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Research Article

Metagenomic and serological evidence of emerging tick-borne viruses in livestock, humans, and rats in Pakistan

Muhammad Ammar [a,b,1], Yaohui Fang [a,1], Muhammad Saqib [c], Jian Xiao [a], Awais-Ur-Rahman Sial [d], Qiaoli Wu [a], Muhammad Khalid Mansoor [e], Xiaoli Wu [a], Muhammad Moaaz [f], Muhammad Usama Butt [a,b], Rehman Hafeez [e], Kashif Iqbal [e], Ali Zohaib [g,h,i,*], Shu Shen [a,*], Fei Deng [a,*]

[a] State Key Laboratory of Virology and Biosafety and National Virus Resource Center, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430071, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China
[c] Faculty of Veterinary Science, University of Agriculture, Faisalabad, 38040, Pakistan
[d] Department of Clinical Studies, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University, Rawalpindi, 46000, Pakistan
[e] Faculty of Veterinary & Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur, 63100, Pakistan
[f] KBCMA, College of Veterinary and Animal Sciences, Narowal, University of Veterinary and Animal Sciences (Sub-Campus), Lahore, 54000, Pakistan
[g] Infectious Disease Research Department, King Abdullah International Medical Research Center, P.O. Box 9515, Jeddah, 21423, Saudi Arabia
[h] King Saud bin Abdulaziz University for Health Sciences, P.O. Box 9515, Jeddah, 21423, Saudi Arabia
[i] Ministry of the National Guard - Health Affairs, P.O. Box 9515, Jeddah, 21423, Saudi Arabia

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ABSTRACT

Tick-borne viruses (TBVs) pose significant emerging threats to public and veterinary health worldwide. In Pakistan, the potential threats posed by TBVs extend far beyond Crimean-Congo hemorrhagic fever virus (CCHFV), which causes outbreaks and severe hemorrhaging with a high fatality rate among humans each year. However, the full extent of the tick-borne virome remains largely unexplored. This study presents the metagenomic profiling of viruses in livestock-associated ticks from Pakistan. Eighty-seven ticks belonging to the genera *Ixodes*, *Rhipicephalus*, *Haemaphysalis*, and *Hyalomma* species from livestock in Punjab. These ticks were subsequently grouped into 11 pools for RNA sequencing. Our analysis revealed extensive viral diversity, identifying sequences related to 31 viruses spanning at least 11 families. New strains of Jingmen tick virus (JMTV), Brown dog tick phlebovirus 2 (BDTPV-2), and Liman tick virus (LMTV) were characterized, confirming their presence in the region. Serological surveys performed among 319 livestock, 253 humans, and 214 rats detected antibodies against these viruses, indicating host exposure. Notably, the presence of JMTV-neutralizing antibodies was confirmed in two livestock animals, one human, and one rat, providing evidence of productive infection. Our findings significantly expand the known diversity and distribution of TBVs in Pakistan, establish the preliminary baseline of the tick virome in the country, and provide serological evidence of cross-species exposure to emerging TBVs. This study highlights the underestimated risk of tick-borne viral zoonoses in Pakistan and underscores the urgent need for enhanced surveillance and risk assessment.

* Corresponding authors.
E-mail addresses: df@wh.iov.cn (F. Deng), shenshu@wh.iov.cn (S. Shen), zohaiba@kaimrc.med.sa (A. Zohaib).
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[1] Muhammad Ammar and Yaohui Fang contributed equally to this work.

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INTRODUCTION

Ticks are a type of hematophagous arthropod that act as important vectors for viral transmission, acquiring and spreading viruses as they feed on humans and animals (Dantas-Torres et al., 2012; Fang et al., 2015; Kilpatrick and Randolph, 2012). Since the identification of the first tick-borne virus (TBV), Nairobi sheep disease virus, in 1912 (Montgomery, 1917), numerous viruses have been discovered in ticks over the past century. A significant proportion of these TBVs have been linked to zoonotic and veterinary diseases (Jia et al., 2020; Kodama et al., 2021; Ma et al., 2021; Ni et al., 2023; Shi et al., 2018; Wang et al., 2019; Xu et al., 2011; Yu et al., 2011). This underscores the necessity of characterizing tick viromes and evaluating their potential public health threats. Notable human pathogenic TBVs, such as Crimean-Congo hemorrhagic fever virus (CCHFV), tick-borne encephalitis virus (TBEV), Heartland Virus (HRTV), Kyasanur forest disease virus (KFDV), and severe fever with thrombocytopenia syndrome virus (SFTSV, alternatively *Bandavirus dabieenes*), are recognized as significant pathogens due to their high virulence, morbidity, and mortality in humans. These viruses have had a profound impact on populations across extensive geographic regions (Mantlo and Haley, 2023; Moming et al., 2024; Shi et al., 2018). In response to the threat posed by emerging TBVs, substantial research efforts have been dedicated to profiling the viral communities present in ticks. Advancements in metagenomics have facilitated the discovery of a broad spectrum of viruses in ticks worldwide, including in Asia (Xiang et al., 2025; Zhang et al., 2026), Europe (Ergunay et al., 2023), Africa (Madison-Antenucci et al., 2020; Zhang et al., 2021), the Americas (Bouquet et al., 2017; Habib et al., 2025) and even Antarctica (Wille et al., 2020). These findings have had a profound impact on the classification of RNA viruses. Nevertheless, the host spillover potential and medical relevance of newly identified tick-associated viruses remain unclear. Furthermore, in numerous regions across the globe, the scarcity of surveillance capabilities has led to an absence of comprehensive baseline knowledge concerning the viruses present within local tick populations. Consequently, the risks associated with tick-borne viral diseases are often underestimated, and preparedness for potential outbreaks remains inadequate.

Pakistan is a country that is located in South Asia, where there is an increasing prevalence of TBVs, representing a growing public health concern (Chen et al., 2024; Zohaib et al., 2020). Previous studies have described the diversity, distribution, and pathogen load of ticks in Pakistan, including recent taxonomic and molecular surveys (Ahmad et al., 2023; ALI et al., 2025; Ali et al., 2023; Karim et al., 2017; Numan et al., 2023). It shares borders with India, Iran, Afghanistan, and the northwestern part of China. Iran and Afghanistan have long been endemic for CCHFV, while India reports annual outbreaks of both CCHFV and KFDV (Neyazi et al., 2024; Soozangar et al., 2025; Verma et al., 2024). In northwest China, in addition to CCHFV, several emerging TBVs have also been identified in ticks, including the Guertu virus (Shi et al., 2018), Karshi virus (Bai et al., 2022), Tacheng tick virus-1 (Ji et al., 2023), and Tamdy virus (Moming et al., 2023). These findings suggest that the diversity of TBVs with public health significance may extend well beyond the currently recognized pathogens. In Pakistan, CCHFV remains the sole TBV known to be responsible for annual epidemics, with reported fatality rates of up to 29% (Wahid et al., 2019; Zohaib et al., 2020). Nevertheless, the threat posed by emerging TBVs in the country is arguably underestimated. Recent serologic studies have provided evidence of human exposure to other TBVs, including SFTSV, TAMV, and KSIV, in addition to CCHFV (Chen et al., 2024), indicating the presence of a broader spectrum of TBVs in Pakistan. Consequently, systematic characterization of the tick virome in Pakistan is imperative to identify TBVs with spillover potential and to evaluate their associated public health risks.

The present study characterized the virome of ticks collected from livestock in the Punjab province of Pakistan, from which new strains of three viruses were identified with genomic sequences. Furthermore, serological responses to the three viruses were assessed in local livestock, humans, and rats, and the epidemiological features were characterized. The results provide a foundation for further research by offering essential baseline data on the genetic diversity and seroprevalence of TBVs in Punjab. This work underscores the necessity for continuous surveillance and risk assessment to inform public health planning.

RESULTS

An overview and virome composition of tick samples collected from Punjab, Pakistan

A total of 87 ticks were morphologically classified into four genera, including *Ixodes* spp. (20, 22.98%), *Rhipicephalus* spp. (53, 60.91%), *Haemaphysalis* spp. (5, 5.74%), and *Hyalomma* spp. (9, 10.34%). Due to the constraints of morphological identification and the lack of molecular validation, the classification of *Ixodes* spp. is to be considered as tentative. They were grouped into 11 pools for RNA-seq according to their species and sampling locations. Consequently, a total of 409,700,843 raw reads were generated (Supplementary Table S1).

The majority of reads were classified as eukaryotic in origin. The presence of bacterial reads was detected in all pools, with their abundance ranging from 0.10% to 0.65% of the total. Archaeal reads were exclusively identified in pool P6 ($n = 54$). Virus-related reads were also identified in all pools, albeit at low proportions ranging from 0.000167% to 0.041595% (Table 1). The assembly of these viral reads resulted in contigs classified into 27 distinct viruses, spanning at least 11 established families: *Orthomyxoviridae, Nairoviridae, Peribunyaviridae, Phenuiviridae, Chuviridae, Spinareoviridae, Flaviviridae, Totiviridae, Rhabdoviridae,* and *Luteoviridae*. Additionally, four viruses remained unclassified and could not be assigned to any known families (Fig. 1, Supplementary Table S2). The genomic organization and phylogenetic relationships of the assembled viruses were then characterized.

Identification of new strains of Jingmen tick virus, Liman tick virus, and Brown dog tick phlebovirus 2

Following the assembly of viral reads, full-length or partial coding sequences of three viruses were obtained, including Jingmen tick virus (JMTV), Liman tick virus (LMTV), and Brown dog tick phlebovirus 2 (BDTPV-2), suggesting the presence of these viruses in Pakistan (Table 2).

A new strain of JMTV (designated PK-202201) was identified in a *Rhipicephalus* tick pool (P8). The complete sequences of its four RNA segments (S1–S4) were acquired. A comparative analysis was conducted, revealing nucleotide identities of 93.85% (S1), 93.69% (S2), 94.74% (S3), and 94.55% (S4) with corresponding segments of reference JMTV strains from China and Japan (Table 2). Phylogenetic analysis of the strain PK-202201 revealed its placement within clade 1, a group predominantly comprising strains from China, Japan, and Kenya (Fig. 2A).

Two strains of LMTV (PK-202204 and PK-202205), belonging to the genus *Mivirus* (*Chuviridae*), were identified in *Ixodes* tick pools (P1 and P6, respectively). Strain PK-202204, from pool P1, contained the nucleoprotein sequence of 1759 nucleotides, which shared 98% identity with the reference strain BSLab (Russia). Strain PK-202205, from pool P6, yielded partial sequences for the RNA-dependent RNA polymerase (RdRp), glycoprotein, nucleoprotein, and a hypothetical protein showing 91.16% nucleotide identity to the reference strain mon1 (Russia) (Table 2). Phylogenetic analysis of the partial RdRp sequence confirmed that PK-202205 clustered with other known LMTV isolates in the genus *Mivirus* (Fig. 2B).

Two distinct new strains of BDTPV-2 (PK-202202 and PK-202203) were identified. Strain PK-202202 was detected in an *Ixodes* pool (P2), with its S (1390 nt) and L (3173 nt) segments showing 94.67% and 94.90% nucleotide identity, respectively, to Chinese reference strains.

Table 1
Taxonomic profile of metagenomic reads from tick pools.

Sample ID	Tick Species	Total reads	Eukaryota	Bacteria	Archaea	Viruses	Unknown
P1	*Rhipicephalus* spp	18,513,531	17,360,975 (93.77%)	31,175 (0.17%)	0	75 (0.000405%)	1,121,306 (6.06%)
P2	*Ixodes* spp	22,539,657	20,927,181 (92.85%)	60,294 (0.27%)	0	3231 (0.014335%)	1,548,951 (6.87%)
P3	*Rhipicephalus* spp	18,922,401	17,381,968 (91.86%)	18,983 (0.10%)	0	1364 (0.007208%)	1,520,086 (8.03%)
P4	*Rhipicephalus* spp	26,313,891	25,225,342 (95.86%)	33,644 (0.13%)	0	126 (0.000479%)	1,054,779 (4.01%)
P5	*Hyalomma* spp	21,164,312	19,409,516 (91.71%)	47,767 (0.23%)	0	1103 (0.005212%)	1,705,926 (8.06%)
P6	*Ixodes* spp	32,292,115	28,404,570 (87.96%)	208,842 (0.65%)	54 (0.0001%)	13,432 (0.041595%)	3,665,217 (11.35%)
P7	*Rhipicephalus* spp	21,608,676	21,247,444 (98.33%)	20,613 (0.10%)	0	525 (0.00243%)	340,094 (1.57%)
P8	*Rhipicephalus* spp	19,148,305	17,634,845 (92.10%)	23,403 (0.12%)	0	5397 (0.028185%)	1,484,660 (7.75%)
P9	*Rhipicephalus* spp	29,389,224	28,492,452 (96.95%)	40,689 (0.14%)	0	1672 (0.005689%)	854,411 (2.91%)
P10	*Rhipicephalus* spp	36,109,750	35,308,797 (97.78%)	77,007 (0.21%)	0	3361 (0.009308%)	720,585 (2.00%)
P11	*Haemaphysalis* spp	31,191,380	29,278,631 (93.87%)	85,456 (0.27%)	0	52 (0.000167%)	1,827,241 (5.86%)

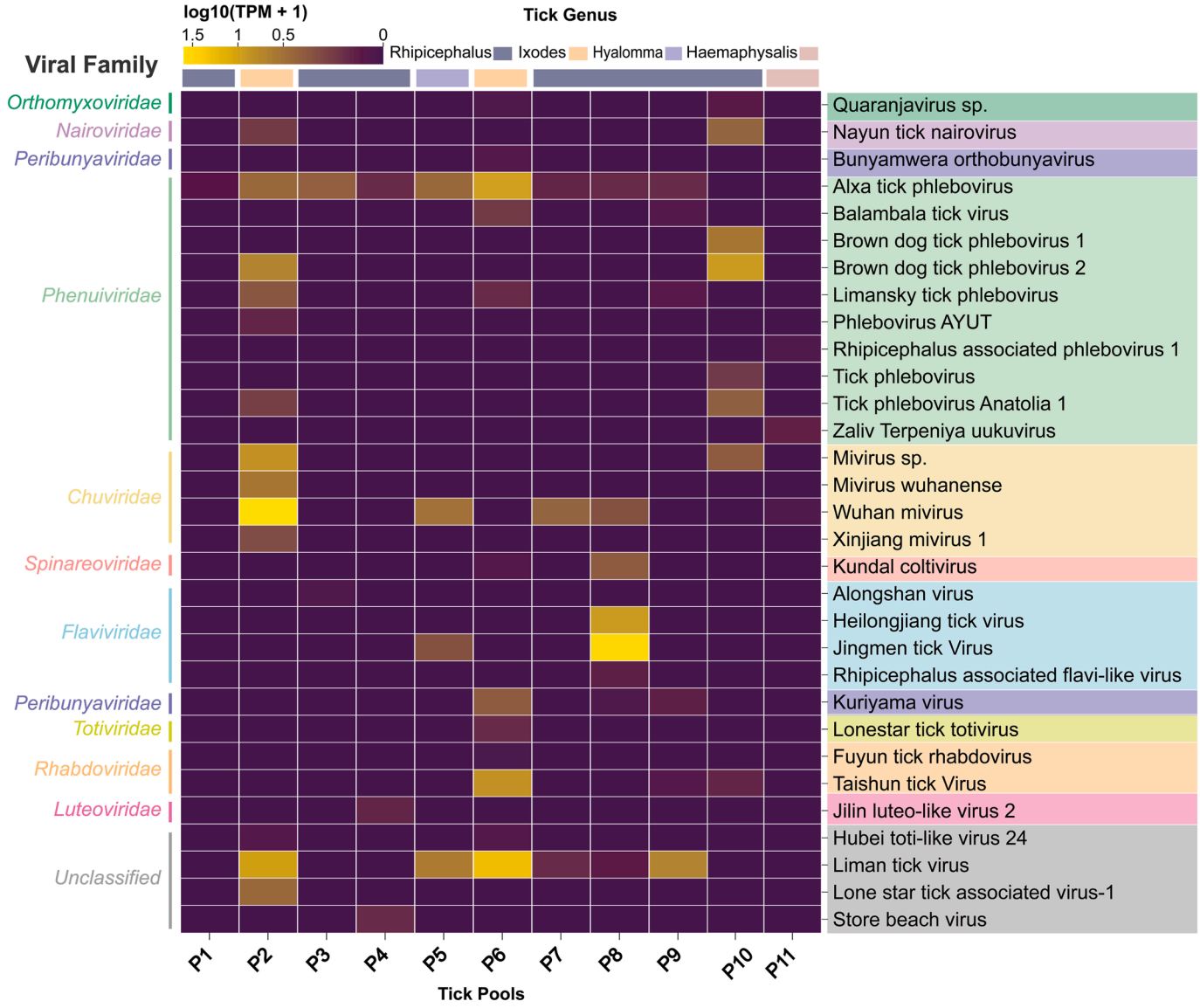

Fig. 1. Composition and abundance of viral communities in tick pools. The heatmap depicts the abundance profiles of different viral sequences across tick pools, expressed as log10-transformed Transcripts Per Million (TPM + 1) values. The data were $\log_{10}$-transformed and clustered based on a Euclidean distance matrix. Tick genera are indicated above the heatmap, viral families are shown on the left, and the corresponding viral species are listed on the right. Viral species were taxonomically classified using BLASTx and are grouped according to family.

The strain PK-202203 was identified in a *Rhipicephalus* pool (P10); its S (1348 nt) and L (6533 nt) segments exhibited 95.25% and 94.68% nucleotide identity with their closest references from China (Table 2). Phylogenetic analysis based on the S and L segments of BDTPV-2 further confirmed the close genetic similarity of the two strains with other known strains, despite being assigned to unclassified phleboviruses

Table 2
New virus strains identified from ticks in Pakistan.

Reference Virus	Positive Pool	Tick species	Strain	Segment	Genome length (nt)	Closest virus Strain	Nucleotide identity (%)	Abundance (TPM)
Jingmen Tick Virus	P8	*Rhipicephalus* spp	PK-202201	Seg 1	3114	PV622648.1	93.85	7.86
				Seg 2	2854	OR652606.1	93.69	16.19
				Seg 3	2843	OR036992.1	93.74	7.92
				Seg 4	3030	LC628151.1	94.55	8.84
Liman tick virus	P1	*Ixodes* spp	PK-202204	Nonsegmented	1759	PQ613839.1	98.00	1.57
	P6	*Ixodes* spp	PK-202205	Nonsegmented	11,333	MN542376.1	91.16	18.07
Brown dog tick phlebovirus 2	P2	*Ixodes* spp	PK-202202	Seg S	1390	OM326753.1	94.67	1.45
				Seg L	3173	ON812439.1	94.90	2.69
	P10	*Rhipicephalus* spp	PK-202203	Seg S	1348	OM263651.1	95.25	1.45
				Seg L	6533	ON812439.1	94.68	6.53

(Fig. 2C and D). Furthermore, the detection of BDTPV-2 in both *Ixodes* and *Rhipicephalus* ticks suggests a broad host adaptation.

Serologic evidence suggests JMTV, LMTV, and BDTPV-2 exposure among livestock, humans, and rats in Pakistan

In order to investigate the spillover potential of the identified viruses, serum samples from 319 livestock, 253 humans, and 214 rats were tested for antibodies against JMTV (VP2), LMTV (NP), and BDTPV-2 (NP) using a luciferase immunoprecipitation system (LIPS) assay (Supplementary Fig. S1A and B; Supplementary Table S3). Overall, 38 out of 786 (4.83%) samples were seropositive for at least one of the three viruses (Table 3). Antibodies against JMTV were detected in 1.88% (6/319) of livestock, 0.79% (2/253) of humans, and 1.86% (4/214) of rats. Seropositivity for LMTV was identified in 1.56% (5/319) of livestock, 2.76% (7/253) of humans, and 0.93% (2/214) of rats. For BDTPV-2, antibodies were detected in 1.56% (5/319) of livestock, 1.58% (4/253) of humans, and 1.40% (3/214) of rats (Supplementary Fig. S2; Table 3). These results obtained provide serological evidence of exposure to JMTV, LMTV, and BDTPV-2 across multiple species in Pakistan.

Serological confirmation, epidemiological features, and a potential tick-livestock transmission link for JMTV

In order to corroborate the findings of the JMTV antibody findings, Western blot analysis was performed on available LIPS-positive samples using purified JMTV particles. This confirmed reactivity in 4 of 6 livestock (66.7%), 1 of 2 human samples (50%), and 2 of 4 rat (50%) samples (Supplementary Fig. S3). The discrepancy in confirmation rates that was observed may be attributed to variations in assay sensitivity between LIPS and Western blot, or alternatively, to the circulation of other viruses with serological correlation to JMTV, which could be detected using LIPS but failed to be recognized under a denaturation condition by Western blot. Subsequently, the seroepidemiological features of JMTV exposure in livestock, humans, and rats were characterized. Despite the identification of multiple trends in seropositivity, the majority did not attain statistical significance. A higher seroprevalence was detected in outdoor rats compared to indoor rats (Table 4), this observation should be interpreted cautiously due to the small sample size and restricted number of positive cases. Of the samples that were analyzed, two from livestock (L7 and L311) and one from rat (R90) exhibited an antibody response to JMTV by both LIPS and Western blot and were further identified as exhibiting neutralizing antibody to this virus (Supplementary Table S4). The results further suggest a specific immune response to JMTV infection is present in livestock and rats.

As the tick samples were collected from the body surfaces of livestock concurrently with the collection of blood samples, the identification of JMTV in a specific tick pool may be linked to the livestock with positive antibody responses to this virus. By tracing the links between the tick pools and livestock samples, it was established that the *Rhipicephalus* ticks of pool P8, from which the JMTV strain PK-202201 was sequenced, were collected from cattle L7, which tested positive for antibody response to JMTV by both LIPS and Western blot, as well as for neutralizing antibodies with a titer of 40 (Supplementary Table S4). The findings indicate the presence of a tick-livestock transmission link and a specific host immune response to JMTV.

DISCUSSION

The zoonotic risks posed by TBVs, in addition to CCHFV, are likely to be underestimated in Pakistan. As evidenced by previous serologic surveys that have demonstrated evidence of human exposure to other TBVs, including SFTSV, TAMV, and KSIV (Chen et al., 2024). Recent studies have reported the presence of diverse bacteria, protozoans, and other tick-borne pathogens in ticks from Pakistan (Ahmad et al., 2023; ALI et al., 2025; Ali et al., 2023; Jamil et al., 2025; Karim et al., 2017; Numan et al., 2023), further indicating that livestock-associated ticks harbor multiple tick-borne pathogens. Notwithstanding these findings, the paucity of knowledge regarding the baseline diversity of known and emerging tick-vectored pathogens continues to impede the establishment of effective surveillance and disease control strategies. Consequently, there is a paucity of comprehensive data on the viral composition of livestock-associated ticks in Pakistan.

This study provides the preliminary metagenomic profile of TBVs in livestock-associated ticks from Pakistan. A comprehensive analysis of viral populations present within *Ixodes, Rhipicephalus, Haemaphysalis*, and *Hyalomma* ticks was conducted, resulting in the identification of sequences associated with a minimum of 31 viruses classified within 11 distinct viral families, of which viruses belonging to the families *Phenuiviridae, Chuviridae*, and *Flaviviridae,* as well as unclassified viruses, were particularly abundant. Interestingly, no sequences related to CCHFV, which is known to be endemic in Pakistan, were found in this investigation. No sequences linked to CCHFV, a well-established endemic tick-borne virus in Pakistan, were identified in this investigation. This absence could be attributed to several factors, including sample size, the diversity of tick genera sampled (predominantly *Rhipicephalus* and *Ixodes*, with only a limited number of *Hyalomma* ticks, which are the primary vectors of CCHFV), and potential geographic or temporal variation in virus circulation within the study area. These findings are consistent with those reported in previous virome studies of ticks from countries in Europe, Asia, and Latin America (Brinkmann et al., 2018; Pettersson et al., 2017; Tufts et al., 2020).

Notably, new strains of JMTV, BDTPV-2, and LMTV were identified at relatively high abundance in several tick pools. Unlike the typical phenuiviruses, which possess three genomic segments (L, M, and S), the M segment of BDTPV-2 was not recovered in the sequencing data. This absence has also been reported in other recently described phenuiviruses, such as Iftin tick virus, Lihan tick virus, Bole tick virus 1, Changping tick virus 1, and Tacheng tick virus 2. It may be attributed to high sequence divergence compared to known viruses (Li et al., 2015). The genomic architecture of the BDTPV-2 strain identified in this study resembles that of other M segment-deficient uukuviruses, such as

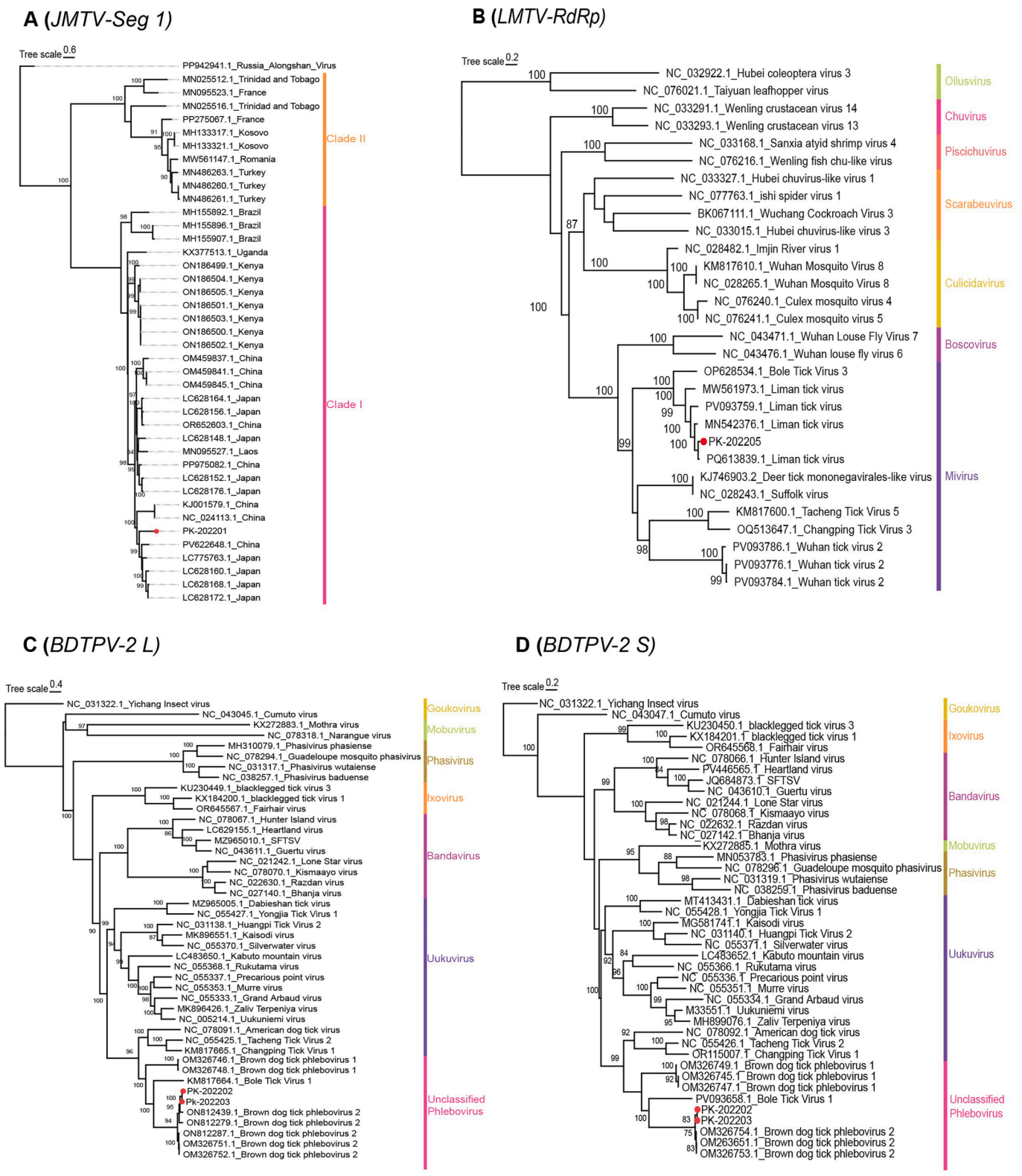

Fig. 2. Phylogenetic analysis of the identified tick-borne virus strains. Maximum-likelihood (ML) phylogenetic trees were constructed to determine the genetic relationships of the new strains of JMTV (**A**), LMTV (**B**), and BDTPV-2 (**C** and **D**) strains identified in this study using the full-length segment 1 (NS5-like protein) sequence, the RdRp sequence, and the L and S segments, respectively. All trees were inferred using the ML method with 10,000 ultrafast bootstrap replicates. The strains from this study are indicated by a red circle.

Table 3
Seroprevalence rates of JMTV, LMTV, and BDTPV-2 across livestock, humans, and rats populations in Pakistan.

Metrics	Livestock (n = 319)			Humans (n = 253)			Rats (n = 214)			TOTAL = 786		
	LIPS	WB	Neutralization (titer)	LIPS	WB	Neutralization (titer)	LIPS	WB	Neutralization (titer)	LIPS	WB	Neutralization
JMTV (n, %)	6, 1.88%	4, 1.25%	2, 0.63% (L7: 40; L311:40)	2, 0.79%	1, 0.40%	1, 0.40% (F90: 20)	4, 1.86%	2, 0.93%	1, 0.47% (R90: 40)	12, 1.52%	7, 0.89%	4, 0.51%
LMTV (n, %)	5, 1.56%	N/A	N/A	7, 2.76%	N/A	N/A	2, 0.93%	N/A	N/A	14, 1.78%	N/A	N/A
BDTPV-2 (n, %)	5, 1.56%	N/A	N/A	4, 1.58%	N/A	N/A	3, 1.40%	N/A	N/A	12, 1.52%	N/A	N/A
Total	16, 5.01%	N/A	N/A	13, 5.13%	N/A	N/A	9, 4.20%	N/A	N/A	38, 4.83%	N/A	N/A

Table 4
Univariable analysis of associations between host demographic factors and JMTV seropositivity.

Category	Positive/Tested	Prevalence (95% CI)	*P*-value
Livestock			
Locations			
Narowal	2/100	2.00% (0.55–7.00)	0.92
Faisalabad	3/145	2.07% (0.71–5.91)	
Lahore	1/74	1.35% (0.24–7.27)	
Animal species			
Buffalo	1/89	1.12% (0.20–6.09)	0.44
Cattle	5/199	2.51% (1.08–5.75)	
Sex			
Male	1/94	1.06% (0.19–5.78)	0.48
Female	5/225	2.22% (0.95–5.1)	
Farming			
Subsistence farming	3/225	1.33% (0.45–3.85)	0.26
Commercial farming	3/94	3.19% (1.09–8.97)	
Humans			
Locations			
Narowal	0/100	0.00% (0.00–3.70)	0.25
Faisalabad	2/153	1.31% (0.36–4.64)	
Age			
>40	0/40	0.00% (0.00–8.76)	0.53
≤40	2/213	0.94% (0.26–3.36)	
Gender			
Male	2/173	1.16% (0.32–4.12)	0.33
Female	0/80	0.00% (0.00–4.58)	
Jobs			
Outdoor job	2/127	1.57% (0.43–5.56)	0.15
Indoor job	0/125	0.00% (0.00–2.98)	
Animal contact			
Yes	1/174	0.57% (0.10–3.18)	0.56
No	1/79	1.27% (0.22–6.83)	
Rats			
Locations			
Narowal	3/97	3.09% (1.06–8.70)	0.38
Faisalabad	1/60	1.67% (0.29–8.86)	
Lahore	0/57	0.00% (0.00–6.31)	
Gender			
Male	1/76	1.32% (0.23–7.08)	0.65
Female	3/138	2.17% (0.74–6.20)	
Environment			
Outdoor	2/23	8.70% (2.42–26.80)	0.01
Indoor	2/191	1.05% (0.29–3.74)	

Humans from Pakistan were divided into the "Indoor job" group (teachers, housewives, governors, doctors, nurses, freelancers, and bankers) and the "Outdoor job" group (merchants, shopkeepers, laborers, and farmers). Contacts with rats were divided into the "Indoor environments" group (godown, shops) and the "Outdoor environment" group (farms, open markets). Livestock was divided into two types: subsistence farming (Household) and commercial farming (Mixed, dairy farms).

Qīnghǎi Lake virus 3, which was identified in *Dermacentor* ticks in China (Fang et al., 2024). Future studies may employ recently developed tools such as SegFinder (Liu et al., 2025) to recover missing segments, which identifies genomic segments through co-occurrence patterns across samples without reliance on sequence similarity.

The presence of JMTV has been documented in a wide range of tick genera, including *Amblyomma, Rhipicephalus, Dermacentor, Hyalomma, and Haemaphysalis,* collected from a diverse array of hosts, including humans, cattle, rodents, bats, monkeys, and tortoises. Accordingly, the geographic distribution of JMTV extends to China, Trinidad and Tobago, Brazil, Uganda, and Kenya (Sameroff et al., 2019; Shi et al., 2016). The JMTV strain identified in this study clusters within the Asian lineage, suggesting either regional introduction or conserved local evolution in *Rhipicephalus* ticks. Furthermore, BDTPV-2 (GenBank: QDW81040.1), initially reported in *R. sanguineus* ticks from Trinidad and Tobago, and LMTV (GenBank: QPD01635.1), identified in *H. anatolicum* ticks from Russia, are reported for the first time in Pakistan, thus extending their known geographic ranges to South Asia and indicating broader dissemination across tick populations on a global scale.

Serological investigation provided further evidence of exposure to JMTV, BDTPV-2, and LMTV in livestock, humans, and rats. The presence of JMTV-neutralizing antibodies in two livestock, one human, and one rat underscores its potential for broad host infection and substantial zoonotic threat. While no significant seroepidemiologic patterns were observed in either livestock or humans, a higher seroprevalence was observed in outdoor rats compared to indoor rats; however, this observation should be interpreted cautiously due to the limited sample size and limited number of positive cases. Furthermore, JMTV seropositivity was found to be higher in cattle (2.51%) than in buffalo (1.12%), and was not detected in dogs, goats, and sheep. This may be indicative of limited sampling, or it may align with prior evidence that cattle develop stronger antibody responses to TBVs, possibly due to higher tick exposure in grazing settings (Zohaib et al., 2020). In humans, seropositivity demonstrated no significant variation across age, gender, occupation, or frequency of animal contact. Despite the findings of a preceding LIPS-based study in Pakistan, which reported demographic risk factors for other TBVs (Chen et al., 2024), the absence of such associations in this study may be indicative of virus-specific transmission patterns. This emphasizes the necessity for further research into JMTV-specific risk factors.

The present study has several limitations. Firstly, the relatively small sample size of ticks (n = 87) precluded the estimation of a robust viral prevalence. In addition, the use of pooled tick samples could obscure individual-level variation and potentially mask low-abundance viruses, hence altering the interpretation of viral diversity and prevalence. Furthermore, the absence of virus isolation limits inferences regarding viral survival, infectivity, and pathogenic potential, and future studies should include virus isolation and experimental analysis. Secondly, tick species identification was based on morphology. Future research should use molecular barcoding to ensure species identity, especially for genera like Ixodes, where morphological identification can be problematic, and species ranges in the region are being refined. Thirdly, we were unable to confirm the seroprevalence of LMTV and BDTPV-2 using alternative serologic assays due to the unavailability of live virus isolates. Although LIPS is a sensitive serological approach, the likelihood of cross-reactivity with antigenically related viruses, notably within the *Phenuiviridae* and *Chuviridae* families, cannot be ignored in the absence of confirming neutralization or virus isolation. Additionally, human seroprevalence values should be taken as typical of the sampled population rather than the general population, owing to the approach of convenience sampling. However, seropositivity of JMTV was corroborated by Western blot and LIPS assays, and further confirmed by the identification of neutralizing

antibodies, thereby reinforcing the importance of continued surveillance. Finally, we did not detect any sequences related to previously reported TBVs in Pakistan. These include CCHFV (Zohaib et al., 2020) and viruses for which serological tests have indicated an association, such as SFTSV, TAMV, and KSIV (Chen et al., 2024). It is imperative that future studies encompass larger samples of ticks from a range of geographic regions, in conjunction with integrated host surveys. Such endeavours are pivotal in comprehensively elucidating the transmission dynamics for TBVs in Pakistan.

CONCLUSIONS

This study presents a comprehensive virome analysis of ticks parasiting livestock in Punjab, Pakistan, revealing a previously undocumented diversity of viruses. New strains of JMTV, BDTPV-2, and LMTV were identified in local tick populations. It is imperative to note that the integration of virome data with serological testing not only confirmed the presence of JMTV in the region but also suggested its transmission activity, as evidenced by specific antibody response across local livestock, humans, and rats.

These findings carry substantial implications for public health in Pakistan. The confirmation exposure of humans and animals to JMTV, in conjunction with the detection of other viruses with unknown zoonotic potential, signals an emerging threat from TBVs that is currently underestimated. This study underscores the pressing necessity to implement enhanced, systematic surveillance for vector-borne diseases and to develop targeted prevention strategies to mitigate the risk of future outbreaks.

MATERIALS AND METHODS

Collection of ticks and serum samples

Between March and October 2022, ticks and serum samples were collected from three districts in Punjab Province, Pakistan: Narowal (32° N, 74.8°E), Lahore (31.5°N, 74.3°E), and Faisalabad (31.5°N, 74°E), all of which are located within a 180-km radius. Ticks were collected from the body surfaces of buffalo, cattle, dogs, goats, and sheep in households, animal selling markets, and farm-based animal setups, while serum samples were also being collected. Personal protective measures were adopted during the collection process to minimize the risk of tick-borne infection. The ticks were identified by an experienced acarologist based on their morphology using standard taxonomic keys. However, owing to the absence of molecular identification and possible morphological resemblances among taxa, identification was confined to the genus level. Ticks identified as *Ixodes* spp. were considered provisional and should be interpreted with caution. They were stored at −80 °C, and the associated metadata (geographic location, species, count) were recorded (Supplementary Table S1).

Human blood sampling was obtained using a convenience sampling approach, necessitated by sociopolitical constraints, limited healthcare infrastructure, and logistical challenges in recruiting farmers. Random selection was applied where feasible. Informed consent was obtained from all participants, either verbally or in writing. Peripheral blood (4 mL) was collected in a gel-clot activator vacutainer (Improvacuter, Germany). Sampling of livestock was conducted concurrently with human sampling in order to establish spatial correlation for comparative exposure analysis. The selection of livestock was based on convenience, with consideration to owner consent and farm accessibility. Rodents (rats) were trapped in areas of high human-animal interaction, including local godowns, shops, livestock farms, and open markets, which serve as hubs for the inter-district movement of goods and animals. Blood (4 mL) from livestock and rats was collected by veterinarians via the jugular or tail vein using identical vacutainers. All blood samples were centrifuged (5000×*g*, 12 min), and the resulting serum was aliquoted into cryovials (Imec, China) and stored at −40 °C until analysis (Supplementary Table S3). The selected districts represent significant livestock-rearing areas in Punjab with high levels of animal mobility and human-animal contact, making them appropriate for investigating the spread of tick-borne viruses. In order to capture representative host-vector interactions in peri-urban and rural contexts, sampling was based on accessibility, owner consent, and the presence of visible tick infestation on animals.

Cells, viruses, antibodies, and reagents

Human embryonic kidney cells (HEK293T; ATCC, CRL-11268) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, NZK Biotech, China), supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (PS). BME/CTVM23 tick cells (Tick Cell Biobank) were maintained in Leibovitz's L-15 medium (Thermo Fisher Scientific, USA). JMTV strain CQ-2021 (CSTR: 16533.06 IVCAS06.9297) was obtained from the National Virus Resource Center (NVRC) in China.

Rabbit polyclonal antibodies against the JMTV capsid protein (VP2) were produced in-house using a method as previously described (Zhang et al., 2026). The commercial antibodies used included: anti-FLAG Tag (Sangon Biotech, Shanghai, China), GAPDH mouse monoclonal antibody (mAb) (abclonal, Wuhan, China), goat anti-Rabbit IgG (H + L) conjugated with horseradish peroxidase (HRP) (Proteintech, Wuhan, China), goat anti-rabbit IgG H&L conjugated with Alexa Fluor 488 (Abcam, Shanghai, China), goat anti-human IgG H&L conjugated with HRP (Abcam), and Protein A conjugated with HRP (Proteintech). Hoechst 33258 (Beyotime, China) was used for nuclear staining. Molecular cloning was performed using the ClonExpress® Ultra One Step Cloning Kit. Plasmid transfections used Lipofectamine™ 3000 (Invitrogen). The Renilla-Lumi™ Plus Luciferase Assay Kit (Beyotime) and Pierce Protein A/G UltraLink Resin beads (Thermo Fisher Scientific) were used for luciferase immunoprecipitation system (LIPS) assays.

Metagenomic sequencing and bioinformatics

Eighty-seven ticks were grouped into 11 pools (3–10 ticks per pool) based on location, species, and engorgement status. Total RNA was extracted from the tick pools as previously described (Fang et al., 2024). RNA sequencing libraries were prepared from 3 μg of total RNA per pool and sequenced on an Illumina HiSeq 3000 platform (Illumina, San Diego, USA). Raw reads were quality-filtered and assembled *de novo*. Viral contigs were identified through DIA MOND-BLASTx and BLASTn searches against viral databases (Fang et al., 2024). Viral abundance was estimated using transcripts per million (TPM) to normalize for gene length and sequencing depth (Xiao et al., 2024). A heatmap of $\log_{10}$ (TPM+1) values was generated to visualize viral distribution across the pools.

Phylogenetic analysis

Viral sequences were aligned using MAFFT v7.505 (Katoh and Standley, 2013), and ambiguous regions were trimmed with trimAl v1.2 (Capella-Gutiérrez et al., 2009). Full-length or partial coding sequences were used to build phylogenetic trees, including JMTV NS5-like protein (Segment 1), LMTV RdRp and BDTPV-2 RdRp, as well as BDTPV-2 nucleoprotein (NP). The best-fit model (GTR + F + G4) was selected using ModelFinder (Kalyaanamoorthy et al., 2017) under the Bayesian Information Criterion. Maximum-likelihood (ML) trees were constructed using IQ-TREE v2.2.0 (Nguyen et al., 2014) with 10,000 ultrafast bootstrap replicates (Minh et al., 2013) and SH-aLRT support (Guindon et al., 2010). All analyses were conducted within the PhyloSuite v1.2.3 environment (Zhang et al., 2019). Heatmaps were generated using TBtools (https://github.com/CJ-Chen/TBtools/releases) (Chen et al., 2020).

Luciferase immunoprecipitation system (LIPS) assays

In previous studies (Zhang et al., 2021; Zhang et al., 2026), a luciferase immunoprecipitation system (LIPS) was established for the detection of antibodies against JMTV VP2 and LMTV NP in serum samples. This study expanded the system to include BDTPV-2, which was identified in tick pools through metagenomic analysis. The BDTPV-2 *NP* gene was cloned into the pREN2 plasmid, in-frame with *Renilla* luciferase and a Flag-tag, using the ClonExpress® Ultra One-step Cloning Kit. This was then verified by Sanger sequencing. Antigen expression was validated in HEK293T cells transfected with the plasmid. The expression of JMTV VP2 and LMTV NP had been previously been validated (Chen et al., 2024; Zhang et al., 2021) and was included as controls. Consequently, serum antibodies to the three viruses were detected as previously described (Chen et al., 2022).

Microneutralization assays

The LIPS-positive serum samples were serially diluted from 1:10 to 1:1280 (v/v) in Leibovitz's L-15 medium, then incubated with 100 $TCID_{50}$ JMTV at 27 °C for 1 h. The virus-serum mixtures were then applied to BME/CTVM23 cells (1×10^4 cells/well) in the 96-well plates. After five days of incubation at 27 °C, infection was assessed using an immunofluorescence assay (IFA). The neutralization titer was expressed as the highest dilution that completely inhibited JMTV infection in all three replicate wells.

Statistical analyses

Statistical analyses were performed using Python 3.12.2 (Python Software Foundation, Wilmington, DE, USA). Seroprevalence was reported with 95% confidence intervals (CIs). Associations between JMTV seropositivity and host risk factors were evaluated using the χ^2 test or Fisher's exact test (or the Fisher-Freeman-Halton exact test with Monte Carlo simulation), as appropriate. A *P*-value of less than 0.05 was considered statistically significant.

DATA AVAILABILITY

The datasets generated and analyzed in this study have been deposited in the GenBank Sequence Read Archive (SRA) under Bioproject PRJNA1313286, and the virus genome sequences have been deposited in GenBank under accession numbers PX289089–PX289090 and PX259824–PX259831. All data are also available in ScienceDB (https://doi.org/10.57760/sciencedb.27117).

ETHICS STATEMENT

The study was approved by the Departmental Bioethics Committee, Department of Microbiology, The Islamia University of Bahawalpur (REC. BEC No. 05-2021-21/2), Pakistan, and by the ethics committees of Wuhan Institute of Virology, Chinese Academy of Sciences (WIVHF33202404).

AUTHOR CONTRIBUTIONS

Muhammad Ammar: formal analysis, investigation, visualization, writing-original draft, writing-review & editing. Yaohui Fang: investigation, resources, visualization. Muhammad Saqib: investigation, visualization. Jian Xiao, Awais-Ur-Rahman Sial, Qiaoli Wu, Muhammad Khalid Mansoor, Xiaoli Wu: investigation, resources. Muhammad Moaaz, Muhammad Usama Butt, Rehman Hafeez, Kashif Iqbal: sample collection, technical assistance. Ali Zohaib: resources, data assistance, visualization, writing-review & editing. Shu Shen, Fei Deng: funding acquisition, project administration, resources, supervision, writing-original draft, writing-review & editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. Prof. Fei Deng is editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.06.001.

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