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MOLECULAR EPIDEMIOLOGY AND PREVALENCE OF HEPATITIS C AND B VIRUS INFECTIONS IN SOUTH PUNJAB, PAKISTAN: MULTICENTER PCR-BASED STUDY

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ABSTRACT

Objective: *To establish the prevalence and geospatial mapping of Polymerase Chain Reaction (PCR) confirmed Hepatitis C (HCV) and Hepatitis B (HBV) infections and to identify the possible demographic and occupational risk factors in South Punjab, Pakistan. A multi-centric cross-sectional study was carried out at different collection sites in South Punjab.*

Method: *Active viral infections were confirmed using real-time PCR (COBAS 5800 system, detection limit 2.7 IU/ml) in 1,000 subjects for both viruses, who were suspected based on clinical presentation. A questionnaire was used to gather information about age, sex, profession and risk factors, which were analyzed with Chi-square tests and Odds Ratios (OR).*

Result: *The overall positivity rate for active infection was 34% (340/1000) for HCV and 53% (530/1000) for HBV. Maps revealed hyper-endemic hotspots, mostly in rural areas, including Jalalpur Peerwala (85% HBV), Alipur (82% HBV), and Mian Channu (70% HCV).There was a clear generational shift: HCV was highest in the age group 40-60 years (67%), whereas HBV was the highest in young adults, 15-39 years (57%).In terms of occupations, the highest prevalence was among laborers (81% HBV) and farmers (70% HBV).Key risk factors included ignorance (74% HCV; 50% HBV), history of surgery (45% HBV; 8% HCV), and blood transfusion (50% HBV). Significantly, there was a 100% association between drug abuse and HBV. Family history was a significant predictor for HBV (46%) but not for HCV (3%, $p < 0.001$).*

Conclusion: *The viral hepatitis burden in South Punjab is a serious one, with a "legacy" HCV epidemic in middle-aged people and an emergency of HBV in rural youth and the labor force. The concentrated infection in rural "exposure points" highlights the need for micro-level public health strategies, vaccination in high-risk workplaces, and improved clinical infection control practices.*

Keywords:

INTRODUCTION

Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) continue to pose a major global burden, with millions of people affected and a significant impact on global health expenditure [1, 2]. HCV infection prevalence is variable globally, with differences in socio-demographic, behavioral and health service-related factors [3]. Pakistan is one of the countries with the highest burden of HCV, with a prevalence of 4.3% [4]. HBV is also very common, with 254 million people infected

globally in 2022 and 1.2 million new infections each year [5]. The most densely populated province of Pakistan, Punjab, has an epidemiological heterogeneity. Punjab has an estimated anti-HCV prevalence of 8.9%, and to eliminate the virus by 2030, 7,045,000 cases need to be treated, save 152,000 lives and prevent 104,000 incident cases [4, 6, and 7]. South Punjab, in particular, is a high-risk area for HBV and HCV [8-10]. But most of the data are based on serological screening and blood donors which may not discriminate between active and past infections. As a result, there is a paucity of surveillance data based on PCR for estimating the true burden of active infection in this area. South Punjab, with a population of mostly rural and semi-urban people, represents an ideal region to explore the socio-demographic, occupational and behavioral risk factors associated with hepatitis. Understanding these factors is essential for addressing the infection. Earlier studies using antigen or antibody-based methods have only given a partial picture of the burden of active infections, often underestimating or misclassifying disease [11, 12]. However, real-time polymerase chain reaction (PCR) enables sensitive and specific detection of viral genetic material, enabling the distinction between active infection and previous exposure. However, multicenter epidemiological studies based on PCR are rare in South Punjab. The current study at the Alkhidmat Diagnostic Center, Multan, is one of the first large-scale, retrospective, multicenter, diagnostic surveillance using real-time PCR to determine the presence of HBV and HCV infections in South Punjab. The study aimed to: (i) estimate PCR-confirmed active infections among clinically referred individuals, (ii) determine the risk factors associated with infection, and (iii) determine the geographic variations of PCR prevalence rate among clinically referred individuals. This study fills a surveillance gap by furnishing PCR-confirmed data for risk factor analysis that can be used to inform targeted prevention interventions, resource allocation, and policy making to control viral hepatitis in South Punjab.

Material and Methods

Study Design:

This was a retrospective cross-sectional study to measure PCR positivity rate among clinically referred cases of Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) infections in South Punjab, Pakistan, between January 2024 and January 2026. Participants clinically referred for testing for HCV or HBV infection from various collection sites of Alkhidmat Diagnostic Centre were identified. Samples of only those who belong to South Punjab region were selected and those who were not on antiviral treatment.

Data Collection:

A total of 2,000 patients (1,000 for HCV and 1,000 for HBV) had their blood samples and socio-demographic information collected. Information was collected on sex, age, site of collection, occupation (businessman, laborer, farmers, other jobs (government and private-sector jobs), unemployed), education, iatrogenic risk factors (blood transfusion, dental treatment, surgical procedure or drug use), knowledge of viral hepatitis, risk factors (smoking, alcohol intake), family history of hepatitis B, tattooing and drinking water source as a socio-economic status indicator . History of previous HBV/HCV infection and co-infection was extracted from the patient data form (data collection form) filled at sample collection centers. Informed consent was taken from all the participants and the study was undertaken according to the ethical guidelines of AlKhidmat Diagnostic Ethical Committee and standard biosafety procedures.

Sample Collection and Processing:

Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes (Greiner BIO-ONE Vacutainer) with sterile phlebotomy procedures. Barcodes were used to uniquely identify each sample. The blood samples were shipped to the central laboratory in cold chain (4-8°C).The supernatant (plasma) was then removed by centrifugation (4,000 rpm for 4-5 minutes).HCV and HBV nucleic acids were detected by the COBAS 5800 System (Roche Diagnostics) that uses nucleic acid extraction from the specimen using magnetic glass particles and real-time PCR amplification. Specific primers were

used to amplify viral RNA/DNA, and the dual detection of viral nucleic acids and internal RNA quality controls were achieved using fluorescent probes. The system automatically interpreted data with pre-set cut-offs and the quality of the run was checked using positive and negative controls.

Data Analysis:
The results of the lab test were linked with the socio-demographic and risk behavior data to assess the association between HCV and HBV PCR positive rate among the referred population. Data were statistically analyzed with GraphPad Prism v.10, with chi-square tests used to compare infection status with categorical risk factors. Confidence intervals were used to assess the significance of differences in age, gender, occupation and geographic location. A p-value <0.05 was deemed significant.

Declaration of Generative AI use:
Generative AI (ChatGPT and Geminie) was used in the preparation of this manuscript to improve the language. The authors determined all scientific content, interpretation and conclusions.

Results:
Geographical Distribution of HCV and HBV:
1,000 samples for HCV from different regions of South Punjab were tested. The PCR positivity rate for HCV was (340/1000, 34%). Multan had the highest number of HCV-positive cases (182/526, 35%) due to the larger sample size tested. But the highest positivity rates were in Farroqpora (3/3, 100%), Khanewal (3/3, 100%), Mianchuno (7/10, 70%), and Jampur (14/21, 67%), suggesting marked geographic variations in HCV burden in the region (Table 1). The overall PCR positivity for HBV was (530/1000, 53%). While the volume of testing was not consistent across the sites, there was tremendous geographic variation in HBV positivity. Multan had the highest absolute burden of disease (278/425, 65%) due to a higher volume of tests, but the highest positivity rates were observed in Jalalpur Peerwala (11/13, 85%), Alipur (9/11, 82%) and Jampur (38/50, 76%) (Table 2).Other districts with high burden include Kot Adu (8/12, 67%) and Muzaffargarh (8/13, 62%) (Table 2). 1,000 individuals referred from clinical settings for HCV real time PCR test, including 700 males (70%) and 300 females (30%).Of the males, 195 were positive for HCV RNA (PCR positivity 28%) while 145 females were positive for HCV PCR (48%)..

Gender-wise Distribution:
Our analysis revealed a significant association between gender and HCV PCR positivity (χ^2 = 39.23, df = 1, $p < 0.001$) with females showing a higher prevalence of infection than males. These results suggest a higher rate of active HCV infection in female patients among clinically referred patients. A sample of 1,000 patients referred by clinicians was tested for HBV real-time PCR, comprising 610 males (61%) and 390 females (39%).Males had a positivity rate of 54% (n = 330) and females 51% (n = 200) for an overall HBV PCR positivity rate of 53%.While a slightly higher proportion of males were positive for HBV compared with females, this was not statistically significant (χ^2 = 0.65, df = 1, p = 0.42).

Age-wise Distribution:
Age ranged from 15 to 101 years and was classified into three groups. The 15-39 years age group followed by 40-60 years and 60-101 years age groups. Stratified analysis by age showed a difference between HCV and HBV (Figure 1). HCV positivity peaked in the 40–60 years age group (228/340, 67%), followed by 15–39 years (102/327, 31%) and was lowest in individuals aged 60–101 years (10/333, 3%). Age-specific analysis of PCR positive for Hepatitis B virus (HBV) showed a high burden in young age population in South Punjab. The 15-39 years age group had the highest rate of positives: 57% (391/687) of those screened were positive for HBV DNA. Notably, after decreasing in the middle-aged category (40-60 years) to 43% (114/263); a moderately high peak was observed in the elderly. Among the elderly (61-101 years), 50% (25/50) were positive, implying a bimodal pattern of risk in the population. This categorized age-distribution suggests that modes of transmission of the two viruses vary in the South Punjab.

Occupational Distribution:

The researchers assessed the occupational distribution of 1,000 clinically referred cases for its association with the prevalence of Hepatitis C virus (HCV) infection. Farmers and laborers had the highest frequency of infection; the highest rate of infection was found among farmers (47.1% (120/255) followed by laborers (40.2% (142/353). A slightly lower frequency of infection was found in businessmen (27.4% positive, 52/190) and the "other jobs" category (including government and private-sector jobs) (20.0% positive, 10/50). The prevalence of HCV infection was least among the unemployed population with 10.5% (16/152) testing positive. These findings suggest that laborers and farmers in South Punjab are at higher risk of HCV infection, implying a significant role of occupational exposures in the spread of HCV. HBV positivity was higher in the farmers and laborers. Laborers exhibited the highest prevalence at 81% (195/240), followed by farmers at 70% (160/230) and businessmen at 60% (84/140).By contrast, the remaining groups showed significantly lower rates of infection; the unemployed group had a 25.3% positivity rate (43/170), while those in "other jobs" (government and private-sector jobs) had the lowest infection rate at 21.8% (48/220). These findings suggest that laborers or farmers in South Punjab are more vulnerable to HBV infection, possibly due to occupational and socio-economic influences.

Risk Factor Analysis:

A thorough screening of behavioral, iatrogenic and awareness factors revealed a number of key factors associated with HCV infection. A lack of awareness was the most striking feature, with a 74% (250/690) positivity rate and a significant association with the presence of virus (OR = 2.041; χ^2= 55.411; $p < 0.001$). Iatrogenic exposures also demonstrated strong statistical significance, including dental treatments at 12% (14/120) (OR = 0.372; $p < 0.001$), blood transfusions at 10% (10/100) (OR = 0.314; $p < 0.001$), and surgical procedures at 8% (16/200) (OR = 0.236; $p < 0.001$). HCV positive case who reported drug abuse was 2.5% (1/40). Additionally, HCV positive cases with family history were 3% (3/110). HCV positive cases with previous HCV diagnosis were 12% (42/350), and with HBV co-infection were 5% (2/40). However, alcohol intake was not a significant factor (10%, 1/10; $p = 0.444$). Environmental studies confirmed that transmission was not water-borne; although the infection rates were varied for different types of water, from 58% (85/147) for filtered water and 47% (28/59) for WASA to 0% (0/118) for mineral water and 0% (0/147) for other, these variations are likely to reflect socio-economic class clustering, rather than direct water-borne transmission. The behavioral and iatrogenic risk factors for Hepatitis B virus (HBV) assessed identified some key drivers of HBV transmission in South Punjab. A lack of awareness was prevalent with 50% (415/820) of the population affected. In particular, drug addiction was a clear risk factor, with a 100% (25/25) positivity rate in this particular group, making it an extremely high-risk group ($p < 0.001$). Other notable iatrogenic factors include blood transfusions (50% (25/50), surgical procedures (45% (59/130) and dental treatment (32.5% (13/40). Biological and family exposures also played a significant role in HBV infection. Many participants reported a history of hepatitis in their families (46%, 138/300) and among those who had been previously diagnosed with HBV, 44% (35/80) were found to be positive again. Alcohol was a less significant factor at 26% (13/50). Regarding environmental demographics, HBV positivity for the various sources of water was 50% (113/225) for WASA and 42.6% (187/438) for pumps, mineral water 40% (50/125) with the highest rates in the "other" category (85%, 180/212). Consistent with global health priorities, the observed correlation between water sources and viral positivity is interpreted as a surrogate marker of socioeconomic status rather than an indication of water-borne transmission, as lower-quality water sources often reflect households with limited financial resources and a corresponding lack of access to sterile medical practices and health literacy. Taken together, the findings indicate that intravenous drug use and iatrogenic exposure, coupled with the lack of awareness, are the main drivers of the HBV epidemic in the population in question.

Statistical Analysis:
The chi-square test (GraphPad Prism v.10) was used to test the association of HCV and HBV positivity with demographic/behavioral risk factors (gender, age, occupation, iatrogenic exposure, family history, awareness, and water source). Stratified analyses according to age, sex, occupation and geographical location was done.

Discussion:
We show a high prevalence of PCR-confirmed HCV and HBV infections in suspected patients from South Punjab, suggesting an ongoing public health challenge of viral hepatitis in this part of Pakistan. In particular, we found that the prevalence of HCV infection was higher in females than males, a finding similar to other studies in Punjab [12], but different from the worldwide pattern in which males predominate. HBV infection was higher in males (54%) than in females (51%) as reported by one such study conducted in Multan [9].

Regional and Age-specific Patterns:
The viral hepatitis epidemiology in South Punjab is characterized by considerable geographic and age-specific variation. While Multan is the main diagnostic epicentre (182/526 for HCV; 278/425 for HBV) [9, 13-15], the maximum number of positive cases were found in rural districts, namely Jalalpur Peerwala (85% HBV), Alipur (82% HBV) and Mian Channu (70% HCV). This map shows that while cities offer mass screening, rural and urban "hotspots" are sites of intense transmission. A recent striking finding in this cohort is the contrasting age at risk while HCV prevalence had a deep peak in the 40-60 years age group (67% [228/340]), it was low (3% [10/333]) in the elderly (61-101 years). This shows that lack of awareness in 40-60 years age group was (158/228, 69%) In contrast, HBV prevalence was highest among the young, productive population with an estimated 57% (391/687) in the 15-39 years age group. By contrast, the HBV epidemic among the young (15-39 years) reflects a contemporary public health failure, with lack of awareness (HBV 50%), current risks such as surgical procedures, drug abuse, blood transfusion and occupational risks in the laborers and agricultural industries (HCV 40.2% - 47.1%; HBV 70% - 81%) contributing to continued transmission. Significantly, the 100% HBV positivity among drug users and the high family history (HBV 46%) suggest peculiar modes of transmission, which are not necessarily linked to health services [16, 17]. Divergent age-related distribution of viral hepatitis was observed; while HBV positivity remains consistently distributed across all age cohorts, HCV prevalence is significantly lower among the elderly. This suggests that the transmission risk for HBV is uniformly established across the population, whereas the HCV burden appears more concentrated in younger and middle-aged groups. These findings highlight the pressing need for age-specific treatment programs: for HCV, middle-aged groups need to be targeted for therapy and, for HBV, massive vaccination and education efforts must be directed towards the rural youth.

Occupational and Behavioral Risk Factors:
The occupational distribution in our study shows a socio-economic gradient in viral hepatitis, with laborers and farming occupations being the most affected. Laborers were the most vulnerable group, especially for HBV, with a staggeringly high 81% (195/240) positivity, in contrast with 40.2% (142/353) HCV positivity in this group. Farmers were the second most vulnerable group, with 70% (160/230) HBV and 47.1% (120/255) HCV positivity, respectively.
These differences have been linked to variations in exposure to informal healthcare providers, transmission at the community level and health seeking behaviors. Moreover, previous research has shown a high proportion of people living with HCV have low knowledge [19, 20]. Recent information demonstrates around 74% of patients have a low level of knowledge about transmission and prevention of viral hepatitis disease, revealing a lack of knowledge [20], and the need for better health education programs among high-risk occupational groups. Family and Co-infection Dynamics Of those surveyed, 46% with a family history of hepatitis B virus (HBV) were also positive for HBV. This finding is consistent with emerging evidence of the role of intra family spread of HBV. One study recently found that of family contacts of HBV infected index cases,

9.23% were HBsAg positive and 32.75% had anti HBc antibodies; shared bedding, personal hygiene products and dining utensils were common risk factors; suggesting the importance of close contact in viral transmission [21]. Analytic evidence shows that family contacts of carriers are a risk group even in non-endemic regions [22]. Based on the history at the time of HCV PCR testing, 5% had documented HBV co-infection. Though the frequency of co-infection positivity varies with the population and location, co-infection remains an issue of global concern [23, 24]. In some studies, the prevalence of HCV-HBV co-infection has been reported to be as low as 1% and as high as 15%, depending on the population and risk factors [25]. Risk factors and iatrogenic exposures risk factor analysis showed that most important risk factor for infection for both viruses was lack of awareness (HCV 74%, HBV 50%). Iatrogenic factors (surgery, dental treatment, blood transfusion, drug abuse) play role in transmission of HCV and HBV infections in line with previous research [26, 27]. These results highlight the ongoing contribution of unsafe health-care practices to the spread of viral hepatitis, especially in resource poor settings. For HBV, drug abuse was striking with 100% positive among those who reported this. History of infection and family history were also significant but to a lesser degree [22]. Importantly, drinking water sources did not seem to be a risk factor, confirming the CDC and WHO guidelines [28, 29].

Epidemiological Findings and Major Observations:

The use of real-time PCR in this study offers a significant advantage over serological methods as it identifies active infection rather than exposure and immunity. This study used the COBAS 5800 system, which has a limit of detection of 2.7 IU/ml to detect low-level viremia that may have been overlooked in other studies [30]. This approach improves the accuracy and precision of the estimates of the proportion of positives and reflects our knowledge of the epidemiology of viral hepatitis disease in South Punjab. The results suggest the need for awareness-raising among farmers, laborers and young adults, and rigorous infection control measures in health and dental care. Multan, D.G. Khan, Sadiqabad, D.G. Khan, Shujaabad and Jampur should be considered priority areas for public health measures. The study also highlights the need for PCR-based surveillance to estimate the prevalence of active viral hepatitis infections to guide evidence-based planning and prevention.

Conclusion:

This is the first multicenter study confirming the epidemiology of HCV and HBV infections in South Punjab, Pakistan, using PCR. Through the use of real-time PCR, a highly sensitive and specific molecular diagnostic method, not only active infections were identified but also the viral load distribution in patients referred to clinical settings. Our study identifies geographic hotspots and high-risk subpopulations, including Multan district and its associated areas, as well as specific high-risk demography and occupations.

Risk factors such as iatrogenic risks (surgery, blood transfusion, dental treatment, and drug abuse), and poor awareness were identified as major risks of transmission. These findings highlight the need for focused public health strategies, such as awareness-raising, training of healthcare professionals, and rigorous infection control practices to control viral hepatitis infections in South Punjab. This research highlights the importance of molecular surveillance in the accurate assessment of active viral hepatitis to inform policy makers and health authorities to plan effective prevention, treatment and resource allocation strategies. This research moves beyond reporting numbers to offering a tactical guide to regional action, making data-driven precision a cornerstone for Hepatitis eradication in South Punjab.

Declaration:

Availability of Data and Material

The datasets analyzed during the current study are not publicly available due to institutional restrictions and patient confidentiality, but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study protocol was reviewed and approved by the Institutional Ethics Committee of Alkhidmat Diagnostic Center, Multan. Data used were anonymized and collected as part of routine clinical and laboratory procedures, with informed consent obtained from all participants or their legal guardians in accordance with ethical guidelines.

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Competing interests
The authors declare no conflicts of interest.
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Conflict of interest statements:
The authors declare that there is no conflict of interest.
One of the co-authors Dr. Muhammad Sarwar Chaudhry serves as the Head of Department at AlKhidmat Diagnostic Center and provided the ethical approval for this study. However, the data collection and analysis were conducted independently to ensure objectivity, and this role did not influence the study's findings.

Authors' contribution:
Ayma Aftab: Data Collection, analysis and writing original draft
Masud Ali Ansari: Reviewed and edited the data
Hijab Imran: Conceptualization
Muhammad Sarwar Chaudhary: Supervision

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Table 1: Multicenter HCV PCR testing in South Punjab demonstrating geographic variation and high-burden areas, with Multan showing the highest positivity.

Collection Centers of AKDC in South Punjab	Total number of person who requested for test	HCV positive cases	Positivity Rate (%)
LAYYAH	36	10	28
MUZAFARGARH	43	10	23
JALALPUR	7	3	43
SADIQABAD	3	0	0
SHUJABAD	94	43	46
SAMIJABAD	3	0	0
KABIRWALA	10	3	30
D.G.KHAN	25	7	28
JAHANIA	43	14	33
MIANCHUNO	10	7	70
ALIPUR	43	14	33
LODHRAN	14	0	0
DUNIYAPUR	21	7	33
FARROQPORA	3	3	100

RAJANPUR	3	0	0
KHANEWAL	3	3	100
JAMPUR	21	14	67
BAHAWALPUR	10	3	30
MULTAN	526	182	35
TOUNSA SHARIF	3	0	0
KEHRORPAKA	25	7	28
MAILSI	18	3	17
BASTI MALOK	29	7	24
HARONABAD	7	0	0
Total	1000	340	34

Table 1 legend:
This table presents the distribution of HCV real-time PCR positivity across multiple collection centers of Al-Khidmat Diagnostic Center in South Punjab. Percentages represent the proportion of HCV PCR–positive cases among clinically referred individuals tested at each center.

Table 2: HBV PCR testing in South Punjab, highlighting hotspots and variation across collection centers.

Collection Centers of AKDC in South Punjab	Total number of person who requested for test	HBV positive cases	Positivity Rate (%)
DYNIYAPUR	25	13	52
MULTAN	425	278	65
MUZAFFARGARH	13	8	62
SADIQABAD	75	25	33
VEHARI	13	6	46
JAMPUR	50	38	76
MAILSI	13	4	31
D.G.KHAN	100	35	35
BAHAWALPUR	38	13	34
JAHANIA	13	7	54
LAYYAH	25	11	44
KABIRWALA	24	9	38
RAJANPUR	38	13	34
KEHRORPAKA	38	10	26
RAHIMYARKHAN	13	5	38
LODHRAN	13	8	62
SHUJABAD	10	3	30
JALALPUR PEERWALA	13	11	85
KOT ADU	12	8	67
BASTIMSLOK	13	5	38
ALIPUR	11	9	82
TOUNSA	13	4	31
HAROONABAD	12	7	58
Total	1000	530	53

Table 2 legend:
This table shows the distribution of HBV real-time PCR positivity across collection centers of Al-Khidmat Diagnostic Center in South Punjab. Percentages represent the proportion of HBV PCR–positive cases among clinically referred individuals tested at each center.

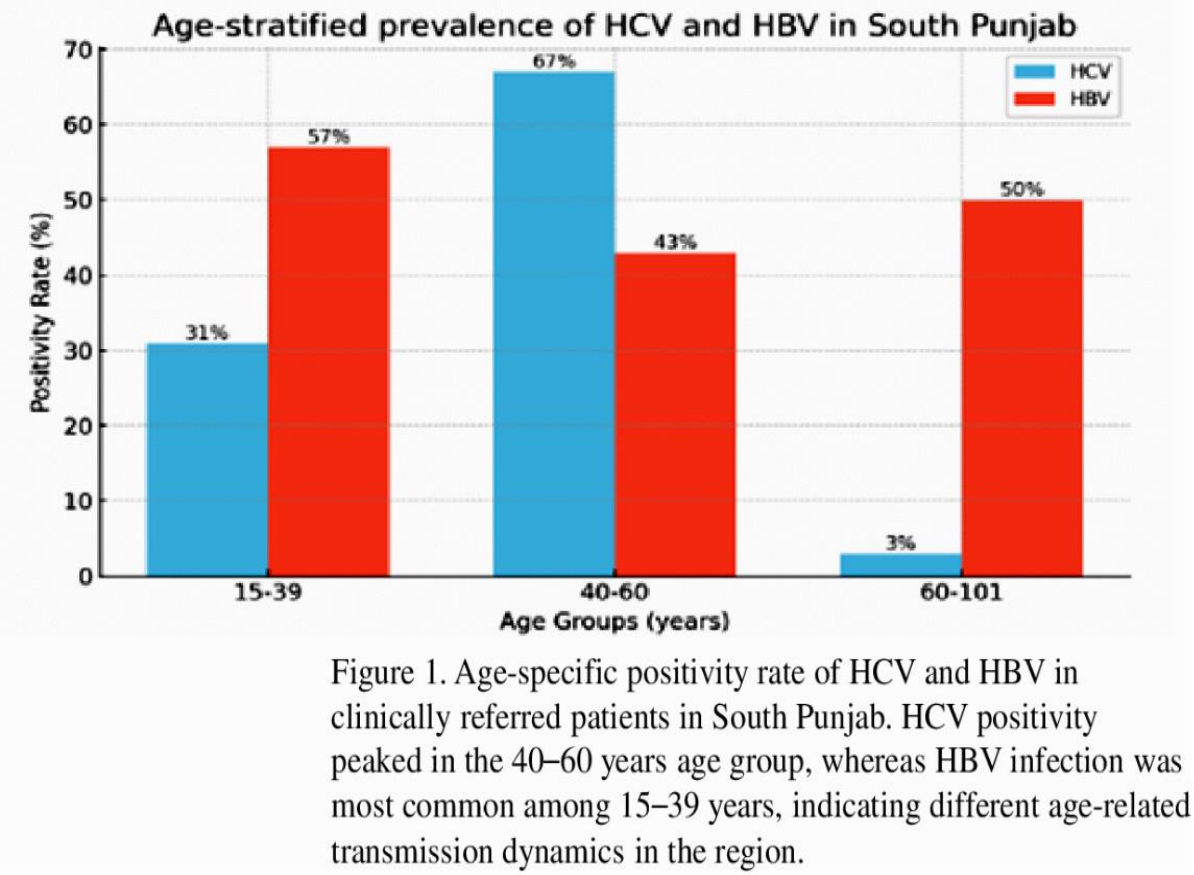

Figure 1. Age-specific positivity rate of HCV and HBV in clinically referred patients in South Punjab. HCV positivity peaked in the 40–60 years age group, whereas HBV infection was most common among 15–39 years, indicating different age-related transmission dynamics in the region.