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Original article

Molecular detection and associated risk factors of infectious bronchitis virus (IBV) isolated from poultry in Khyber Pakhtunkhwa, Pakistan

F. Islam[1], S. Khan[1], Rafiullah[1], F.A. Khan[3], S.S.A. Shah[2], M. Shahid[2], K. Khan[2], S.U. Hassan[3], H.U. Rahman[2]

[1] Department of Poultry Science, University of Agriculture, Peshawar, Khyber Pakhtunkhwa, 25130, Pakistan
[2] Veterinary Research Institute, Peshawar, Khyber Pakhtunkhwa, Pakistan
[3] College of Veterinary Sciences, Faculty of Animal Husbandry and Veterinary Sciences, University of Agriculture, Peshawar, Khyber Pakhtunkhwa, 25130, Pakistan

Correspondence to: H.U. Rahman, e-mail: drhanif001tanha@gmail.com, tel.: +92 333 9698115, +92 346 9418728

Abstract

Infectious bronchitis, caused by the infectious bronchitis virus (IBV), is a highly contagious multisystem disease affecting poultry of all ages and leading to significant economic losses worldwide. IBV exhibits considerable genetic variability, with numerous genotypes and lineages associated with respiratory, reproductive and kidney issues. This study aimed to investigate the molecular epidemiology of IBV in poultry-dense areas of Khyber Pakhtunkhwa, Pakistan. An outbreak-driven sampling method was used to collect a total of 425 clinical samples, including tracheal swabs and tissue specimens, from suspected flocks across different districts. Specifically, 100 samples were collected from Abbottabad and Mansehra, 90 from Mardan and Peshawar, and 45 from Malakand Division, proportionate to regional poultry populations. Viral RNA was extracted and tested using RT-PCR targeting the IBV S1 gene. Selected positive samples underwent Sanger sequencing. Sequence analysis was carried out using ClustalW in MEGA 12, and phylogenetic relationships were inferred via the Maximum Likelihood method based on the T92+G Tamura 3-parameter model with gamma distribution. The overall IBV prevalence was 29.6%. The highest prevalence was observed in Mansehra (39%), whereas Malakand Division had the lowest prevalence (7.7%). IBV detection was more frequent in young birds (35.2%) and layer flocks (33.7%) compared to broilers (30.7%). Phylogenetic analysis revealed that most circulating IBV strains belong to genotype GI-1, indicating ongoing circulation of classical IBV strains in the area. These findings highlight the importance of ongoing molecular surveillance and the development of region-specific vaccines. Implementing improved biosecurity measures, such as enhanced ventilation, thorough disinfection between production cycles, and all-in-all-out flock management, is essential to reduce IBV outbreaks and improve poultry health and productivity in Khyber Pakhtunkhwa.

Keywords: infectious bronchitis virus (IBV), Pakistan, poultry, phylogenetic analysis, spike gene1 (S1)

Introduction

Infectious bronchitis (IB) is a major viral disease affecting poultry worldwide, resulting in significant economic losses due to reduced productivity, increased mortality and higher control costs (Zhang et al. 2021). The disease is caused by infectious bronchitis virus (IBV), a highly contagious and rapidly evolving avian coronavirus that infects chickens of all ages and production types (Jackwood 2012, Bhuiyan et al. 2021). Since its initial identification in 1931, IBV has spread worldwide and remains an ongoing threat to poultry health and food security (Chen et al. 2013).

IBV exhibits significant genetic and antigenic diversity, primarily driven by mutations and recombination within the spike (S) glycoprotein gene, which is responsible for host attachment and immune recognition (Chhabra et al. 2015). The virus is classified within the Gammacoronavirus genus (family Coronaviridae, order Nidovirales) and encodes structural proteins, including the spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins. The highly variable S1 subunit contains critical neutralizing epitopes and is commonly used for genotyping and molecular epidemiology studies (Valastro et al. 2016). Clinically, IBV strains exhibit variability in tissue tropism, affecting the respiratory, renal, reproductive, and, in some cases, gastrointestinal systems, leading to a range of disease symptoms (Cavanagh et al. 2002, Bande et al. 2016, Bande et al. 2017).

Pakistan's rapidly growing poultry industry is a key source of affordable animal protein. However, IB continues to impose significant economic and health challenges (Ahmed et al. 2007, Khan 2019, Khan et al. 2024). Few surveillance studies have documented the co-circulation of Massachusetts and several European IBV variants, along with evidence that commonly used vaccines like D-1466 (GII-1), 4/91 (GI-13), D-274 (GI-12), and Mass-41 (GI-1) provide limited protection, indicating the presence of uncharacterized field strains (Ahmed et al. 2007). Molecular studies confirmed the dominance of the GI-1 and GI-13 lineages, identified unique Pakistani isolates such as Pak-973 and Pak-786, and revealed genetic differences relative to vaccine strains (Rafique et al. 2018a, Rafique et al. 2018b). Recent phylogenetic analyses show the emergence and increasing prevalence of the GI-24 lineage, which is genetically distinct from the widely used GI-23 vaccine strains, suggesting ongoing viral evolution and potential vaccine escape in Pakistan (Saleem et al. 2024).

Although IBV diagnosis is crucial, many regions in Pakistan still rely on clinical and postmortem assessments due to limited access to advanced molecular tools. In Khyber Pakhtunkhwa, a key poultry-producing province, there is a lack of region-specific molecular epidemiological data, which hampers evidence-based vaccine choice and disease control efforts. Consequently, this study used S1 gene-based molecular detection and genetic analysis to examine the IBV strains present in poultry populations across Khyber Pakhtunkhwa, Pakistan.

Materials and Methods

Study area

The study was conducted in major poultry-producing districts of Khyber Pakhtunkhwa (KP), Pakistan, including Abbottabad and Mansehra (Hazara Division), Mardan and Peshawar (Central KP), and Malakand (Malakand Division), selected based on high commercial poultry density and epidemiological relevance to infectious bronchitis virus (IBV) circulation. These districts represent diverse agro-climatic zones that may influence IBV transmission dynamics. Abbottabad and Mansehra are characterized by a humid Usx climate, with more than 50 inches of annual rainfall and relative humidity of 55-60%, indicating uniform rainfall with summer concentration (Usx). Malakand falls under the Uwx category, characterized by uniform rainfall with winter concentration and relatively higher humidity (Uwx) (50-55%). In contrast, Mardan and Peshawar are classified as Uwy, which is characterized by winter-dominant rainfall and comparatively lower relative humidity (Uwy) (45-50%) (Khan 2019). The inclusion of geographically and climatically distinct poultry hubs enabled a representative assessment of IBV occurrence across major production regions of KP (Fig. 1).

Sample collection

An outbreak-based sampling strategy was used to collect clinical specimens from poultry flocks suspected of infectious bronchitis (IB) in selected districts of Khyber Pakhtunkhwa, Pakistan. A total of 425 samples were collected, including tracheal swabs and trachea/lung tissues from birds showing symptoms. These samples were proportionally distributed based on poultry density: Abbottabad (100), Mansehra (100), Peshawar (90), Mardan (90), and Malakand (45). They were aseptically collected, placed in viral transport medium (Biogen GmbH; Lot No. 20211027), kept on ice, and transported to the Virology Laboratory at the Veterinary Research Institute in Peshawar. There, they were stored at -40°C until molecular analysis. At the same time, epidemiological and management data, such as flock age, production type, vaccination history and clinical

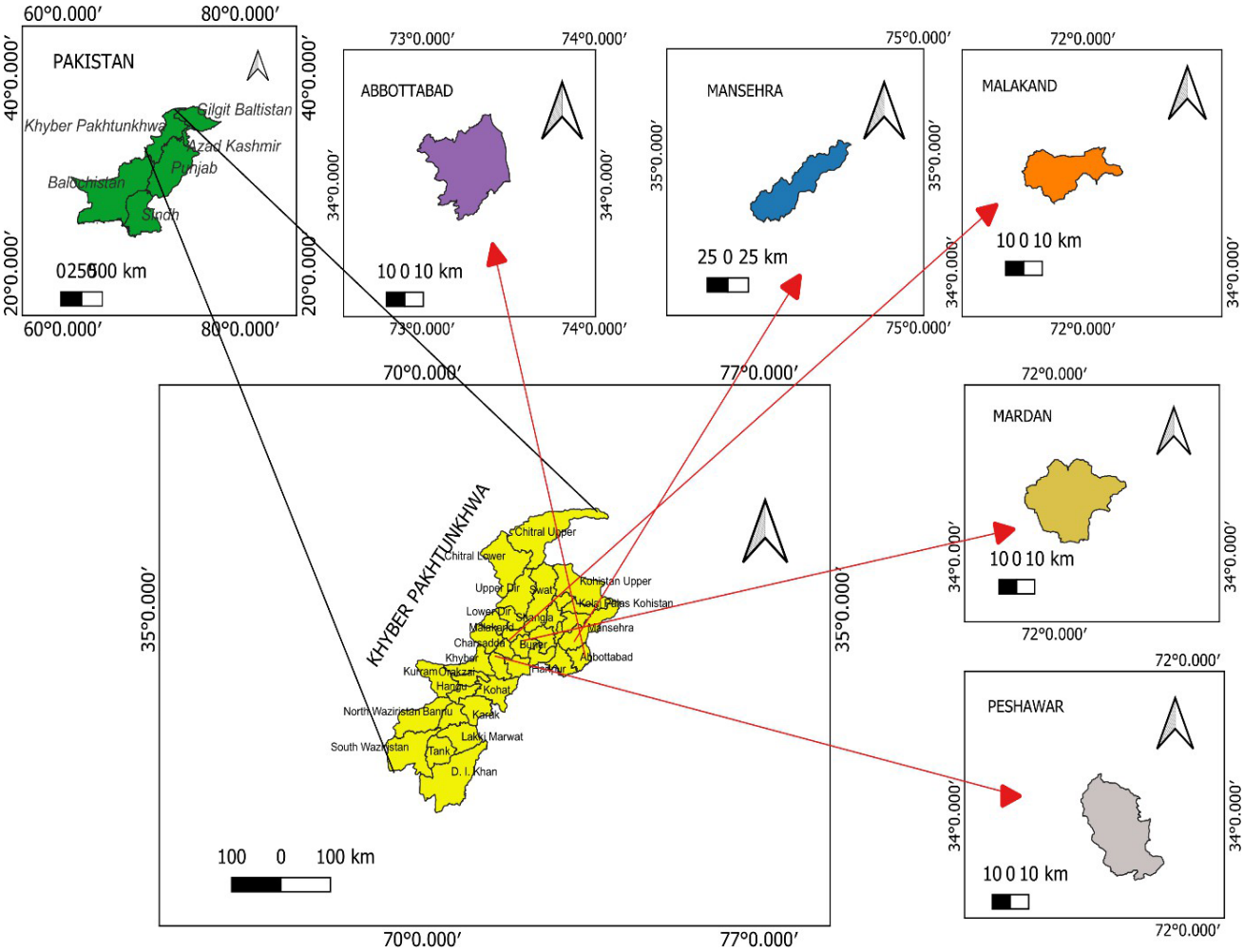

Fig. 1. Interpolated map showing Peshawar, Mardan, Malakand, Mansehra, and Abbottabad Districts of Khyber Pakhtunkhwa, Pakistan (generated by QGIS 3.32.1)

signs, were recorded using a structured questionnaire during sampling.

Viral nucleic acid extraction

Viral RNA was extracted from tracheal swabs and tissues using a commercial spin-column kit (Favorgen Viral RNA Extraction Kit; Cat. No. FATRK 001; Favorgen, Taiwan), following the manufacturer's instructions. Approximately 0.1 g of tissue was homogenized under sterile conditions, then centrifuged at 3,000 X g for 5 minutes at 4°C; the supernatant was used for extraction. Tracheal swabs were processed directly from the viral transport medium. Samples were lysed in buffer with β-mercaptoethanol and RNA was captured on silica columns with ethanol and then washed to remove impurities. The purified RNA was eluted in 50 µl RNase-free water and stored at -80°C for further molecular analysis.

S1 Gene amplification via two-step RT-PCR

Complementary DNA (cDNA) was synthesized from viral RNA extracted using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; Cat. No. K1622), following the manufacturer's protocol. Briefly, 5 µl of RNA was primed with random hexamers, denatured at 65°C for 5 minutes, and immediately cooled on ice. Reverse transcription was performed in a 20 µl reaction mixture containing 5x reaction buffer, RiboLock RNase inhibitor, 10 mM dNTPs, and RevertAid M-MuLV reverse transcriptase. The reaction was incubated at 25°C for 5 minutes, then at 42°C for 60 minutes, with enzyme inactivation at 72°C for 5 minutes. The resulting cDNA was stored at -80°C until further use. Subsequently, conventional PCR was conducted to amplify a partial segment of the IBV S1 gene using primers designed with NCBI Primer3-BLAST and validated for specificity by BLAST (Table 1). The 25 µl PCR mixture included 12.5 µl of DreamTaq Master Mix (Thermo Fisher, K1081), 1 µl of each primer (10 µM), 3 µl of cDNA template, and

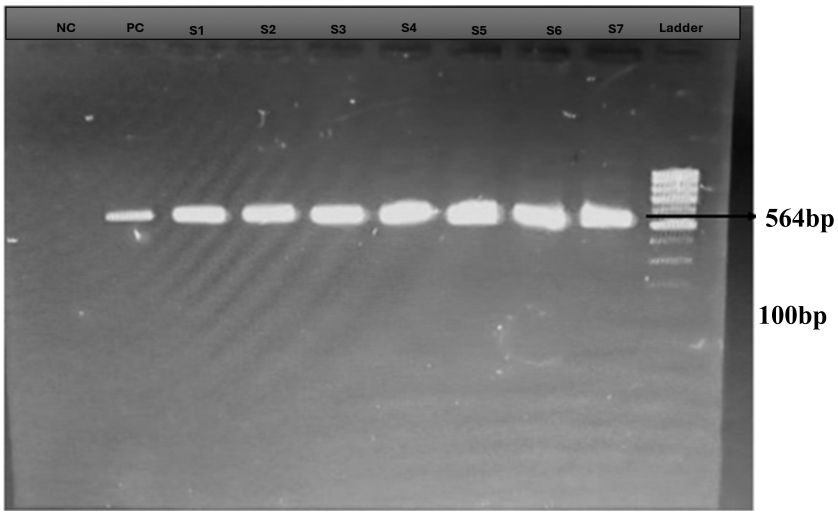

Fig. 2. Gel picture showing 100 bp DNA ladder and IBV positive samples having amplicon size of 564 bp (NC – Negative control, PC – Positive control, and positive samples (S1, S2, S3, S4, S5, S6, S7)

Table 1. Primer sets used for PCR analysis of infectious bronchitis virus (IBV).

Target Gene	Primer direction	Sequence 5′ to 3′	Product Size (bp)	Reference
IBV-S-1	Forward	CGGTCCTCTTCAAGGTGGTT	564	Primer designed using NCBI Primer BLAST
	Reverse	TCGTATAGACGCTAACGACGTG		

7.5 µl nuclease-free water. The thermal cycling protocol started with an initial denaturation at 95°C for 5 minutes. This was followed by 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 60 seconds. A final extension was carried out at 72°C for 5 minutes. This method describes a standard end-point two-step RT-PCR without probe-based detection or Ct value analysis. Amplified products were verified by agarose gel electrophoresis for expected size and quality before sequencing (Fig. 2).

Sequence and phylogenetic analysis

A total of thirteen IBV-positive amplicons, each showing a clear, single band of the expected size on an agarose gel, were selected for nucleotide sequencing to ensure representative coverage across study segments. The purified S1 gene PCR products underwent bidirectional Sanger sequencing through Excel Scientific Lahore Pvt. Ltd., Pakistan, via Macrogen Inc., Korea. Raw chromatograms were quality-checked and trimmed with BioEdit, and sequence identities confirmed by BLASTn against the NCBI database, with closely related reference sequences retrieved. Multiple sequence alignments were performed using ClustalW in MEGA 12, and phylogenetic relationships were inferred using the Maximum Likelihood method with the Tamura-3-parameter model (T92+G) incorporating gamma-distributed rate variation. The tree's robustness was assessed with 1,000 bootstrap replicates (Valastro et al. 2016). The resulting nucleotide sequences were deposited in GenBank under accession numbers PX229739-PX229745 and PV416838-PV416843.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics v21. IBV detection by RT-PCR (positive/negative) was categorized as a binary outcome. Binary logistic regression was used to assess the association between IBV prevalence and variables including geographic location (district/division), age group and production type (broiler vs. layer). Overall prevalence was reported as percentages with 95% confidence intervals (CI), and odds ratios (OR) with 95% CI were used to estimate the strength of associations. A p-value < 0.05 was considered statistically significant, in accordance with standard epidemiological practice (Shah et al. 2022).

Results

Overall prevalence and risk factors

Analysis of 425 samples identified notable differences in IBV positivity among districts $p \leq 0.04$. The highest prevalence was seen in Mansehra (39%), followed by Mardan (31.1%), Abbottabad (31.0%),

Table 2. Analysis of various risk factors for the prevalence of infectious bronchitis of poultry in the study area.

Variables	Categories	Total Samples (n=425)	Positive, n (%)	OR	95% CI (Lower bound-Upper bound)	p value
Districts	Abbottabad	100	31 (31%)	1.33	1.12-1.57	0.04
	Mansehra	100	39 (39%)			
	Mardan	90	28 (31.1%)			
	Peshawar	90	20 (22.2%)			
	Malakand	45	8 (7.7%)			
Months	Jan-March	73	25 (34.2%)	0.802	0.62-1.03	0.18
	April-June	172	44 (25.5%)			
	July-Sep	99	36 (36.3%)			
	Oct-Dec	81	21 (25.9%)			
Age	Young	290	102(35.2%)	2.89	1.61-5.17	0.001
	Adult	135	24 (17.7%)			
Bird type	Layer	205	69 (33.7%)	1.39	0.98-1.97	0.02
	Broiler	140	43 (30.7%)			
	Mix	80	14 (17.5%)			

OR – odd ratio, CI – confidence interval, significance at p-value <0.05

Table 3. Analysis of various risk factors associated with farm management practices and the prevalence of Infectious Bronchitis in poultry.

Variables	Categories	Total Samples	Positive	OR	95% CI (Lower bound-Upper bound)	p value
Ventilation	Poor	186	85 (45.6%)	5.78	3.41-9.8	0.001
	Good	239	41 (17.2%)			
Disinfection of the farm	Yes	208	69 (33.2%)	1.07	0.66-1.76	0.07
	No	217	57 (26.2%)			
Carcass management	Burial or Burning	188	63 (33.5%)	1.39	0.85-2.27	0.07
	Removal to a nearby area	237	63 (26.5%)			
Bird rearing practices	All-in all-out	213	57 (26.7%)	0.63	0.39-1.02	0.115
	Kept in different batches	212	69 (32.5%)			

OR – odd ratio, CI – confidence interval, significance at p-value <0.05

Peshawar (22.2%), with Malakand having the lowest at 7.7%. Geographic location was a significant predictor of IBV presence (OR=1.33; 95% CI: 1.12-1.57). Seasonal variation was observed but not statistically significant (p=0.18), with increased positivity during July to September (36.3%) and January to March (34.2%). Age was strongly associated with IBV detection (p≤0.001), with younger birds having a higher prevalence (35.2%) than adults (17.7%) and nearly triple the odds of infection (OR=2.89; 95% CI: 1.61-5.17). Bird type also affected prevalence (p≤0.02), with layers (33.7%) and broilers (30.7%) exhibiting higher rates than mixed flocks (17.5%) (Table 2).

Farm management factors

Ventilation was strongly linked to IBV positivity among management variables (p≤0.001). Farms with poor ventilation had a much higher prevalence (45.6%) than those with good ventilation (17.2%), and the odds of infection were significantly higher (OR=5.78; 95% CI: 3.41-9.80). In contrast, disinfection practices, carcass disposal methods and rearing systems were not significantly associated with IBV occurrence, although there was a slight increase in prevalence among farms that did not disinfect, disposed of carcasses nearby or kept multi-age flocks (Table 3).

Table 4, Sequences of IBV submission and Accession Number assigned by the NCBI GenBank.

Submission ID	Isolate Name	Accession Number
BankIt2996983	IBV-1/Poultry/Mansehra/PK/2025	PX229739
BankIt2996983	IBV-2/Poultry/Mansehra/PK/2025	PX229740
BankIt2996983	IBV-3/Poultry/Abbottabad/PK/2025	PX229741
BankIt2996983	IBV-4/Poultry/Abbottabad/PK/2025	PX229742
BankIt2996983	IBV-5/Poultry/Mardan/PK/2025	PX229743
BankIt2996983	IBV-6/Poultry/Mardan/PK/2025	PX229744
BankIt2996983	IBV-8/Poultry/Peshawar/PK/2025	PX229745
BankIt2940959	CK_FI_VRI/Pak/IBV-1	PV416838
BankIt2940959	CK_FI_VRI/Pak/IBV-11	PV416839
BankIt2940959	CK_FI_VRI/Pak/IBV-12	PV416840
BankIt2940959	CK_FI_VRI/Pak/IBV-13	PV416841
BankIt2940959	CK_FI_VRI/Pak/IBV-14	PV416842
BankIt2940959	CK_FI_VRI/Pak/IBV-15	PV416843

Phylogenetic analysis

Phylogenetic analysis based on partial S1 gene sequences was performed using the Maximum Likelihood method with the Tamura-3-parameter + Gamma (T92+G) model, incorporating 1,000 bootstrap replicates. All study isolates clustered within genotype I, lineage GI-1, forming a distinct subcluster closely related to classical Massachusetts vaccine strains (H120, M41, Beaudette), with strong bootstrap support (>90%). No isolates were found within lineages GI-13, GI-22, or GI-24. The Pakistani isolates showed close genetic relationships to previously reported local strains, indicating ongoing circulation of Mass-like viruses rather than the recent introduction of divergent genotypes. Sequences have been deposited in GenBank, with accession numbers provided (Table 4, Fig. 3).

Discussion

Infectious bronchitis virus (IBV) continues to pose a major constraint to global poultry production due to its high transmissibility, extensive genetic variability and frequent vaccine escape. The present study provides updated molecular epidemiological insights into IBV prevalence, phylogenetic characteristics and associated risk factors in major poultry-producing districts of Khyber Pakhtunkhwa (KP), Pakistan, thereby addressing an important regional data gap. The molecular prevalence detected in this investigation is consistent with recent reports from other Pakistani poultry production zones, including Punjab Province (Shahid et al. 2024), confirming sustained IBV circulation within the country. However, the prevalence observed here is lower than that reported in several earlier Pakistani studies (Muneer et al. 1999, Mustafa 2005, Ahmed et al. 2007, Kanwal et al. 2018, Rafique et al. 2018b), but higher than some localized surveys (Abbas et al. 2015). Such discrepancies likely reflect differences in diagnostic methodologies, sampling frameworks, vaccination coverage, and farm management systems. Earlier serology-based investigations may have overestimated exposure due to long-lasting antibodies, whereas the RT-PCR approach employed in this study more accurately reflects active viral infection and ongoing transmission (Meir et al. 2010, Bhuiyan et al. 2021). Molecular surveillance of IBV in Pakistan remains limited but indicates considerable genetic diversity. Early work documented the co-circulation of Massachusetts and several European variant strains (Ahmed et al. 2007), alongside evidence of inadequate protection conferred by widely used vaccines, including D-1466 (GII-1), 4/91 (GI-13), D-274 (GI-12), and Mass-41 (GI-1), suggesting the presence of uncharacterized field variants. Subsequent molecular investigations confirmed the predominance of GI-1 and GI-13 lineages, identified unique indigenous isolates such as Pak-973 and Pak-786, and demonstrated genetic divergence from vaccine strains (Rafique et al. 2018a, Rafique et al. 2018b). More recently, phylogenetic analyses have highlighted the emergence and increasing dominance of the GI-24 lineage, genetically distinct from the commonly used GI-23 vaccines, indicating ongoing viral evolution and potential vaccine escape in Pakistani IBV populations (Saleem et al. 2024).

In contrast to these national trends, phylogenetic analysis in the present study revealed that all sequenced isolates clustered exclusively within genotype I, lineage GI-1, which is closely related to classical Massachusetts vaccine strains (H120, M41, Beaudette) and to previously reported Pakistani Mass-like isolates. The limited sequenced isolates in this study indicate

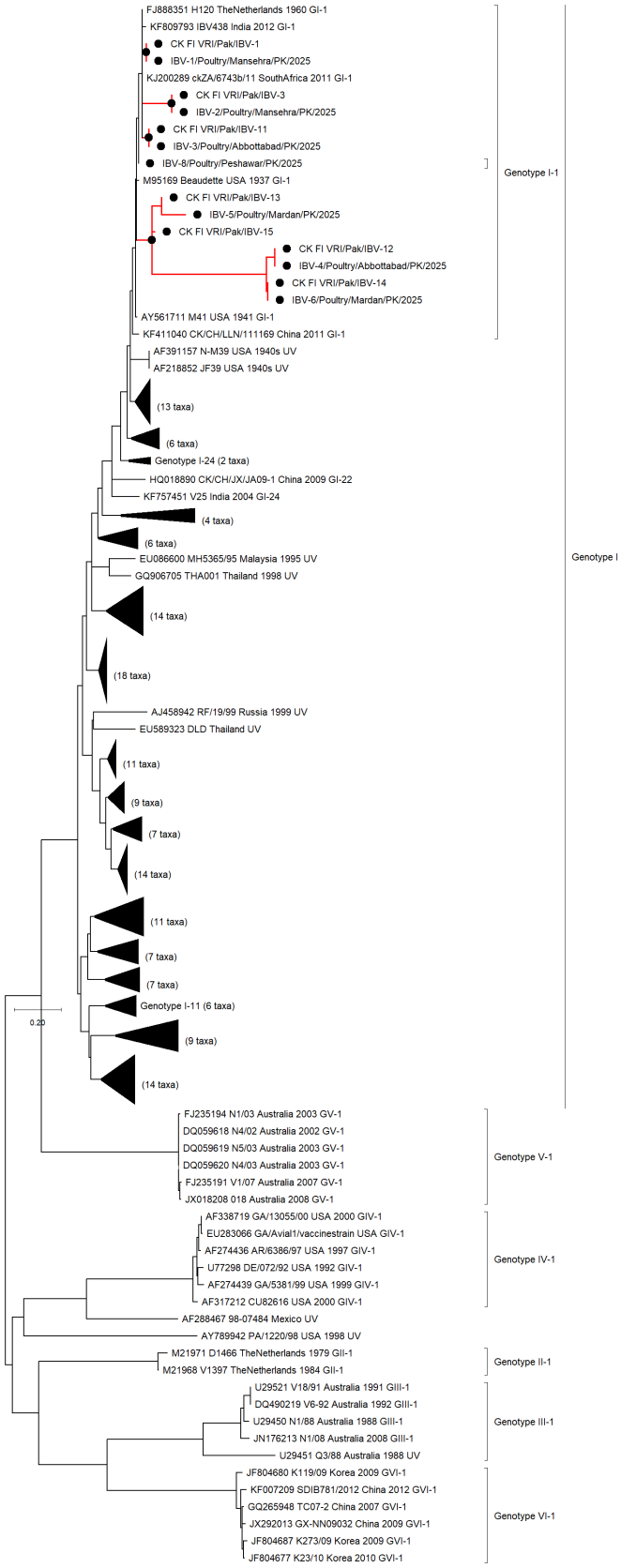

Fig. 3. A maximum likelihood phylogenetic tree was constructed from partial S1 glycoprotein gene sequences of IBV isolates from Pakistan (2025) and reference strains from GenBank. The tree was generated in MEGA12 using the Tamura-3 model with 1,000 bootstrap replicates. Bootstrap values over 70% are indicated at branch nodes. Pakistani field isolates identified in this study are marked with blue circles, previously reported Pakistani isolates with green triangles, and reference vaccine strains (H120, M41, Beaudette) with red squares. The scale bar represents the number of nucleotide substitutions per site.

low genetic diversity of IBV strains in KP during the study period, as the GI-13, GI-22 (QX-like), and GI-24 lineages were not observed. This finding contrasts with reports from other Pakistani provinces where multiple genotypes co-circulate, including emerging GI-24 variants (Saleem et al. 2024). The observed genetic uniformity may reflect regional vaccine pressure, limited viral introductions, or localized transmission dynamics; however, the relatively small sequencing dataset necessitates cautious interpretation and underscores the need for expanded genomic surveillance.

Spatial analysis demonstrated significant district-level variation in IBV prevalence, with higher positivity in Mansehra, Abbottabad and Mardan. Logistic regression confirmed geographic location as a significant predictor of infection, indicating the influence of local epidemiological drivers. Similar spatial heterogeneity has been linked to poultry density, farm clustering, and bird movement networks in other epidemiological investigations (Zeleke et al. 2005, Birhan et al. 2021). Districts characterized by intensive poultry production and frequent flock turnover may facilitate sustained viral circulation and inter-farm transmission. Seasonal variation in IBV detection was observed but was not statistically significant, suggesting year-round viral circulation. While some studies report increased IBV incidence during colder months (Shiferaw et al. 2022), the absence of a seasonal association in KP may reflect continuous bird placement, overlapping production cycles and inconsistent vaccination schedules, reinforcing the importance of continuous surveillance rather than season-targeted control strategies.

Host factors played a critical role in IBV epidemiology. Younger birds exhibited significantly higher infection rates and nearly threefold greater odds of positivity, supporting previous findings that immature immunity and incomplete vaccine protection increase susceptibility (Cavanagh and Naqi 2003, Mungadi et al. 2015, Birhan et al. 2021). Although some studies have reported higher infection rates in adult birds due to cumulative exposure (Jackwood 2012, Shiferaw et al. 2022), the present findings highlight the vulnerability of early production stages within the regional management context. Production type also influenced infection dynamics, with higher prevalence in layer flocks than broilers or mixed flocks. This pattern supports existing epidemiological findings that longer production cycles, extended farm residence, and repeated exposure to the virus elevate the risk of IBV in layers (Jackwood 2012, Shettima et al. 2016, Birhan et al. 2021). Among farm management variables, inadequate ventilation emerged as the most significant risk factor, with markedly elevated positivity and infection odds. Poor ventilation likely enhances aerosol persistence, viral load accumulation, and respiratory stress, thereby facilitating IBV transmission (Dhama et al. 2014). In contrast, disinfection methods, carcass disposal and rearing systems were not statistically significant predictors. This indicates that standalone biosecurity measures might be inadequate unless combined with environmental management and vaccination strategies. Similarly, Ardicli et al. (2025) emphasized that vaccination, effective disinfectants, and additional antiviral measures should work together within a comprehensive IBV control program. This underscores the importance of multifaceted approaches to disease prevention (Bhuiyan et al. 2021, Zhang et al. 2021, Ardicli et al. 2025).

Overall, this study confirms the continued circulation of a Massachusetts-type IBV lineage in Khyber Pakhtunkhwa and demonstrates that geographic location, host age, production type and ventilation quality significantly shape infection patterns. While the genetic homogeneity observed contrasts with reports of multi-lineage circulation and emerging GI-24 dominance elsewhere in Pakistan, ongoing molecular surveillance is essential to detect future genotype incursions. Strengthening farm infrastructure, particularly ventilation, and implementing genotype-matched vaccination programs remain critical for mitigating the impact of IBV on Pakistan's poultry industry.

Conclusion

This study shows that IBV strains circulating among poultry in northern Pakistan mainly belong to genotype I, lineage GI-1, which corresponds to the traditional Massachusetts-type lineage. Phylogenetic analysis revealed a close genetic relationship among the isolates, common vaccine strains such as H120 and M41, and earlier Pakistani IBV sequences, suggesting that a Mass-like lineage remains endemic in Khyber Pakhtunkhwa. The absence of other genotypes in the data indicates a relatively uniform viral population in the region at the time of sampling. These findings suggest that the virus persists locally with little recent introduction of significantly different IBV lineages. However, the limited number of sequenced samples is a drawback, and the presence of undetected or novel variants cannot be ruled out. Therefore, continuous molecular surveillance with expanded sequencing efforts, along with regular assessment of genotype and vaccine compatibility, is crucial for improving IBV control and early detection of emerging strains.

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Author Declarations

Ethics approval

The experiments were approved by the Ethical Committee of the Faculty of Animal Husbandry and Veterinary Sciences at the University of Agriculture, Peshawar, vide letter No. 703/PS/UAP dated 21/3/2024.

Use of generative artificial intelligence

No AI generative tools were used in manuscript preparation.

Conflict of interest

The authors declare no competing interests.

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