Journal of Animal & Plant Sciences, 35(6): 2025, Page: 1613-1629
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2025.6.0136

PREVALENCE, GENETIC VARIABILITY AND POPULATION DIVERSITY OF ALLEXIVIRUSES INFECTING GARLIC IN PAKISTAN

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ABSTRACT

Garlic (*Allium sativum* L.) is an important bulbous vegetable, vulnerable to viral infections of different genera particularly *Allexivirus* genus, known to cause economically significant diseases, leading to havoc yield losses worldwide. This study aimed to assess potential prevalence, genetic variability and population diversity of Allexiviruses in Pakistan. Field surveys were carried out in 2021 and 2022 growing seasons and leaf samples, both symptomatic and asymptomatic were tested using reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from leaf tissue and used to synthesize complementary DNA (cDNA) with primers targeting *Allexivirus* CP and NABP gene and selected samples were further confirmed through PCR amplification. Out of 700, 296 samples were tested positive for Allexiviruses infection and disease prevalence for *Garlic virus* C (GarV-C) was recorded at 20.85% followed by *Garlic virus* A (GarV-A) at 11.4% and *Garlic virus* B (GarV-B) at 7.7% during 2021 season. In 2022 season, GarV-C again showed highest prevalence at 21.14% followed by GarV-B at 14% and GarV-A at 9.4%. PCR amplifications were visualised on a 1% agarose gel, resulting bands of 750 bp and amplified DNA was subsequently purified and cloned for sequencing. Sequence analysis identified two sequences, each comprising of 819 nucleotides corresponding to *Garlic virus* C (GarV-C) were submitted in Genbank with accession number PP759407 and PP759408. Comparative analysis of nucleotide and amino acid sequence of CP and NABP gene revealed 88%-98% identity between this study isolates and GenBank sequences from Pakistan, China, Japan, Australia, Czech Republic, Poland, and Serbia. Phylogenetic analysis based on nucleotides and amino acid sequences of CP and NABP genes showed that isolates of this study were closely related with GenBank isolates. Analysis of population diversity and selection pressure revealed that GarV-C (CP) gene was under balancing selection associated with contracting population, NABP gene appears to be under negative selection suggesting adaptation in the context of population expansion. This study highlighted the disease prevalence, genetic variability and population diversity of *Allexivirus* species affecting garlic in Pakistan. These findings will provide valuable insights into the GarV-C population structure and contribute to ongoing efforts focusing virus-free garlic production in Pakistan.

Keywords: Garlic, Allexiviruses, Prevalence, Genetic variability, Population diversity

INTRODUCTION

Garlic (*Allium sativum* L.) locally known as lehsan is a widely used culinary spice and medicinal plant cultivated in Pakistan and worldwide. It is highly rich source of essential nutrients and minerals that offers numerous health benefits, and its extracts are frequently utilized in the treatment of various human diseases (Bouhenni *et al*., 2021; Karavelioğlu and Hoca, 2022). In Pakistan, garlic is cultivated over an area of 12.9 thousand hectares, yielding a total production of 115.3 thousand tonnes (Government of Pakistan Statistics, 2022-23). Due to its vegetative propagation, garlic is susceptibble to various pathogens, particularly viruses, which are responsible for diseases, that cause yield and economic losses (Anum *et al*., 2024; Shevchenko *et al*., 2024; Zhu *et al*., 2025). Garlic plant is infected by viruses of highly aggarassive members of different texa's including the *Potyvirus* genus which contains *onion yellow dwarf virus* (OYDV), *leek yellow stripe virus* (LYSV) and *shallot yellow stripe virus* (SYSV), other viruses includes *Garlic latent virus* (GLV) and *shallot latent virus* (SLV) from *Carlavirus* genus, *Iris yellow spot virus* (IYSV) from *Tospovirus* genus as well as *Garlic virus* X (GarV-X), *Garlic virus* C (GarV-C), *Garlic virus* B (GarV-B), *Garlic virus* A (GarV-A), *Garlic virus* E (GarV-E) and *shallot virus* X (ShV-X) from *Allexivirus* genus (Chodorska *et al*., 2014;Bereda *et al*., 2017; Gupta *et al*., 2017; Rao and Reddy, 2020; Koczor *et al*., 2024; Nurulita *et al*., 2024). The transmission of these viruses is facilitated by insect vectors, particularly aphid, thrips and mite, which contribute to their widespread dissemination. These viruses often occurs as mixed infection in garlic, leading to a viral complex known as garlic virus complex, resulting in yield losses up to 50% (Abraham *et al*., 2019; Godena *et al*., 2020; Bhusal *et al*., 2021; Cremer *et al*., 2021; Santosa and Ertunc, 2023).

Allexivirus species are characterized by non enveloped rod-shaped, flexible filamentous particles and virion particles ranges in length from 800 nm to 12 nm in diameter. Their genome consists single-stranded, positive-sense, RNA with a genome size typically between 8,000 and 9,000 nucleotides depending upon the species. The genome encodes several proteins including a coat protein (CP), a nucleic acid binding protein (NABP) and a serine-rich protein, the function of which remains unidentified (Bereda *et al*., 2017; Celli *et al*., 2019; Roylawar *et al*., 2021; Prajapati *et al*., 2022a). The coat proteins (CP) are believed to evolve more rapidly than the protiens involved in replication and expression of viral genome, because coat protiens recognized as the most significant region within genus *Allexivirus* as earlier described by International Committee on Taxonomy of Viruses (ICTV) for species demarcation (Adams *et al*., 2012). Allexiviruses induce granule like flexible particles and small inclusion bodies in the cytoplasm of garlic plant and the symptoms of infection include mosaic patterns, leaf distortion, chlorotic streaks and young leaf deromation. These viruses cause a significant decrease in bulb weight up to 49% and bulb size as much as 16%. Allexiviruses have been identified and documented in garlic plants across the globe including USA, Europe, Australia, Japan and many other countries (Perotto *et al*., 2010; Ghaemizadeh *et al*., 2014; Celli *et al*., 2015; Bereda *et al*., 2017; Mitiku *et al*., 2020; Kreuze *et al*., 2020; Prajapati *et al*., 2022; Amir *et al*., 2024; Neupane *et al*., 2025). Allexiviruses transmission occurs during bulb storage, facilitated by vector mite *Aceria tulipae* in a semi-persistent manners (Dąbrowska *et al*., 2020; Mansouri *et al*., 2021).

Pakistan ranked among top garlic producing countries but its average yield remains low owing to impact of various plant viruses. The species of genus *Allexivirus* are well known in regions surrounding Pakistan including China, India, Iran and Nepal. Existing literature indicates that limited research has been conducted on garlic and restricted to initial documentation of OYDV, IYSV and GarV-X in Pakistan (Gilani *et al*., 2016; Ahsan and Ashfaq, 2018; Hamza *et al*., 2023; Shahid *et al*., 2024). Previous reports related to the prevalence of Allexiviruses highlights the best scope of investigation in garlic crop. The development of advanced diagnostic measures are crucial in understanding the biology of Allexiviruses as well as their genetic makeup and population diversity for sustainable garlic production and reduced yield losses in Pakistan. This study aimed to assess the potential prevalence, genetic diversity and population density of Allexiviruses in relation to their geographical distribution in Punjab, Pakistan.

MATERIALS AND METHODS

Sample collection: Field surveys of garlic crop were conducted across 14 geographically distinct districts in Punjab province, including Multan, Vehari, Khanewal, Jhang, Sahiwal, Kasur, Sheikhupura, Gujranwala, Sialkot, Narowal, Rawalpindi, Chakwal, Attock, Jhelum, and the capital territory Islamabad, Pakistan, over two consecutive years, 2021 and 2022. Sample collection was done using a random stratified design (RSD) implemented in a zigzag pattern considering each district as a stratum and visited randomly selected farmer fields (Iqbal *et al*., 2012; Ahsan *et al*., 2021). A total of 10 samples including asymptomatic and symptomatic ones were collected from each field at mature crop stage, resulting in 700 samples grouped from distinct geographic locations within studied region. All samples were placed in polyethylene zipper bags and kept on ice, while they were divided in two equal halves and stored at -20°C for further analysis.

Statistical Analysis: Geographical distribution of Allexiviruses and their association among each other was linked by Chi-square test. Analysis was performed in Statistix-10 software (https://www.statistix.com/) and results were deliberated significant, if p-value seems <0.05.

Molecular Characterization

RNA isolation and RT-PCR: A total of 100 mg infected and healthy leaf tissue was used for RNA isolation through RNA extraction kit by following manufacturer standard protocol (Thermo Fisher Scientific, USA). RNA quantification was performed by measuring the *A*260/280 ng ratio using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). One step RT-PCR was used for Allexiviruses group test and a total of 500 ng RNA was used for complementary DNA synthesis (cDNA) by employing previously described CP+NABP gene based degenerate and specific primers (Table 1). cDNA assembly was performed using a first strand (cDNA) synthesis kit followed by manufacturer instructions (Thermo Fisher Scientific, USA). Reaction was achieved in a total volume of 20 µL, consisting of 2 µL RNA, 1 µL reverse primer, 10 µL RT-PCR master mix and nucleic acid free water to reach final volume. Primer conditions were optimized and results were validated using primer pair AL-N30/AL-C30 as described in literature (Nam *et al*., 2015). The disease incidence based on RT-PCR results was computed using the following formula (Ashfaq *et al*., 2017; Asad *et al*., 2022).

Disease Prevalence %= $\frac{\text{Number of infected samples}}{\text{Total number of examined samples}} \text{x}100$

Polymerase chain reaction (PCR): PCR amplification was done in a total volume of 25 µL, including 2 µL cDNA, 1 µL each primer forward and reverse (Table 1), 12 µL of 2X PCR Go Taq Green Master Mix (Thermo Fisher Scientific, USA) and 10 µL nuclease-free water. PCR conditions was set as follows: an initial denaturation at 94 °C for 5 minutes, followed by denaturation at 94 °C for 1 minute, annealing at 57 °C for 1 minute and extension at 72 °C for 1 minute, with a final elongation at 72 °C for 10 minutes for 35 cycles. For all PCR tests an equal portion of total RNA from each infected sample was used as positive control and water was used as negative control (Ahsan *et al*., 2020). The amplified PCR products were separated on 1% w/v agarose gel pre-stained with ethidium bromide using 10X TBE buffer for gel electrophoresis. A 1 kb GeneRuler DNA ladder was used to confirm the expected fragment size. The bands corresponding to desired fragment length were pictured using a gel documentation system (Thermo Fisher Scientific, USA). PCR product was purified through a PCR purification kit following the manufacturer guidelines (Thermo Fisher Scientific, USA). The purified product was transformed into *E. coli* with commercially available XL1-Blue chemically competent cells (Wang *et al*., 2020) for cloning into the pTZ57R/T cloning vector according to manufacturer information (Thermo Fisher Scientific, USA). The recombinant plasmid DNA was isolated using the plasmid DNA Kit (Thermo Fisher Scientific, USA) as per manufacturer guidelines and previously narrated by Ashfaq *et al*., 2015). The presence of inserts in the transformants was verified by digestion with restriction enzyme EcoRI and selected amplicons were sequenced in both directions using Sangar sequencing method by MacroGen (North Korea).

Sequence comparison and phylogenetic analysis: The resulted sequences were compared based on CP and NABP gene nucleotides and amino acid identities with previously documented isolates from the GenBank database. These sequences were aligned using ClustalW tool in MEGA version XI, nucleotides and amino acid identities were calculated using a sequence identity matrix in BioEdit program. The selected isolates showing higher relationship among CP and NABP genes nucleotide and amino acid sequences were compared with previously reported GenBank isolates using Sequence Demarcation Tool v5 (SDT). Phylogenetic analysis of CP and NABP gene nucleotides and amino acid sequences showing close relationship was conducted using the maximum likelihood method for their ancestral relationships. A phylogenetic tree was constructed in MEGA version XI using neighbour-joining method with support from 1000 bootstrap replicates, considering only values above 50% as significant (Kumar *et al*., 2018).

Population diversity analysis: The analysis of neutrality and demographic characteristics for the GarV-C population was performed using statistical tests, including Tajima's D, Fu and Li's D and F tests, within DnaSP version 6.12. Tajima's D test was used to evaluate the variations in the number of nucleotide differences relative to the sites of segregation. Fu and Li's D test assessed the variation between the total number of mutations and the count of singletons, while Fu and Li's F test was applied to examine the differences between the average number of nucleotides and the number of singletons in the sequences (Tajima, 1989; Fu and Li, 1993). The mutation rates of the CP gene were documented by calculating nucleotide diversity per site (π), haplotype diversity (Hd), insertions and deletions (INDELs), single nucleotide polymorphisms (SNPs), and theta (Θ) based on segregation per site (S) and haplotypes (h). Additionally, synonymous (Ka) and non-synonymous (Ks) mutations were also calculated (Rozas *et al*., 2017).

RESULTS

Disease prevelance and geographical distribution of Allexiviruses: Field surveys for Allexiviruses prevalence in garlic was carried out during 2021 and 2022 seasons. Samples were collected from geographically distinct districts including Rawalpindi, Chakwal, Jehlum, Attock, Kasur, Gujranwala, Sheikhupura, Sialkot, Narowal, Sahiwal, Khanewal, Vehari, Multan and capital territory Islamabad, Pakistan (Figure 1). Plants showing Allexiviruses suspected symptoms such as mosaic patterns, leaf distortion, chlorotic streaks and young leaf deformation (Figure 2). A total of 296 symptomatic garlic samples, representing for 42.28% of the 700 tested samples, showed positive reactions for the presence of allexiviruses, primarily identified through RT-PCR.

In terms of prevalence, GarV-C infection was notably high during 2021 season, with the following geographical distribution: Attock 36%, Chakwal 28%, Jehlum 16%, Rawalpindi 40%, Islamabad 24%, Narowal 12%, Sialkot 16%, Gujranwala 20%, Sheikhupura 12%, Kasur 24%, Multan 20%, Vehari 16%, Khanewal 12%, Jhang 8%, and Sahiwal 8%. The disease prevalence and geographical distribution of GarV-B was as follows: Attock 24%, Chakwal 8%, Jehlum 4%, Rawalpindi 16%, Islamabad 8%, Narowal 4%, Sialkot 4%, Gujranwala 8%, Sheikhupura 4%, Kasur 12%, Multan 4%, Vehari 8%, Khanewal 0%, Jhang 4%, and Sahiwal 0% respectively. GarV-A was identified as the least infective virus among the tested samples, with the following distribution: Attock 16%, Chakwal 4%, Jehlum 8%, Rawalpindi 12%, Islamabad 16%, Narowal 8%, Sialkot 16%, Gujranwala 20%, Sheikhupura 12%, Kasur 16%, Multan 8%, Vehari

12%, Khanewal 8%, Jhang 0%, and Sahiwal 4% (Figure 3). In the 2022 season disease prevalence was higher compared to 2021 season with GarV-C remaining the predominant virus. Its geographical distribution was as follows: Attock 40%, Chakwal 20%, Jhelum 16%, Rawalpindi 32%, Islamabad 20%, Narowal 12%, Sialkot 16%, Gujranwala 24%, Sheikhupura 20%, Kasur 28%, Multan 20%, Vehari 16%, Khanewal 12%, Jhang 12% and Sahiwal 8%. GarV-B was the second most prevalent virus with following geographical distribution: Attock 28%, Chakwal 16%, Jhelum 8%, Rawalpindi 12%, Islamabad 16%, Narowal 12%, Sialkot 16%, Gujranwala 20%, Sheikhupura 8%, Kasur 12%, Multan 20%, Vehari 12%, Khanewal 4%, Jhang 0% and Sahiwal 12%. Additionally, GarV-A was observed as least prevalent virus with following geographical distribution: Attock 20%, Chakwal 12%, Jhelum 4%, Rawalpindi 16%, Islamabad 8%, Narowal 4%, Sialkot 8%, Gujranwala 12%, Sheikhupura 4%, Kasur 8%, Multan 16%, Vehari 4%, Khanewal 0%, Jhang 12% and Sahiwal 8% (Figure 3).

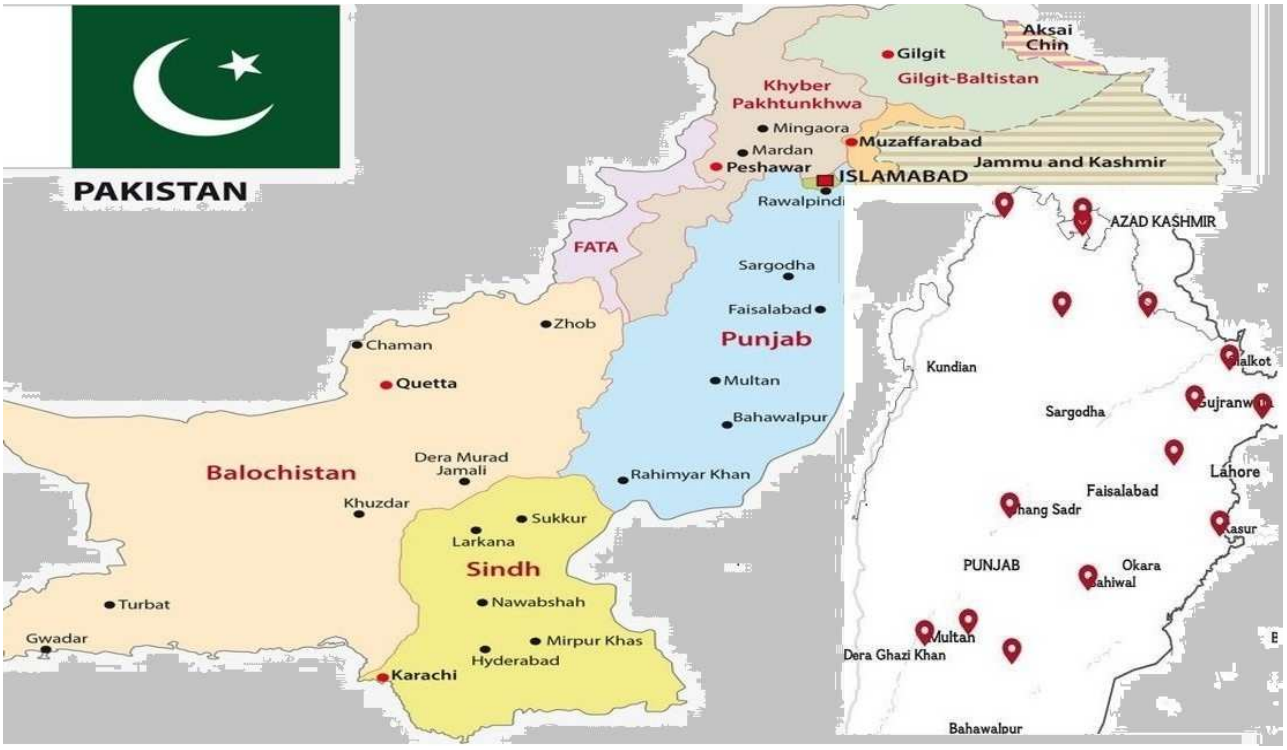
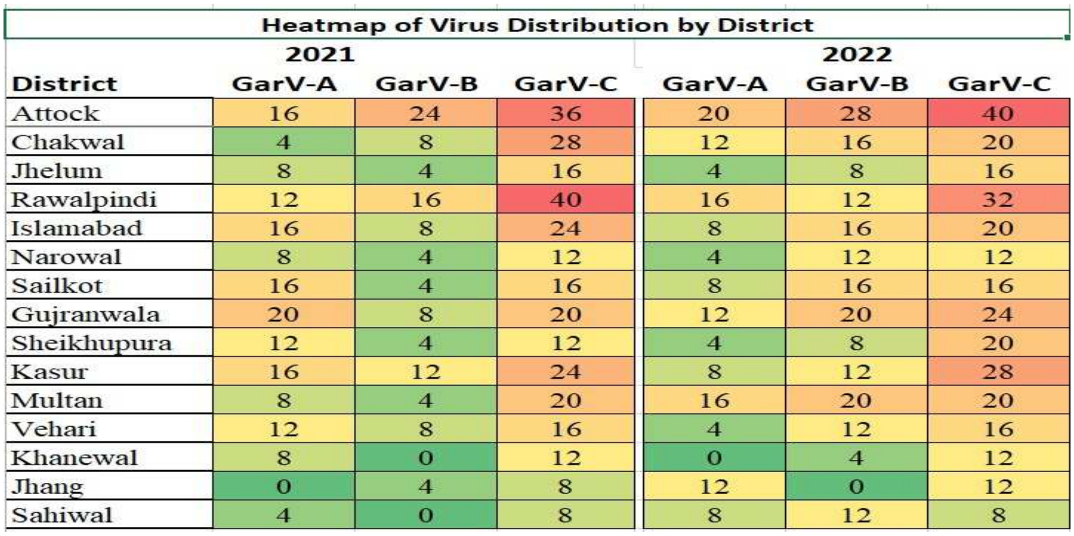


Figure 1: Map of surveyed districts in Punjab Pakistan

Figure 2: Characteristic symptoms of Allexiviruses observed in garlic during field visits:A. Infected plant**, B.** Pale leaf stripes **,C.** Mosaic pattern**, D.** Leaf distortion**.**



Heatmap of Virus Distribution by District

	2021			2022		
District	**GarV-A**	**GarV-B**	**GarV-C**	**GarV-A**	**GarV-B**	**GarV-C**
Attock	16	24	36	20	28	40
Chakwal	4	8	28	12	16	20
Jhelum	8	4	16	4	8	16
Rawalpindi	12	16	40	16	12	32
Islamabad	16	8	24	8	16	20
Narowal	8	4	12	4	12	12
Sailkot	16	4	16	8	16	16
Gujranwala	20	8	20	12	20	24
Sheikhupura	12	4	12	4	8	20
Kasur	16	12	24	8	12	28
Multan	8	4	20	16	20	20
Vehari	12	8	16	4	12	16
Khanewal	8	0	12	0	4	12
Jhang	0	4	8	12	0	12
Sahiwal	4	0	8	8	12	8

Figure 3: Heatmap showing district wise prevalence and geographical distribution of Allexiviruses in garlic during both seasons in Punjab, Pakistan

The higher prevalence of GarV-C was recorded in Rawalpindi at 40% followed by GarV-B in Attock at 24% and GarV-A in Gujranwala at 20% during the 2021 season. Subsequently, increased prevelance of Allexiviruses was observed as GarV-C 40%, GarV-B 28% and GarV-A 20% respectively in district Attock in 2022 season. The results also highlighted the variable prevalence across the surveyed region and five districts including Kasur, Gujranwala, Attock, Rawalpindi and the capital territory Islamabad were recognized as most infected areas by GarV-C infection following GarV-B and GarV-A during both seasons (Figure 4). Disease prevalence results indicated a variable and significant increased in Allexiviruses infection during both seasons. GarV-C was the most prevalent virus during both seasons, while GarV-B was most prevalent virus in 2022 as compared to GarV-A, which showed the significant and strong association among these viruses (Figure 5). This phenomenon is directly linked to the common vector mite, *Aceria tulipae*, faciltating their transmission during bulb storage. The diverse climatic conditions and established vector population in studied region underscore the fluctuating prevalence and distribution patterns of allexiviruses and the results were consistent with earlier findings (Oliveira *et al*., 2014; Abraham *et al*., 2019).

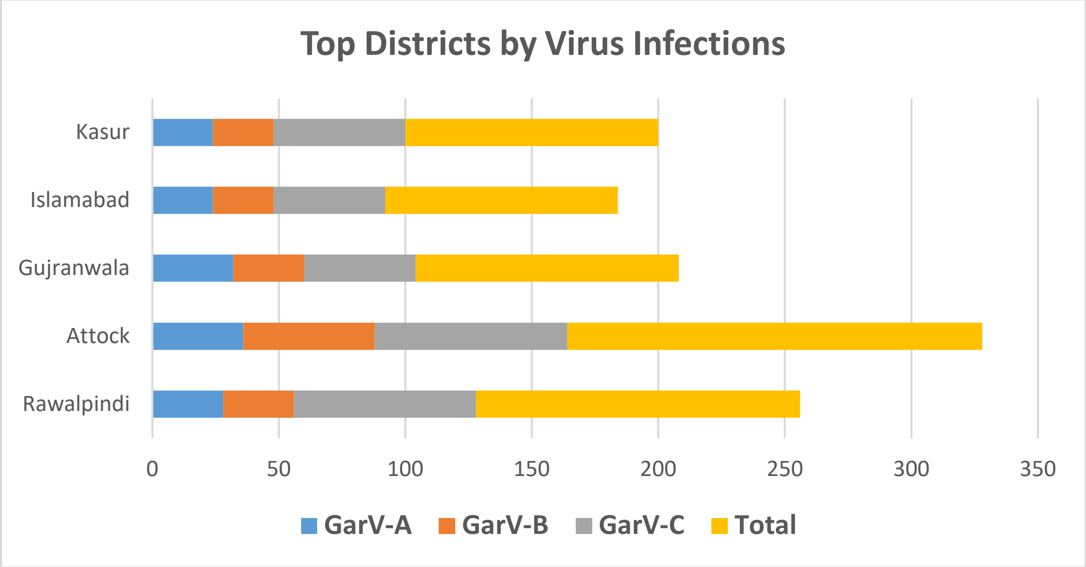


Figure 4: District wise comparison of Allexiviruses infection in garlic crop during both seasons in Punjab, Pakistan

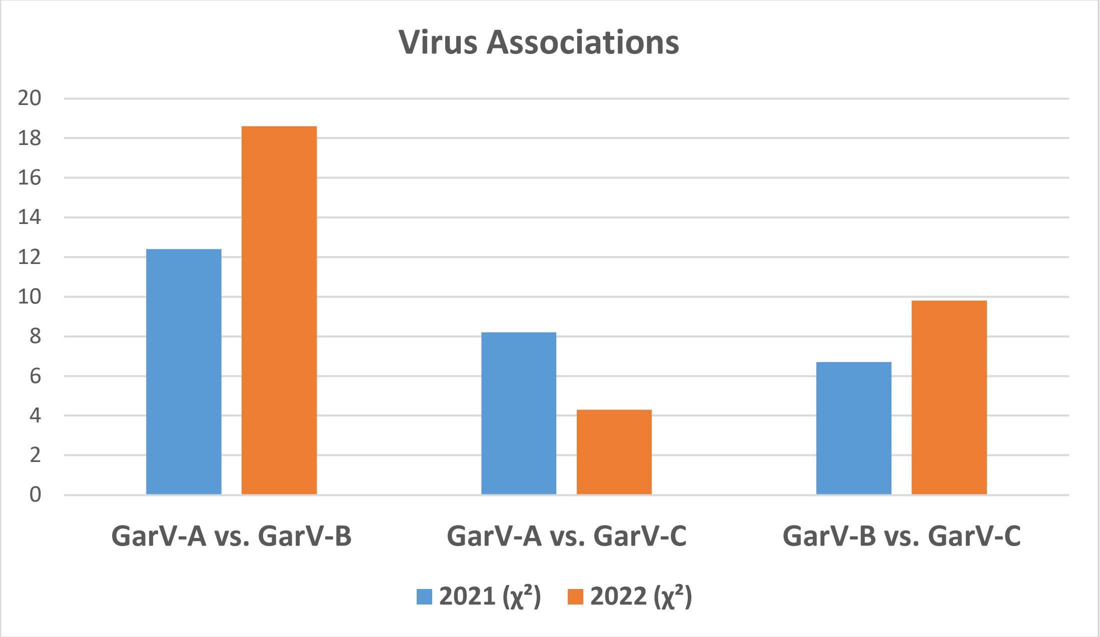


Figure 5: Comparison of Allexiviruses association in garlic during both seasons in Punjab, Pakistan

Molecular characterization: Allexiviruses infection was confirmed by using RT-PCR with group and specie-specific primers targeting CP+NABP gene, however GarV-A, GarV-B and GarV-C specific primers did not amplify target DNA fragment size. *Allexivirus* genus specific primer AllexCP-74/AllexNABP-75 yielded a 750 bp product corresponding to the CP+NABP gene, while positive samples were further validated through PCR amplification using primer pair AL-N30/AL-C30 (Table 1). The obtained sequences were analysed using the NCBI tool BLASTn (www.ncbi.nlm.nih.gov) and compared with closely related globally reported GarV-C CP+NABP nucleotide and amino acid sequences and submitted to GenBank database for accession numbers. RT-PCR results confirmed the prevalence of GarV-A, GarV-B and GarV-C in garlic plants, but PCR amplicons from selected samples identified two sequences of GarV-C with GenBank corresponding accession numbers PP759407 and PP759408. Each sequence comprised of 819 nucleotides, including 417 nucleotides of coat protein (CP) gene encoding 138 amino acids and 387 nucleotides of nucleic acid binding protein (NABP) gene, corresponding to 128 amino acids. The CP gene of PP759407 isolate exhibits a nucleotide composition of 28.3% Adenine, 26.7% Cytosine, 20.1% Guanine, and 24.7% Uracil. In contrast, the PP759408 isolate shows a composition of 27.7% Adenine, 26.8% Cytosine, 20.3% Guanine, and 25% Uracil (Table 2).

Table 1: List of previously described primers used for detection of Allexiviruses infecting garlic crop in Pakistan

Virus group	Primer pair	Sequence 5\`-3\`	Target Gene	Amplicons size	Reference
	AL-N30	CAYTCHATGAAYGCBAARATGTC	CP+NABP	281/580bp	Nam *et al.*, 2015
	AL-C30	GGCTTATTYTGWCTAGYYTTACG			
Allexiviruses	AllexCP+-74	TGGRCXTGCTACCACAAYGG	CP+NABP	750bp	Chen *et al.*, 2004
	AllexNABP-R-75	CCYTTCAGCATATAGCTTAGC			
Garlic virus A	GarV-A-F	TGTCTCGCGCTCCTACATCAGAA	CP+NABP	1330 bp	
	GarV-A-R	TCTGGGGACAATAGTTGTTGCAAGGT			
Garlic virus B	GarV-B-F	TTGTGTTAAGTTTGGAYTTGGGTTGA	CP+NABP	1216 bp	Bereda *et al.*, 2017
	GarV-B-R	TGATATCAACAGCATGGGTGTCTT			
Garlic virus C	GarV-C-F	AGTGATTTGSAMCCATAYCAAGC	CP+NABP	1557bp	
	GarV-C-R	AGTAATATCAACAAGCATGGGTGT			

Table 2: CP+NABP gene nucleotide and amino acid features of GarV-C sequences of current study isolates deposited in GenBank database

Viral specie	Accession no.	Isolate	Crop	cDNA (nt)	Features of (CP) gene						NABP (nt)
					CP (nt)	CP (aa)	A (%)	C (%)	G (%)	U (%)	
GarV-C	PP759407	ZAAHG	Garlic	819	417	138	28.3	26.7	20.1	24.7	387
	PP759408	ZAMNG	Garlic	819	417	138	27.7	26.8	20.3	25	387

Sequence comparison: These sequences were blasted in NCBI database and BLASTn analysis based on CP+NABP gene indicated that both isolates share highest nucleotides identity range of 97-99% with each other. They also exhibited the highest nucleotides identity with previously submitted GenBank sequences from various countries including two isolates MH021116-17 (98.6%) from Pakistan followed by one isolates JQ899448 (93.6%) from Australia, two isolates MN059145-46 (92.9%) from China, five isolates (NC003376, AB010302, D49443, LC097170-71) with identities ranging 92.5-92.1% from Japan, two isolates JX682944-55 (91.5-91.2%) from the Czech Republic, one isolate KX034780 (89%) from Poland and one isolate MK814851 (88.5%) from Serbia. Subsequently, the isolates of current study shared the lowest nucleotides identity of 83% with a previously found isolate HQ681937 from Iran followed by 82.1% identity with an isolate HQ724848 from Spain (Figure 6). When analyzing the amino acid sequences of CP gene, it was found that isolates from current study demonstrated a remarkable similarity of 98.8% with previously identified GarV-C (MH021116-17) isolates from Pakistan, followed by Australia JQ899448 (98.5%), China MN059141-45 (99%) and Japan NC003376, AB010302, D49443, LC097170-71 (98.2%) respectively detected from garlic. The lowest amino acid identity was observed at 80%-87%, with reported isolates from Iran, Poland, Serbia, the Czech Republic and Spain (Figure 6).

The BLASTp analysis of partial coat protein (CP) amino acid sequences of GarV-C isolates from current study indicated a strong affiliation within Flexi-CP group of Alphaflexiviridae superfamily. ORF1 encodes polypeptides which is thought to be a virus specific RNA replicase and a conserved motif known for proposed active site for RNA dependent RNA polymerase with 14 amino acids (KFAAFDFNAVSSDS) were identified located at codon positions 63-77 in present study isolates. However variations were observed in CP conserved region at codon positions G190, where it was substituted by A and L198, when compared to previously described sequences (Majumder and Baranwal, 2011). Furthermore, a conserved region comprising 16 amino acids, "KFAAFDFFNAVSSDSS" was identified at codon positions 63-79 in the CP gene of both GarV-C isolates examined in this study and these results were consistent with earlier findings by Prajapati *et al*., (2022). Similarly, the amino acid sequence of NABP gene was classified within the Flexi-NABP superfamily, featuring a conserved region including 16 amino acids (KFQGTSKCAARRAKRY) were observed at codon positions 45-62 in the NABP gene of present study isolates, as well as in previously reported isolates available in GenBank. A conserved sequence of 14 amino acid "CFDCGGYLLNNHVC" was also identified at similar codon positions (65-79) present in NABP gene of both examined isolates of current study. The NCBI conserved domain database (CDD) analysis of coat protein (CP) of current study isolates classified them under the pfam0286 protein family, a well-known member of the Flexi-CP superfamily, which was also found in *Potyvirus* and *Carlavirus* species. The NABP protein of present study GarV-C isolates was categorized under Pfam 05515, a viral NABP superfamily protein with a zinc-finger motif associated with nucleic acid binding features. These dominant features were found among most ssRNA viruses and results were aligned with the findings of previous studies (Majumder and Baranwal, 2014). Furthermore, sequence analysis of GarV-C isolates revealed a non-translated region (NTR) situated within the coding region of both CP and NABP gene. These observation have also been documented in previously reported isolates of GarV-B, GarV-X and GarV-C (Chen *et al*., 2004; Salih *et al*., 2013).

Phylogenetic analysis: The evolutionary relationship analysis of GarV-C isolates was performed on the basis of CP+NABP gene nucleotide and amino acid sequences homology. A phylogenetic tree was constructed by two isolates of current study, along with 16 closely related previously reported isolates from NCBI database in MEGA v11 using neighbor joining algorithm and isolates from current study were placed on the same branch with a previously found unpublished isolate (MH021116) from Pakistan. The present study isolates also showed a strong affiliation with these reported isolates from China (MN059145-46), five isolates from Japan (NC003376, AB010302, D49443, LC097170-71) and Australia (JQ899448). Both isolates from the present study revealed the highest homology with unpublished isolate MH021116, but presented a smaller relationship with the other two isolates MH021117-18 from Pakistan as well as isolates HQ681937 from Iran, JX682953 from the Czech Republic and HQ724848 from Spain (Figure 7).

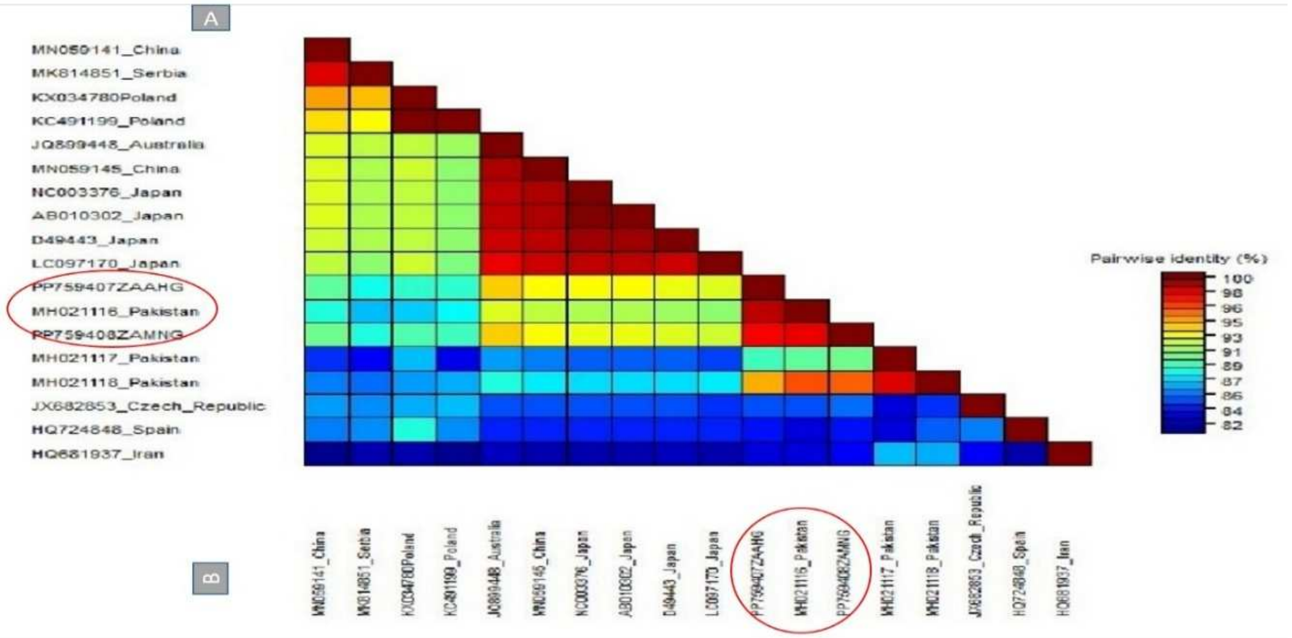


Figure 6: CP+NABP gene nucleotides and amino acid based sequences comparison of present study GarV-C isolates with previously reported GenBank retrieved sequencees. A. Nucleotides**, B.** Amino acid: closely related sequences indicating red to yellow color showing highest identity, while blue color showing lowest identity.

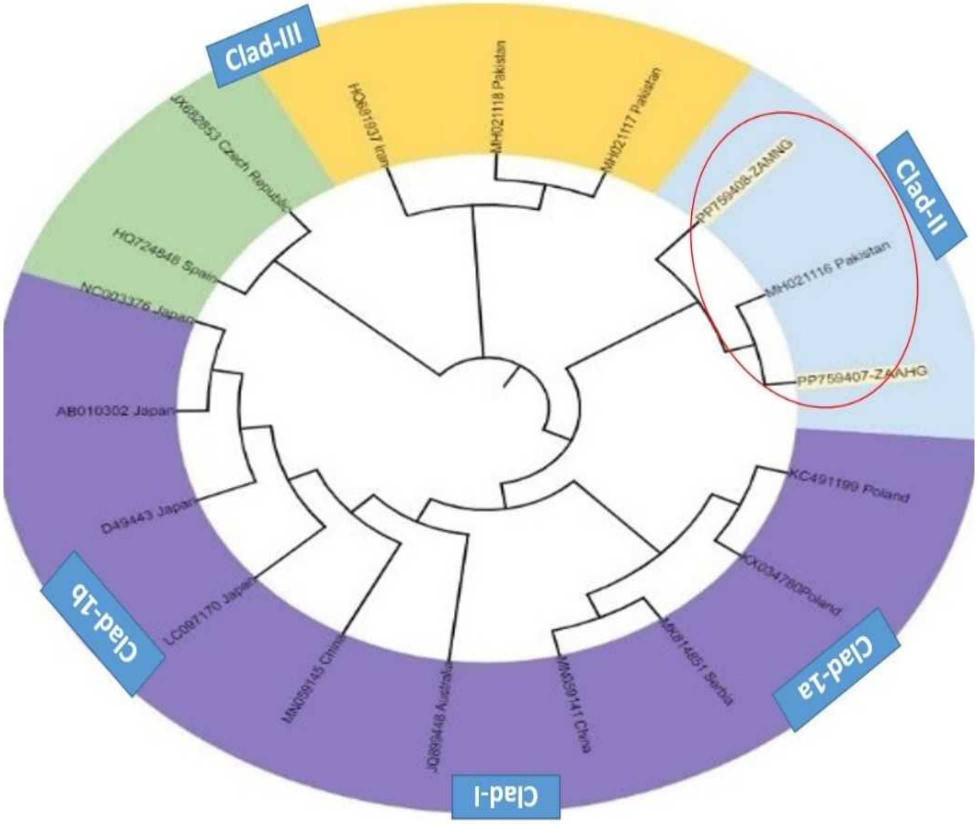


Figure 7: A consensus tree displaying the evolutionary connections among GarV-C CP+NABP gene nucleotide sequences from present study in comparison to previously documented GenBank sequences (only above 50% bootstrap values were recorded)

Phylogenetic analysis based on CP+NABP gene amino acid sequences showed that isolates from current study were grouped with previously identified Pakistani GarV-C isolate MH021116. These isolates also exhibited the close relationship with isolates from China (MN059145), Australia (JQ899448), and five isolates (NC003376, AB010302, D49443 and LC097170) from Japan, MK814851 from Serbia, MN059141 from China, and KC491199 from Poland. In contrast isolates MH021117-18 from Pakistan, HQ681937 from Iran, JX682953 from Czech Republic, KX034780 from Poland and HQ724848 from Spain showed variation and formed a separate group (Figure 8). These variations may be attributed to the evolutionary relationship among different isolates of GarV-C and results were consistent with earlier findings. This divergence is likely due to host adaptation in viruses, virus evolution, mutations and genetic recombination or re-assortment in the CP gene and CP plays a pivotal role in host adaptation of many plant viruses as previously described. Earlier studies also demonstrated that specific mutation occurred in NABP gene can limit virus accumulation and prevention of symptoms appearance (Salih *et al*., 2013; Nam *et al*., 2015; Bereda *et al*., 2017; Parajapati *et al*., 2022).

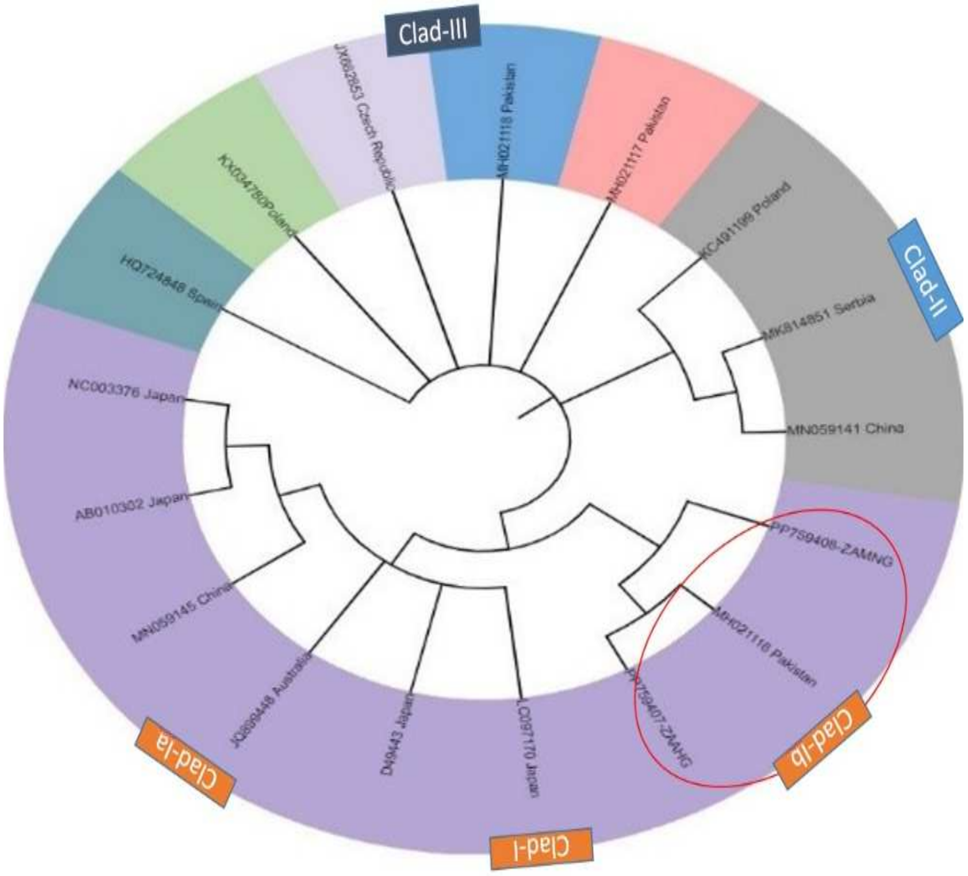


Figure 8: A consensus tree displaying the evolutionary connections among GarV-C CP+NABP gene amino acid sequences from present study in comparison to previously documented GenBank sequences (only above 50% bootstrap values were recorded)

Population diversity analysis: Analysis of two genomic regions of GarV-C indicated that CP gene nucleotide diversity was lower than NABP gene, suggesting that NABP gene represents a more conserved region. The variable nucleotide diversity of present study isolates indicated a reduced mutation frequency within the CP gene, whereas the NABP gene exhibited greater divergence compared to the CP gene and it is consistent with earlier findings (Chen *et al*., 2004; Oliveira *et al*., 2014; Bereda *et al*., 2015; Bereda and Cichal, 2019). The evolutionary distances among the present study isolates ranged from 0.045 to 0.113, while the distances with other isolates available in GenBank varied between 0.086 and 0.248. The present study isolates PP759407 and PP759408 demonstrated the smallest evolutionary distances of 0.045 and 0.019 respectively and showed the greatest evolutionary distance with the Japanese isolate LC097169, as indicated by phylogenetic analysis (Table 3).

Table 3: Nucleotide Identity percentage of Pakistani GarV-C isolates with GenBank reported isolates and the Ka/Ks ratio of CP gene.

Accession No.	Location	Ka	Ks	Ka/Ks	Nucleotides identity % of Pakistani isolates	
					PP759407	PP759408
MH021116	Pakistan	0.0063	0.9139	0.0069	98.6	98
MH021117	Pakistan	0.0060	0.9135	0.0065	98.3	98.1
JQ899448	Australia	0.0063	0.9483	0.0066	93.5	93.6
MN059146	China	0.0063	0.9139	0.0069	92.7	92.9
MN059145	China	0.0127	0.6405	0.0198	92.7	92.9
NC_003376	Japan	0.0305	1.5268	0.0200	92.5	92.5
AB010302	Japan	0.0240	1.3245	0.0181	92.5	92.5
D49443	Japan	0.0304	1.4684	0.0207	92.3	92.2
LC097171	Japan	0.0175	0.7612	0.0230	92.1	92.1
MN059141	China	0.0127	0.7730	0.0164	89.7	89.9
LC097170	Japan	0.0159	0.7730	0.0206	91.9	91.7
JX682844	Czech Republic	0.0159	0.7730	0.0206	91.5	91.3
JX682855	Czech Republic	0.0159	0.7730	0.0206	91.3	91.2
KX034780	Poland	0.0123	0.7725	0.0160	89	88.9
MK814851	Serbia	0.0120	0.07720	0.0150	88.5	88.3
HQ681937	Iran	0.080	0.07650	0.0100	83	82.8
HQ724848	Spain	0.060	0.07600	0.0080	82.1	82

The CP gene Ka/Ks ratio of both isolates indicated that Ka values ranged from 0.0063 to 0.0305, while Ks values varied between 0.6405 and 1.5268 concerning nucleotide transitions in the amino acid sequence. The recorded Ka/Ks values fell between 0.0069 and 0.0230 and notably, the maximum Ka value of 0.0305 and a Ks value of 1.5268 were observed in the CP gene amino acid sequence of this study isolates, aligning with the NC_003376 from Japan. In contrast, isolate LC097171from Japan exhibited the Ka/Ks value of 0.0230, which is significantly higher in comparison to GarV-C isolates of current study (Table 3).

We estimated the Population diversity to discriminate between population demographic expansion and contraction of GarV-C population from Pakistan and from other countries using statistical methods Tajima's D and Fu & Li's D and F tests based on CP+NABP gene sequences. Sequence polymorphisms of GarV-C was analyzed in an 819 nt segment of present study sequences, alongside 16 other reported isolates from various countries, encompassing CP gene (417 nt), NABP gene (387 nt) and a 15 nt intergenic NCR region.The Tajima's D, Fu & Li’s D and Fu & Li’s F results from CP+NABP gene sequences of present study isolates indicated variation and diversity in this genome region was observed with a mean value of 0.0029± 0.0030 nt mutations/site and the results were consistent with earlier findings (Table 4) (Bereda *et al*., 2015).

Table 4: Selection pressure test and haplotype diversity analysis of GarV-C populations from Pakistan and different countries of the World

Viral specie	Target gene	Isolate	Fu & Li`s D	Fu & Li`s F	Tajima`s D	Haplotype	Haplotype diversity
GarV-C	CP	All	0.12512	0.23725	0.36599	25	1.000 (0.00014)
		PK	0.53308	0.60329	0.55050	9	1.000 (0.00300)
	NABP	All	-0.19218	-0.19303	-0.10493	24	0.990 (0.00012)
		PK	-0.43550	-0.49660	-0.45510	10	0.980 (0.00295)

Sequence diversity results revealed 61 single nucleoride substitutions sites, with 27 located in the CP gene and 34 in the NABP gene among the isolates of this study and nucleotide diversity (π) of 0.12706, whereas the haplotype diversity (Hd) was determined to be 1.0 which was comparable to the reported GarV-C isolates from GenBank (Table 5). Population diversity results for the CP+NABP gene sequences of present study revealed that all values for the CP gene sequence were positive, suggesting a trend of purifying selection or a contraction within the GarV-C population. In contrast, the NABP gene sequence exhibited all values negative, which indicated statistically non-significant results. The negative selection observed in the GarV-C NABP gene sequence suggests a growing population of GarV-C in Pakistan.

Table 5: Overview of DNA polymorphism in GarV-C sequences from current study.

Parameters	**CP**	**NCR**	**NABP**	**Entire region**
Total length of amplicons (bp)	417	15	387	819
Single Nucleotide Substitution (bp)	27	0	34	61
Frequency of substitution sites/bp	0.0647	0.00	0.0879	0.0749
INDEL events	2	0	3	5
Frequency of INDEL event	0.0048	0.00	0.0078	0.0061
Total Variations (S)	29	0	37	66
Frequency of all seq. Variations	0.0695	0.00	0.0956	0.0806

DISCUSSION

Garlic is one of the most popular condiment and herbal medicine cultivated worldwide, including Pakistan. However its successful production and bulb quality is significantly impacted by viruses of genus *Allexivirus,* causing low quality yield and economic losses (Cremer *et al*., 2021; Peng *et al*., 2023; Gontijo *et al*., 2025; Neupane *et al*., 2025). Despite the demand for garlic production in Pakistan does not meet its consumption level, leading to the need for importing garlic bulbs from neighboring countries like China, Iran, and India. These imported bulbs are then cultivated over multiple years to reduce production costs. Reports indicated the presence of *Allium* viruses in these exporting countries, and to our knowledge, some studies on garlic viruses have been conducted in Pakistan (Ahsan and Ashfaq, 2018; Hamza *et al*., 2023; Shahid *et al*., 2024). Vegetative propagation of garlic crop through infected bulbs contribute to the disease multiplication and spread over several seasons making it a key challenge for both farmers and researchers (Bereda *et al*., 2017; Bhusal *et al*., 2021) Allexiviruses are a significant problem in garlic plants due to their widespread nature and their synergistic effects with Potyviruses and Carlaviruses leading to the development of a garlic viral complex, which further exacerbates the impact on garlic health and productivity (Benke *et al*., 2023; Lee *et al*., 2024; Melnyk *et al*., 2024).

The present study demonstrated the prevalence and genetic diversity of Allexiviruses using general and specific primers through RT-PCR. The findings revealed the prevalence of GarV-A, GarV-B, GarV-C in the Punjab region and capital territory Islamabad Pakistan. RT-PCR techniques has proven to be highly sensitive in the detection of Allexiviruses and several other plant viruses by employing genus-specific primers. Furthermore, disease incidence of GarV-C, GarV-B, and GarV-A has been documented in garlic fields indicating a significant increase in the prevalence of these viruses. These results validated the findings of previous studies in Pakistan and align with earlier studies worldwide, whereas further confirming the widespread impact of Allexiviruses on garlic production in this region (Fayad *et al*., 2011; Wylie *et al*., 2014; Oliveira *et al*., 2014; Gawande *et al*., 2015; Jemal *et al*., 2015; Abraham *et al*., 2019).The variable disease prevalence recorded in this study can be attributed to the, vegetative propagation, rate of virus population, virus-vector interaction and changing climatic conditions in this region (Nam *et al*., 2015; Bereda *et al*., 2017). The semi-persistent transmission of GarV-C, GarV-B, and GarV-A by vector contributes to the proliferation of these viruses, and efforts to control the vector mite using insecticides, the efficacy of such methods remains limited (Bereda *et al*., 2017; Dąbrowska *et al*., 2020; Mansouri *et al*., 2021). Additionally, understanding the genetic diversity and evolution in these viruses allows us for better forecasting of disease outbreaks and informed decision in agriculture practices (Khan *et al*., 2016; Taglienti *et al*., 2018). Molecular characterization results of present study revealed two GarV-C sequences with CP gene comprising 417 nt followed by NABP gene at 387 nt and a 15 nt NCR region evidenced the genetic variability of present study isolates comparative to previously reported sequences and results were consistent with earlier findings (Bereda *et al*., 2015; Bereda *et al*., 2017; Amir *et al*., 2024).

Sequence comparison demonstrated the 98% identity in both nucleotides and amino acid sequences of CP+NABP gene between the present study isolates and previously reported GarV-C isolates from several countries. Some nucleotide variations were noted, likely due to natural genetic differences in these isolates. Furthermore, changes were observed in the conserved motif of CP gene amino acid sequences in the current GarV-C isolates, aligning with those found in the previously mentioned isolates (Salih *et al*., 2013; Majumder and Baranwal, 2014; Celli *et al*., 2019). Multiple studies conducted in various countries have highlighted a strong link between the rapid evolution of diverse *Allium* viruses and the trade of vegetative material. The amino acid sequence of the NABP gene from current GarV-C isolates showed significant similarities to the previously identified NABP gene of GarV-C isolate (Bereda and Cichal, 2019; Cremer *et al*., 2021; Prajapati *et al*., 2022). Phylogenetic analysis of CP+NABP gene nucleotide sequences revealed a close relationship between the current isolates and previously identified unpublished GarV-C isolates from Pakistan as well as isolates from China, Japan and Australia. A similar pattern was observed in the phylogenetic analysis of CP+NABP gene amino acid sequences showed the divergence and lower nucleotides and amino acid identity compared to previously identified GarV-C sequences from Czech Republic, Poland, Serbia, Iran and Spain. Genetic differentiation and frequent gene flow was also observed in previously identified *Allexivirus* species from several countries and these observations were consistent to the present study findings (Chen *et al*., 2004; Wylie *et al*., 2012; Mohammed *et al*., 2013; Oliveira *et al*., 2014; Wylie *et al*., 2014).

ORF1 encodes a polypeptide believed to function as a virus-specific RNA replicase, a conserved motif, proposed to serve as the active site of the RNA-dependent RNA polymerase, has been identified at amino acid positions 7-42 and19-54 in GarV-X and GarV-E, respectively (King *et al*., 2018). Additionally, the NABP binding motif, thought to be involved in helicase activity, was located at positions 48-55 and 60-67. A leucine zipper pattern was observed at positions 159-180 in both GarV-X and GarV-E, which is also present in other members of the genus. ORF2 encodes a protein that exhibits similarities to TGB1 found in all Carla-potyviruses. Although two proteins share minimal similarity otherwise, an NABP binding motif similar to that in ORF1 has been identified at positions 37-44 and 30-37 in GarV-X and GarV-E, respectively, indicating that this protein may act as an NABP-dependent helicase.

This motif has also been noted in the TGB1 of carla-poty and hordeiviruses. The protein is abundant in leucine and may potentially form amphipathic α-helices, which are believed to bind to DNAs or RNAs (Yoshida *et al*., 2018). ORF3 encodes a TGB2 protein that is homologous to those found in other allexi-carla and potyviruses. Secondary structure analysis has revealed that TGB2 across all allexi-carla and potyviruses features a helix-coil-helix structure. By analogy with other viruses, TGB proteins may facilitate cell-to-cell movement. ORF4 of Allexiviruses is significantly different from the third ORF in the triple gene block of carla- and potyviruses and all these observations were consistent with findings of present study (Zhang *et al*., 2018; Kim *et al*., 2023). This protein, which appears to be characteristic of Allexiviruses, is exceptionally rich in serine and threonine and contains approximately 30 phosphorylation sites that may be targeted by kinases and phosphatases, while ORF5 is highly conserved, with the exception of its N-terminus. ORF6 encodes a nucleic acid binding protein that is rich in leucine, featuring a similar putative zinc binding consensus, suggesting its role in the regulation of RNA replication. The findings of current study suggest that GarV-C isolates were influenced by their geographical origin, climatic conditions and host adaptation, which are consistent with observations made by other researchers (Bereda *et al*., 2017; Taglienti *et al*., 2018; Celli *et al*., 2019; Bereda and Cichal, 2019). This variation may be linked to the trade of garlic cloves and seed with key factors contributing to genetic variability in RNA viruses including mutation, rearrangement, and genetic recombination. These processes promote the integration of diverse sequence elements, repetition and exchange of existing viral components (Akinyemi *et al*., 2016; Araújo *et al*., 2018).

The single nucleotide polymorphisms (SNPs) analysis of present study revealed two insertion-deletion (INDEL) events in the haplotypes of GarV-C isolates CP gene. The CP gene displayed the lowest variability compared to NABP gene, indicating that it is a more conserved region relative to other genes used in species differentiation (Holmes, 2011; King *et al*., 2018). The population demographic and selection pressure analysis was used to distinguish between neutral and non-neutral isolates. Neutral isolates exhibited random evolution without any selection influence, while non-neutral isolates underwent evolution driven by a selection process influenced by genetic drift (Acosta *et al*., 2011; Zhang *et al*., 2013; Bereda *et al*., 2015a; Mitiku *et al*., 2020; Holmes, 2022). Population demographic analysis across the entire region, as well as specific regions of CP and NABP genes, showed that Ka/Ks values of CP gene sequence of current GarV-C isolates were elevated, suggesting positive selection. This was accompained by a balanced selection pressure and a reduction in population size. In contrast, the Ka/Ks ratio of NABP gene from the present GarV-C isolates was lower, indicating negative selection with increasing population selection pressure, as noted in previous studies (Bereda *et al*., 2015a; Bereda and Cichal, 2019).

The changes in CP+NABP gene nucleotide sequences of GarV-C isolates substitutions, as RNA viruses tend to undergo a large number of INDEL events during gene replication. Allexiviruses being smaller in size, replicate much faster than wild type viruses and as a result these mutations are commonly found in most viral populations (Moutailler *et al*., 2011). Selection pressure analysis revealed that GarV-C population based on CP gene underwent the negative selection, as the dN/dS values were below one. In contrast higher dN/dS values were observed for GarV-C population based on the NABP gene. We also identified a common site at codon 41 in the CP gene detected by all four models, where the GarV-C population underwent positive selection and these results were consistent with earlier findings (Bereda *et al*., 2015). The presence of this codon recognized as part of Zinc-finger motif, known as defining feature for amino acid changes at this site likely influence the proteins function particularly in nucleic acid binding proteins (Zhang *et al*., 2018). The analysis supports the hypothesis of purifying selection in Allexiviruses suggests that most of the mutations observed in the sequences of present study were deleterious. Furthermore, previous studies were reinforced by population neutrality assessment of CP and NABP gene. It was concluded that GarV-C population experienced either an increase in size or balancing selection as noted in earlier findings (Chodorska *et al*., 2014; Wijayasekara *et al*., 2019; Ayed *et al*., 2022; Altieri *et al*., 2022). Allexiviruses were only recently identified and remain an under studied area, leading to a lack of data on the genetic population, while a single study has just primarily focused on initial identification of unpublished GarV-C isolate from Pakistan. Reduced garlic yields in Pakistan is inferred the trade of garlic bulbs between different regions and may be key factor contributing to these yield discrepancies. This practice facilitates the introduction of new viral species, posing a significant threat to indigenous garlic varieties and the extensive exchange of propagating materials plays a vital role in Allexiviruses spread. This underscore the need for integrated pest management strategies to control vector population and highlights the challenges faced in managing the spread of Allexiviruses and reduced their impact on garlic crop The PCR amplification of Allexiviruses plays a crucial role in enhancing our understanding of virus biology and genomic structure. This deeper insight is essential in the development of more effective management strategies for garlic crop protection. By accurate detection and biological characterization of these viruses, we can implement targeted control measure and improve overall sustainability of garlic production.

Conclusion: In conclusion present study underscores the impact of geographical factors in shaping the genetic diversity and spread of Allexiviruses infecting garlic in Pakistan. Molecular analysis identified variations in the CP and NABP genes highlighting both conserved regions and their evolving genetic traits within the GarV-C population and results suggest the purifying selection plays a dominant role in the evolution of Allexiviruses. However, to fully understand the genetic diversity, origins and mechanism of genetic exchange of these viruses is needed further research on whole genome studies of each *Allexivirus* species. These findings stress the importance of producing virus free garlic seeds by adopting a strict seed certification program and quarantine protocols to restrict the spread of these viruses for improved garlic production in Pakistan.

Acknowledgements: The work was supported by International Foundation for Science (IFS) under the Research Award No. IFS-1-3-C-5259-2 to M. Ashfaq.

Authors Contributions: Conceptualization & Supervision: M. Ashfaq, Experiments designed & performed: M. Ashfaq, M.Z. Hamza, H. Riaz & M. Ahsan, Writing-original draft preparation: M.Z. Hamza, S. Saeed & M. Ahsan, Review & editing: M. Ashfaq, M.Z. Hamza, H. Riaz, & M. Ahsan, Visualization & Statistical analysis: M.Z. Hamza & N. Ahmad.

Conflict of interest: The authors declare no conflict of interest.

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