor of the Pakistani genotype 3a is estimated to be in the late nineteenth and early twentieth century (in between 1896 and 1914, with a range of 1851-1932 CE) under Bayesian estimates. This is a period of high mobility at the same time as the area is undergoing intensive population shifts and in terms of health services and development. Introduction of HCV in Pakistan seems to be ramped up in the twentieth century with increasing trends of healthcare infrastructure, blood transfusion and medical interventions involving injections. The population increase trend between 1905 and 1970 is in accordance with these developments, indicating that iatrogenic transmission appears to have contributed to dissemination. The effective population size was stable after 1970, indicating saturation of the diversity of HCV 3a with the reduced turnover rate[37].

6.5 Linking phylogeography with epidemiological evidence

Phylogeographic and epidemiological data can be combined to get a fuller picture of HCV transmission dynamics. The high proportion of genotype 3a has been seen in all provinces, and this is likely attributable to the historic founding of this genotype in the region, followed by its spread via linked transmission chains. The finding of different subclusters within genotype 3a in different geographic locations may be an indication of localised transmission patterns that may be the focus for intervention. Phylogeographic analysis revealed a correlation between the distribution of viral lineages and known risk factors (e.g., community or HAT transmission in rural and urban areas, respectively), which further support a targeted prevention approach. Global Hepatitis Outbreak and Surveillance Technology (GHOST) studies with next-generation sequencing (NGS) are starting to identify genetic relatedness among HCV cases and have the potential to identify incipient outbreaks among non-high-risk populations. The results corroborate the need for careful investigation of outbreaks on a country-wide level to locate clusters of transmission and their sources[38].

7. Molecular Epidemiology of HCV Transmission Networks

7.1 Transmission clusters and genetic relatedness

Molecular epidemiological approaches can help to identify HCV transmission clusters, defined as clusters of infections with very similar viral sequences, which suggest recent infection transmission. Systematic transmission network analysis has not been frequently used in Pakistan, but the existing phylogenetic data indicates evidence for clustering. The observed high prevalence of the genotype 3a in all populations is indicative of a high dissemination at the population level with interconnected transmission networks rather than with isolated outbreaks. NGS analysis of the E1-HVR1 region has been able to show genetic relatedness of HCV cases in non-high-risk populations, with 6.6% of cases showing possible evidence of possible transmission linkage. The results indicate that even people who are not traditionally at risk for transmission can have clusters[39].

7.2 Linking viral lineages with risk factors

Specific viral lineages are associated with risk factors, based on the finding of similar distribution of genotypes between different populations with a different exposure history. Genotype 3a was dominant in IDUs, thalassemia patients, and hemodialysis patients and in the general population, indicating that transmission is not risk-group specific. There are studies that show genotype concordance within the families, providing direct molecular evidence for intrafamilial transmission. Familial clustering into HCV cases (30.1%) versus controls (17.4%) and 17% of patients who had infected spouses suggests a contribution of household transmission networks. The same strategies to infections acquired in settings like hospitals and the community could help find connections and guide interventions[40].

7.3 Healthcare- and community-associated transmission networks

HCA-HCV transmission and CCHC transmission are poorly estimated in the burden of HCV in Pakistan. High levels of healthcare-associated HCV transmission are suggested by the high associations with surgical history, injection frequency, and dental procedures. However, community-based transmission is also important, as evidenced by the abundance of barbering and personal-service exposures. Distinguishing these transmission networks might be possible with molecular characterization of isolates from healthcare setting populations and community populations. Targeted interventions could be developed if different viral lineages are found to be preferentially circulating in healthcare environments versus community environments. The current evidence, however, shows a very wide overlap of lineages and mixing across these environments, and thus the transmission system is very entangled in high-prevalence environments[41].

7.4 Super-spreading environments and hidden transmission

Some environments can serve as super-spreading sites which allow efficient HCV transmission to occur. Potential amplification points are informal healthcare settings, unregulated blood banks, and barbershops with insufficiently cleaned-up instruments. One study found that the most common conventional risk factor reported was dental treatment at 32.55%. Control is complicated by "hidden transmission" (infections that arise outside of recognized risk groups). In one study 23.77% of them were not found to have any risk factor, and another 15.92% were only found to have unifactorial transfer through unconventional routes. These groups together accounted for 39.69% of patients with apparently non-conventional routes of infection (most likely by person-to-person contact). The results highlight the requirement for strategies addressing wider transmission routes that have not been considered as yet[42].

7.5 Molecular evidence versus epidemiological inference

However, there are significant gaps in the existing knowledge on HCV transmission in Pakistan, particularly regarding the lack of integration of molecular and epidemiological data. Most studies publish either genotyping results or associations of risk factors but few of them combine these two approaches to obtain a reconstruction of transmission networks. In the absence of sequence data that could be correlated with detailed epidemiological data, inferences about transmission routes are probabilistic. More robust transmission network analysis could be supported by having a national sequence database with linked epidemiological metadata. This type of resource could enable outbreak investigation, identification of new lineages, and assessment of the effectiveness of interventions. The work showing how GHOST technology can be used to recognize transmission clusters in Pakistan represents a proof of concept for this method[43].

8. One Health Interfaces Shaping HCV Persistence

8.1 Healthcare systems and infection-control practices

There are various points of contact at which HCV can be transmitted in the Pakistani health sector. Nosocomial spread is associated with inadequate sterilization of surgical instruments, re-use of syringes and needles and low compliance with standard precautions. The extremely high HCV prevalence in patients attending hospitals with various non-liver disease-related complaints (up to 30%) suggests a rise in the incidence of nosocomial HCV spread. An HSS strategy, which involves embedding HCV services into the infrastructure of primary health care, could help solve the issues of access to treatment and prevention for people living with HCV. The high nosocomial transmission rate provides a rationale for prompt action to ensure that as many safe medical practices as possible are used. Systematic training on infection control and promotion of injection safety and sterilization of medical equipment are among the necessary elements of such an approach[44].

8.2 Occupational and community interfaces

An underappreciated transmission interface is occupational exposure. The use of unsterile equipment for medication has been identified as a main risk factor for HCV transmission. The healthcare workers are not as widely studied as other groups in Pakistan and are also at risk of exposure to blood-borne pathogens via needlestick injuries and due to a lack of protective equipment. Community interfaces involve beauty salons, barbershops and other personal service institutions that re-use instruments that could be contaminated with blood. Street barbers are clearly mentioned in the major mode of transmission in Pakistan. These practices are strongly entrenched in the culture and are difficult to regulate and need to be addressed with community education and engagement with service providers as well as punitive measures[45].

8.3 Informal healthcare and personal-service practices

Informal healthcare is considered a key transmission interface in Pakistan since it offers a significant share of primary care. Unqualified practitioners may not be trained in infection control and may reuse injection equipment. The reuse of syringes and needles has been identified as a major contributing factor towards increased HCV prevalence. Some personal service providers, such as barbers, ear piercers and tattoo artists, use instruments that penetrate the skin and may not be properly sanitized. Facial shaving at barbershops is a key mode of transmission and should be targeted for intervention. Awareness programmes are required to decrease the future burden of HCV in the Pakistani population[46].

8.4 Biomedical waste and environmental risk pathways

The management of biomedical waste in Pakistan is facing a serious issue in which there is a lack of segregation, safe disposal and treatment. Needles and syringes that are not properly disposed of can cause community exposures. Increased HCV prevalence has been attributed to the re-use of injection syringes. Alternative epidemiological methods including wastewater surveillance may supply additional information on HCV transmission in the community. Whereas enteric pathogens can be used to track wastewater, HCV cannot be used because its presence can also be attributed to human contamination[47].

8.5 Socioeconomic and structural determinants

The endemicity of HCV in Pakistan is closely related to the socioeconomic and structural factors affecting exposure risks and access to health care. Poor infrastructure, poor education and poverty all contribute towards substandard health care provision, informal providers and late diagnosis. Poor educational and health indicators as reflected by Pakistan\'s Human Development Index (HDI) have a negative impact on the transmission of HCV. Sustainable elimination requires structural interventions such as improving healthcare infrastructure, regulating informal providers, and improving public health education. Increase of awareness and economic growth are necessary to contribute in reduction of HCV infection and betterment of health standards in Pakistan. But these interventions need to consider the upstream drivers of transmission as well as just individual behaviour change[48].

9. Integrating Genomics, Spatial Epidemiology, and Risk Mapping

9.1 GIS-based HCV epidemiology

Geographic information systems (GIS) can provide platforms to visualize and analyze the spatial distribution of HCV infection in Pakistan. District-level prevalence data can be used to map hotspots to target more intense interventions. The quality of available data is variable, however, and limited georeferencing of case reporting is a constraint on spatial analyses. The prevalence estimates at the provincial level from the systematic reviews (5.46% in Punjab, 2.55% in Sindh, 6.07% in Khyber Pakhtunkhwa, 25.77% in Balochistan, and 3.37% in the federally administered tribal areas) provided a basis for spatial analysis, but district-level data would help to further refine this mapping. Risk maps can be made more informative when combined with GIS data on risk factors, such as healthcare facility density, patterns of injecting drug use, and socioeconomic factors[49].

9.2 Genomic surveillance and lineage tracking

Genomic Surveillance of HCV consists of regular sequencing of HCV isolates in order to monitor the circulation of lineages, emerging variants and transmission clusters. Research studies have produced good sequence information from Pakistan, but these have been time-limited and smaller-scale not a continuous survey. A national framework of genomic surveillance would provide dynamic monitoring of the evolution and transmission dynamics of viruses. Sequencing of the NS5A and NS5B regions would be useful for resistance monitoring and full genome sequencing would aid in the resolution of transmission network analysis. Combining sequence data with epidemiological data is important in order to interpret the sequence data meaningfully. NGS

and GHOST technology to identify transmission clusters should be a blueprint for use in Pakistan[50].

9.3 Spatial–phylogenetic integration

The incorporation of spatial and phylogenetic information can uncover a geographical pattern of viral spread that would otherwise only be evident using one data source. Phylogeographic analysis can be used to pinpoint the most probable pathways for viral dispersal from one region to another and hence direct intervention along the transmission corridors. The use of Bayesian phylodynamic methods allows to estimate transmission rates and reproductive numbers from sequence data. Using these methods with Pakistani HCV data is hampered by the scarcity of georeferenced sequences. More extensive geographical collection of sequences would enable more powerful spatial-phylogenetic analyses. The finding that genotype 3a variants of the Pakistani population are just as genetically diverse as the rest of the world, and extensively intermixing indicates complex spatial dynamics which should be studied[51].

9.4 Predictive mapping of transmission hotspots

Predictive risk mapping includes information on risk factors, environmental factors and population features to pin point areas with high risk of HCV transmission. These maps can be used to direct screening outreach, resource allocation, and prevention initiatives. Data relating to the distribution of healthcare facilities, injection practices, barber density and socioeconomic indicators would be required to make predictive mapping for Pakistan. The high correlation between general population prevalence and liver-related disease populations indicate that general population prevalence data may be used to predict disease burden in liver-related disease populations. It is important to validate predictions with observed prevalence data for model refinement[52].

9.5 Digital and wastewater surveillance: opportunities and limitations

Digital surveillance methods such as mobile health apps that facilitate case reporting and geolocation of test services can help enhance data collection and real-time monitoring. Wastewater surveillance is useful for enteric pathogens, but not very useful for HCV with the primarily blood-borne route of transmission. The integration of digital tools into the health system must consider data privacy, connectivity challenges, and capacity of health workers. These tools should be used in addition to the traditional methods of surveillance[53].

10. Implications for Diagnosis, Treatment, and HCV Elimination

10.1 Risk-stratified screening and diagnosis

It is recommended that risk-stratified screening strategies be implemented, given the high prevalence of HCV in Pakistan and the large number of undiagnosed cases. High-prevalence districts may benefit from universal screening – and high-risk groups such as IDUs, hemodialysis patients and thalassemia patients or adults who have household contacts of infected people should be screened if resources are limited. More than 20% of the infections were untypeable, with consequences for the diagnostic algorithms. Methods used in confirmatory testing should be effective at detecting all genotypes, and the sequencing should be used in cases where a genotype cannot be detected by genotyping. The genetic polymorphisms in the 5'UTR region, causing failure of the RFLP method, emphasize the importance of the use of sequencing-based genotyping analysis[54].

10.2 Genotype-informed therapeutic strategies

Although the use of pan-genotypic DAA regimens is becoming more available, information about genotype continues to be important when making treatment decisions. This is a useful way of estimating genotypes, which can be useful in determining the length and type of antiviral treatment, since some genotypes of HCV may respond to some antiviral treatments better than others. Although genotype 3a predominates in Pakistan, it has demonstrated an unpredictable response to specific DAA combinations in the past, and this genotype should be considered with respect to choice of therapy and follow-up after therapy.

Several mixed and untypable infections are present, which makes genotype-guided therapy difficult. In patients who are non-informative on standard genotyping, deep sequencing approaches to detect minority variants might be needed to maximise the chances to manage the patient effectively[55].

10.3 Surveillance of treatment failure and resistance

Scale up of DAA therapy in Pakistan poses a selection pressure at the population level for resistance associated substitutions. Continuous monitoring to track emergence of resistance and treatment failure is crucial to maintain therapeutic effectiveness. A huge gap is in the lack of a national resistance monitoring programme. Resistance surveillance should be done on patients who failed treatment, breakthrough infections and to assess natural polymorphisms that might influence DAA susceptibility through periodic sampling of treatment-naive persons[56].

10.4 Targeted prevention of transmission

Multiple modes of transmission have been reported in Pakistan and prevention strategies should cover all such possibilities. Infection control measures in healthcare settings need to be improved as do injection safety and the sterilization of medical equipment. Addressing community-based transmission will involve working with barbershops, beauty salons and other types of community personal-service businesses to encourage non-contaminative practices. Needle and syringe programs and opioid substitution therapy are all necessary parts of a comprehensive approach to prevention for IDUs. Intrafamilial transmission can be reduced by interventions at the household level such as sharing education and contact tracing. This discovery of two distinct risk factors (family history and sexual history) for transmission emphasizes the need for household-level interventions[57].

10.5 Toward precision elimination strategies

Precision approaches focused on the geography and demographics to address specific drivers of transmission will be needed to eliminate HCV in Pakistan. Combining genomic, spatial and epidemiological information can help prioritize resources to regions and populations with potential for concentrated transmission. In addition to treatment availability, testing and treatment of populations with liver-related diseases are crucial to minimizing the burden and transmission of the disease and helping to achieve HCV elimination by 2030. The decentralized, integrated delivery model is a flexible approach that can be scaled up to expand access to HCV treatment. Integrating prevention, screening, and treatment into community health systems, and not starting as a standalone programme, is critical for sustainability[58].

11. Research Gaps and Future One Health Priorities

11.1 Fragmented epidemiological and genomic datasets

There is no comprehensive evidence base for HCV in Pakistan with cohesive data sets. There are various methodologies and manifold definitions of populations in prevalence studies which restrict the comparability. The genome studies are not surveillance programmes, and so they have limited geographic and temporal sampling of the genomes. A national data platform to connect epidemiological, clinical and genomic data would allow for more robust analyses and for understanding research and translating findings to policy. Such integration requires standardization of data collection and reporting[59].

11.2 Under-representation of regional populations

Geographic analysis of HCV research in Pakistan revealed an uneven distribution with a relatively high rate of research in the Punjab and Sindh provinces, while Balochistan, Gilgit-Baltistan and the rural areas of Khyber Pakhtunkhwa are under-represented. In addition, the total sample size of thalassemia studies is not more than 10% of the estimated thalassemic patient population, and further studies or a national baseline survey is clearly needed to verify the real frequency of HCV in thalassemic patients nationwide. Increased

research in under-researched areas involves investments in laboratory facilities, training of local scientists and community engagement to enable involvement[60].

11.3 Limited longitudinal genomic surveillance

Lack of longitudinal genomic surveillance limits the tracking of viral evolution, monitoring for emerging variants, and resistance monitoring. The majority of data from Pakistan are from single-time-point cross-sectional samples, and thus, temporal trends cannot be analysed. The requirements for the necessary investments in sequencing techniques, bioinformatics and specimen archiving to create sustainable genomic surveillance. Linkage with pre-existing diagnostic and treatment services can offer a viable platform for specimen gathering[61].

11.4 Standardization of molecular epidemiological studies

Comparability of molecular epidemiological data is constrained by the different ways in which studies design problems, genotyping tests and reporting procedures are conducted. Genotype distribution cannot be compared directly since different genomic regions have been used for geneticization (5'UTR, NS5B and core). There is a need for the development of standardized protocols for HCV molecular epidemiology studies in Pakistan, such as standardization of genotypic regions of interest, sequencing methods, and standardization of minimum metadata[62].

12. Conclusions and Future Perspectives

Hepatitis C virus infection in Pakistan is a multifaceted public health issue that is influenced by virological, ecological, and structural elements that surpass the usual biomedical paradigm. The predominance of the century long endemic genotype 3a, which has an established transmission pattern in South Asia, is evident of several exposure routes including healthcare, community and work. A large number of untypeable and mixed infections make diagnosis and therapy more difficult and the appearance of resistance-associated substitutions poses a serious challenge to therapeutic developments. The blood-borne transmission ecology of HCV provides a rationale for integrated control efforts based on a One Health framework adapted to fit the HCV context. This framework is based on the fact that the complex issue of HCV persistence is related to several interconnected systems including healthcare infection control, informal service provision, socioeconomic and environmental exposures. To be effective, however, interventions need to tackle these systemic drivers and not just risk behaviors per se.

Disabled health systems, inadequate access to diagnosis and treatment, and lack of targeted prevention strategies are necessary conditions on the road to elimination. Combined with spatial and epidemiological data, genomic surveillance can help inform precision interventions and track progress. Evidence of positive extinction signals and saturating diversity of HCV 3a suggests that transmission is likely ongoing via risk exposures and not necessarily through epidemic expansion, highlighting the need for continuous intervention. The challenges are great but so are the opportunities. Given political will, continued investment and evidence-based interventions, Pakistan can strive to make HCV a thing of the past. Molecular epidemiology can be a tool towards this goal, through its linkage to health systems strengthening.

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