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The S1 gene study of avian infectious bronchitis virus (IBV) isolated from local disease outbreaks

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Contribution All authors made equal contributions to the conception and design of the project, data analysis and preparation of the manuscript.

ABSTRACT

Infectious bronchitis (IB) is an acute and highly contagious respiratory, renal, and reproductive organ infection of chickens. Infectious bronchitis virus (IBV) that replicates in epithelial cells of the trachea resulting in respiratory signs (sneezing, cough, tracheal rales, gasping and nasal discharge. This study was carried out to monitor Infectious bronchitis virus (IBV) infection in local disease outbreaks in Khyber Pakhtunkhwa (KP), Pakistan. A total of 57 chickens (broilers & layers) were examined for the presence of IBV. Tissue samples were screened through reverse transcriptase PCR (RT-PCR) to detect IBV S- glycoprotein gene (surface glycoprotein gene) and to amplify the hypervariable region of the S1-glycoprotein gene. Ten samples were found to be positive for the infectious bronchitis virus (IBV) after the initial PCR screening from the original 57 samples. An expected approximately 298bp band was noticed in detection of RT-PCRs. The samples found to be positive by the detection of PCR were further processed for the genotyping RT-PCR of the S1 gene. An approximately 700 bp band was seen in all the 10 cases. The bands were gel-purified and then sent for DNA sequencing. HKY model for Neighbour joining with 1000 bootstrap replicates in Program Geneious (10.2.3 version) was used to construct a phylogenetic tree. Next, we performed amino acid sequence alignment of these positive samples with the amino acid sequences of the previously reported two sequences of the S-1 gene of M41(Massachusetts 41) strain in Pakistan which were used in Phylogenetic analysis. This revealed that 4 sample amino acid sequences were closely related (93-100%) with Pakistan reported IBV sequences (KY588135 and KU145467). Our current study of IBV isolates revealed an identity of 60% with the isolates reported from China, 30% identity with the isolates reported in Pakistan and 10% identity with the isolates reported from India.

Keywords: Infectious bronchitis, hypervariable region, reverse transcriptase-polymerase chain reaction, 1000 bootstrap replicates, Khyber Pakhtunkhwa (KP), Pakistan.

INTRODUCTION: Infectious bronchitis (IB) is an acute and highly contagious respiratory, renal, and reproductive organ infection of chickens. IB disease is caused by Infectious bronchitis virus (IBV) that replicates in epithelial cells of trachea resulting in respiratory signs (sneezing, cough, tracheal rales, gasping and nasal discharge (Dewit *et al.*, 2019). It is a member of Coronaviridae, order Nidovirales that causes acute highly contagious respiratory, renal and reproductive infections in domestic and commercial poultry (Santos *et al.*, 2017). It is the most prominent cause of economic loss within the poultry industry, affecting the production of both meat type and egg-laying birds. IBV financial loss includes decreased egg quality and quantity in layers, reduced growth and poor body weight and high mortality rate in broiler chickens (Villanueva-Pérez *et al.*, 2024). It is generally accepted that chickens are the most important natural hosts of IBV; chickens of all ages can be affected. IBV has also been isolated from other species such as pheasants, quail and partridge (Jackwood and Jordan, 2021). IBV genome contains single stranded positive sense RNA, its length is about 27.6 Kb and its 5' end is covered with cap while '3 end is covered with poly "A" tail. There are 10 open reading frames (ORFs) in the IBV genome while the first 20 Kb genomes contain only ORF1. ORF1 is a replicas gene, the replicas gene produces a single poly-protein 1a (pp1a). During the translation process, ribosomal frame shifting is occurs at the junction of ORF1a and ORF1b as a result of which large protein 1b is produced. Basically, there is a jumping sequence UUUAAAC at the junction of 1a and 1b which causes frame shifting. Now these two large polyproteins are cleaved by a Cis-acting viral enzyme, auto-processing of 1a and 1b produces 16 non-structural proteins (NSPs) (nsp1-nsp 16) including the RNA dependent RNA polymerase enzyme also. In case of IBV, there are 15 non-structural proteins (nsp2-nsp16). IBV genome is encoded for four major structural proteins; spike (S) glycoprotein, envelope (E) protein, the membrane (M) glycoprotein and the nucleocapsid (N) protein. Beside these proteins there is another non-structural protein gene present among structural genes (Salarpour *et al.*, 2019). These non-structural proteins 3a, 3b, 3c, 5a and 5b are playing an important role in pathogenesis. Deep analysis of the structural protein region of the genome, the S gene protein is post-translationally cleaved into S1 and S2 sub-units comprising approximately 500 and 600 amino acids. In mature virion S1 protein is anchored with S2 protein in membrane to form a multimeric coiled-coil S protein. N gene is encoded for nucleocapsid protein which forms a nucleocapsid of the virus and interestingly N gene is highly conserved and show only 2-6% differences at the amino acid level (Lopes *et al.*, 2018). There is an untranslated region (UTR) present downstream of the N gene. This is an important region for the initiation of the replication process and this region is highly conserved among different strains of IBV (Majdani, 2024).

Many antigenic types of the virus has been existed, for example Massachusetts, D274, 793/B, and B1648 serotypes. In 1940s Mass type serotype was isolated in Europe; D274 was isolated in Western European countries in the 1980s. 793/B was first time isolated in UK (Carranza *et al.*, 2017). It becomes clear that many countries deal with many types of IBV and the most common way of classification is genotyping while there is another way through which we can find the function of the virus. In non-functional tests, we can find genotypes of viruses while in functional tests we can find biological function of the virus. Test that looks at genome results of viruses in genotypes. In protecto-typing the complete immune response of a bird against virus studied (Dewit *et al.*, 2019). IBV strains of Japan are classified into six genotypes, JP-1, JP-2, JP3, Mass, grey, 4/91 based on differences in hypervariable regions. Continuous molecular epidemiology of the IBV strains will help us in finding new strains (Dasilva *et al.*, 2021). Strains of IBV were isolated in India, their S1 protein sequencing results were like the S1 protein sequencing results of D1466, Mex/1765/99, DE/072/92 but shared 68% relatedness with strain 6/82 (Wibowo *et al.*, 2019). In 2015, sequence alignment of H120, Ma5 and Mass41 reference strains with Anand strain exhibited 97.7% identity. When they aligned the vaccine, the virus deduced amino acids with the Anand strain then there were no variations. Continuous deletion and insertion bring mutation in the S1 gene and nowadays it is reported that the same mutations are occurred in M gene also (Patel *et al.*, 2015).

Very few mutations occur in N gene sequences; therefore N gene sequences are also used for the detection of IBV in all types of strains. The smaller degree of variations in conserved regions indicates that the variations are produced due to immunological pressure (Lounas *et al.*, 2018). Avian infectious bronchitis virus has bad economic impact on chicken disease in Pakistan, despite the use of live attenuated and inactivated IB virus vaccines. It may cause high mortality in chickens, laying a soft shell, misshaped and uneven-sized eggs. IBV infection in layer and breeder flocks causes significant economic defeats to the farmers. These over throws in

the form of poor egg quality and low egg production while in case of breeder, flocks are lower hatchability and some of the newly hatched baby chicks die immediately after hatching (Saba *et al.*, 2018). About 88% of the flocks were seropositive to M-41 antibodies in Pakistan. The high prevalence of IBV in local chickens shows that it is field virus (Hadipour *et al.*, 2011).

OBJECTIVES: The objective of the study was to develop a reverse-transcriptase PCR (RT-PCR) for the detection of (IBV) from recent outbreak in various regions of Khyber Pakhtunkhwa (KP), Pakistan. After detection, the hypervariable region was amplified by RT-PCR for sequencing and phylogenetic analysis.

MATERIALS AND METHODS: The study was conducted at Poultry Research Institute (PRI) Rawalpindi. The research was carried out from September 2016 to July 2017 outbreaks in Khyber-Pakhtunkhwa KP. Tissue samples were collected from outbreaks in various regions of the KP based on clinical signs and post-mortem analysis of the birds (broilers and layers) that were suspected to have (IBV) infection. These birds were brought to PRI for post-mortem examination from various commercial poultry farms in KP province. Tissue samples: A total of 57 tissue samples were assembled from various investigated outbreaks as described in table 1 in Khyber Pakhtunkhwa Pakistan, where samples were gathered.

Serial No	Location	No of sample 2016-2017
1	Kohat	8
2	Abbottabad	9
3	Peshawar	7
4	Mardan	7
5	Karak	4
6	Noshera	7
7	Dera Ismail Khan	7
8	Bannu	8
Total		57

Table 1: Location and years of samples used in this study.

Tissue samples were collected from lungs, spleen, caecal tonsils, kidneys and trachea after post-mortem and then all these tissue samples were labelled properly with date, amount, and location of the outbreak. After collection, the tissues were stored at -80°C in PRI Rawalpindi.

Sample processing: About 100mg tissue was obtained from each sample with lungs, caecal, tonsils, trachea, kidney and spleen parts of broilers and layers from different KP poultry forms. RNase-free mortars and pestles were used in combination with liquid nitrogen to disrupt and lyse frozen and fibrous tissue samples. For the extraction of RNA, we used the RNA extraction kit (Pure Link RNA Mini Kit, Cat.No.12183026, and USA). The cDNA kit (Revert Aid First Strand cDNA Synthesis Kit. Lot: 00453373, California) was used for cDNA synthesis. Infectious bronchitis virus primers (IBV-UTR 1: 5' GCTCTAACTCTATACTAGCCTAT 3' and IBV-UTR 2: 5' AAGGAAGATA GGCATGTAGCTT 3') were detected from previous research paper of (Adzhar *et al.*, 1996). About 35 cycles of PCR were performed. Cycling conditions were as follows: an initial denaturation step of 1 min at 94 °C, followed by 35 cycles of 56 °C for 1 min as annealing step which is followed by extension step for 72 °C for 2 min. of the PCR product. To carry out genotyping of IBV we designed our own primer based on modification of previous old genotype primers that were given in OIE manual of IBV. We aligned the old primers through muscle sequence alignment programme with reference sequences (accession numbers, KR902510, KY588135, KT203557, KJ128295, KM58618, Kf853202, JX840411, JF732903, EU637854, KT946798 and KU145467) which were taken from NCBI and then we made modification in old genotype primers (IBV-MOD-S15: 5'KRAARAMWGAACAAAAGAS'3 IBV-MOD-CK2: 5'CNGTRTTRT AYT GRCA '3) without changing their positions as shown in table 3. Following cycling parameters were used: initial denaturation at 95°C for 2 min, then 95°C for 30 sec. This was followed by 45 cycles of 52°C for 30 sec as annealing and an extension temperature of 68°C for 30 sec and final extension at 68°C for 12 min was done.

RESULTS: Ten samples were found to be positive for the infectious bronchitis virus (IBV) after the initial PCR screening from the original 57 samples. An expected 298bp band was noticed in detection RT-PCRs. The samples found to be positive by the detection PCR were further processed for the genotyping RT-PCR of S1 gene. An approximately 700bp band was seen in all the 10 cases. The bands were gel-purified and then sent for DNA sequencing. Results for detection PCR are shown in (figure 1).

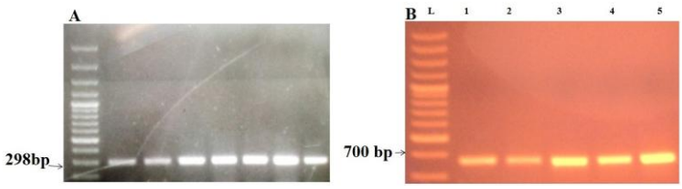

Figure 1: (A) Results of RT-PCR for detection of IBV. L: DNA size marker (100 bp DNA ladder, Thermofisher 128 Scientific). Lanes 1-7: IBV detection RT-PCR amplicons of 298 bp. (B) Results of RT-PCR for the amplification of partial S1 gene of IBV. Lane 1-5: IBV genotypic detection 132 RT-PCR amplicons of 700bp.

Sequencing: Ten out of the total samples were sent to Macrogen (Korea) for sequencing. Table 2 describes the samples from different locations of KPK which were positive. The DNA sequences were analysed by BLAST and verified as those of the partial region of the S1 gene of IBV.

Serial No	Location	No of sample	+ive samples	Labelled sequences
1	Kohat	8	3	IBV-KPK-1, IBV-KPK-2, IBV-KPK-3
2	Abbottabad	9	2	IBV-KPK-4, IBV-KPK-5
3	Peshawar	7	3	IBV-KPK-6, IBV-KPK-7, IBV-KPK-8
4	Mardan	7	1	IBV-KPK-9
5	Karak	4	_	
6	Noshera	7	_	
7	Dera Ismail Khan	7	1	IBV-KPK-10
8	Bannu	8	_	
Total		57	10	

Table 2: The positive samples by sequencing.

Sample sequences were labelled with IBV-KPK-1 to IBV-KPK-10. Geneious program (version 10.2.3) was used for the translation, % identity analysis and alignment of nucleotide sequences of samples. Multiple alignments of all ten positive samples along amino acid sequences to find out similarities and differences among all of them analysed. All 10 positive IBV samples amino acid sequences were 53.55 % similar to each other while in numbers, 128 amino acids were similar out of 239 amino acids. Besides this we did pair-wise alignment of each sample amino acid sequence with previously reported Pakistani IBV partial S1 gene sequences (accession numbers KY588135 and KU145467). IBV-KPK-1 amino acid sequences were aligned with KY588135 amino acid sequences, in 62 amino acids out of 237changes were reported at different places in an alignment; both sequences were 73.84 % identical to each other. While pair-wise alignment of IBV-KPK-1 was performed with KU145467, total of 70 amino acids out of 237 were different according to the percentage distance graph both sequences were 70.46 % identical to one another. IBV-KPK-2 amino acid alignment was done with KY588135 amino acid sequence, 60 amino acid variations were found at different places in alignment with 74.79 % identity. Pair wise alignment of the same sample amino acid sequence with KU145467 amino acid sequence has shown 73.52 % identity with 63 amino acid differences. IBV-KPK-3 amino acid sequences were pair-wise aligned along KY588135 it is noticed that 80 amino acids were different between two sequences while percentage identity was 66.25% and the other percentage identity was 66.81 % alignment of sample sequences of KU145467. Percentage identities of IBV-KPK-4 alignment amino acid sequences were quite stronger as KY588135 with 74.37 % than KU145467 sequence with 73.10 %. Alignment results described 61 amino acid differences in case of alignment of sample sequence with KY588135, while 64 amino acid differences in case of sample sequences alignment with KU145467 sequences. Amino acid sequences alignment of IBV-KPK-5 demonstrated highly significant result with KU145467 as a 99.16 % identity was observed with reported sequence. The same sample sequences' alignment with KY588135 was not significant because several amino acid changes were detected in sample sequences; the identity percentage value was quite less than KU145467's sequence's alignment value due to 70 amino acids alteration in alignment; the along identity percentage value was 70.46 %. Pair wise alignment of IBV-KPK-6 amino acid sequences with KU145467, 63 amino acid differences were observed with 73.53 % identity, another trial sample amino acid sequence of this samples aligned with KY588135 similarity index was74.37 %. The contrasts of 68 amino acid sequences out of 237 were observed in the alignment result of IBV-KPK-7 and KU145467

while their percentage identity was 70.89 %. IBV-KPK-7 amino acid sequences aligned along KY588135 had a 93.19 % identity while the differences in 16 amino acids were shown in alignment. Pair wise alignment of amino acid sequences of IBV-KPK-8 and KY588135 was 99.57 % identical with 1 amino acid change. Alignment result of IBV-KPK-8 was not significant with KU145467, 66 amino acid changes out of 237 and percentage identity with 71.73 %. Another positive sample, 71.84 % identity was found in IBV-KPK-9 amino acid sequences with KU145467 while in the case of KY588135 amino acid sequences alignments were 74.26 % identities. In the sample IBV-KPK-10 amino acid sequences were 100% identical to KY588135 but 68 amino acid differences were found in the alignment result of IBV-KPK-10 and KU145467, percentage identity was 71.30 % noticed.

Sample sequences nucleotide alignment was performed through Generous (version 10.2.3). According to the distance percentage, samples, IBV-KPK-1, IBV-KPK-2, IBV-KPK-3 and IBV-KPK-4 nucleotide sequences had shown approximately 80% similarities. On the other hand, nucleotide sequences of IBV-KPK-5, IBV-KPK-6, IBV-KPK-7, IBV-KPK-8, IBV-KPK-9 and IBV-KPK-10 revealed approximately a 73-75% similarity index. Pakistan reported IBV nucleotide sequences that were aligned with all our positive samples to view their alignment as well as percentage distance. IBV-KPK-1, IBV-KPK-2, IBV-KPK-3 and IBV-KPK-4 were approximately 68-75% similar with KU145467 nucleotide sequence while IBV-KPK-5 was 99.71 % identical; IBV-KPK-6, IBV-KPK-7, IBV-KPK-8, IBV-KPK-9 and IBV-KPK-10 showed 73-76% similarities with KU145467. Samples sequences were also aligned with KY588135 nucleotide sequence and then concluded that IBV-KPK-7; IBV-KPK-8, IBV-KPK-9 and IBV-KPK-10 were taking similarities of 95.99 %, 99.09 %, 76.75 %, and 99.35 %.

Phylogenetic analysis: All positive sample sequences were used in the construction of the phylogenetic tree. For construction of the phylogenetic tree, 43 previously published sequences from China, India, the USA, Iran, Pakistan, Jordan, Taiwan and Thailand were utilized to conduct phylogenetic analysis. Previously published sequences were downloaded from NCBI's website (www.ncbi.nlm.nih.gov). HKY model for Neighbour joining with 1000 bootstrap replicates in Program Geneious (10.2.3 version) was used to construct phylogenetic tree. Phylogenetic tree demonstrated IBV-KPK-1 in close relation with sequences from China (EU637854, EF213568). IBV-KPK-3 and IBV-KPK-4 represented similarities with Chinese sequences. IBV-KPK-5 was totally different and showed significant similarity with Pakistan reported IBV sequence (KU145467). The similarity index of IBV-KPK-6 was determined with China reported sequence (KM586818) while other China sequences were relative to IBV-KPK-6. Phylogeny tree also demonstrated that IBV-KPK-7 was closely related to KR902510 of India. IBV-KPK-8, IBV-KPK-9 and IBV-KPK-10 were compelling alike with China, India and Pakistan reported sequences. Our positive sample sequences exhibited no direct relation with USA, Jordan, Brazil, Pakistan, Thailand and Taiwan while some of our sample sequences were in compelling resemblance with majority of china and India reported sequences. Similarities and dissimilarities among reference sequences of sample sequences were shown in figure 2.

DISCUSSION: Infectious bronchitis virus (IBV) is, by definition, the coronavirus, the causative agent of avian infectious bronchitis, which is characterized by respiratory, reproductive, and renal signs worldwide. IBV is a highly variable virus with a large number of genotypes and 20 serotypes of IBV have been documented worldwide (Brown *et al.*, 2016). S1 gene sequencing has been used for molecular epidemiological studies and genotypic characterization of IBV. To better understand the molecular epidemiology of IBV, we sequenced the S1 gene of IBV field isolates; a total of 10 trachea, lungs, caecal tonsils and kidney tissue specimens from different commercial broiler and layers of Pakistan were collected from KPK areas. Studies also suggests that IBV infection has undesirable effects on morphology and hatchability of embryos (Benazir *et al.*, 2018). Because of rapid recombination, insertions, deletions, point mutation events new strains predominantly in the S1 (the spike protein gene) gene result in the generation of Massachusetts-like and Arkansas-like IBV strains. There are three HVRs located in the S1 gene where continuous mutations occur. There are few conserved regions in the S1 gene for which primers are designed to sequence the HVRs. HVR regions are as follows: amino acids 38-67 (HVR1), 91-141 (HVR2) and 274-387 (HVR3) are HVR (Zanaty *et al.*, 2016). For control and eradication of infection it is necessary to understand the genetic structure of prevalent strains (Xu *et al.*, 2018).

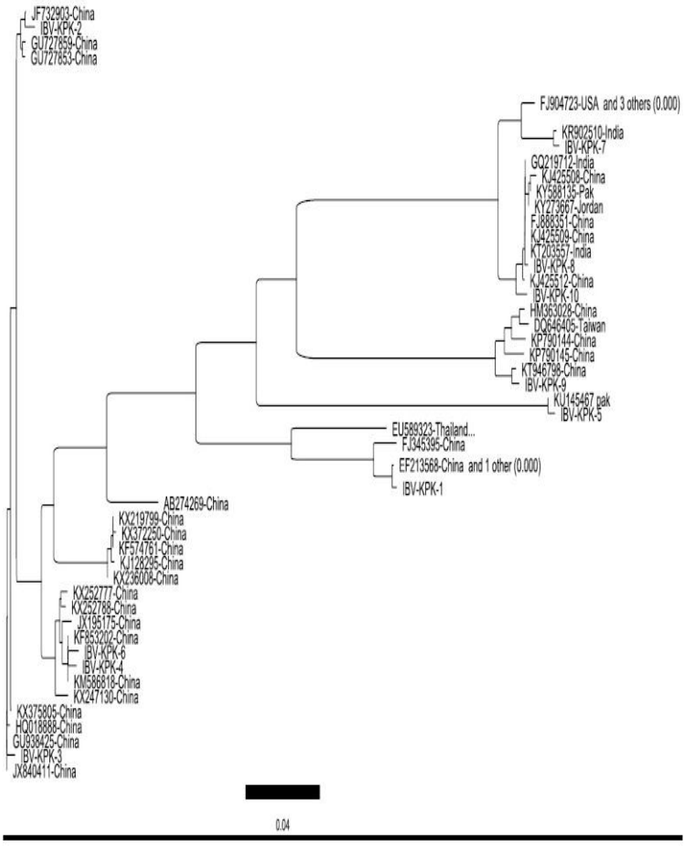

Figure 2: Neighbour joining-Geneious-HKY-Model.

The objectives of this study were to investigate that the phylogenetic analysis of our IBV-positive samples was based on HVRs of the S1 gene. In phylogenetic tree construction we have taken 43 reported IBV sequences HVRs from Gen Bank by comparing the selected regions of nucleotide sequences of the PCR product amplified from the S1 gene. Besides this we have aligned IBV-positive sample nucleotide and amino acid sequences with previously reported two Pakistani S1 gene IBV sequences with accession numbers (KU145467 and KY588135). Muneer et al., 2000 conducted IBV, HI antibody titre tests in commercial broilers and layers to conduct antibodies titter in serum against various strains of IBV like Mass-41, JMK, D-274 and D-1466 but they did not conduct molecular study of IBV and their study was limited to serology so we analysed our positive sample sequences as IBV-KPK-1 was closely related to CK/CH/LSD/05I strain, IBV-KPK-2 related with Sczy3 on the other hand IBV-KPK-3, IBV-KPK-4 and IBV-KPK-6 belong to the QX-IBV strain reported in China (EF213568). While IBV-KPK-5 was closed similar to the previously reported Pakistan IBV sequence (KU145467) and IBV-KPK-8 and IBV-KPK-10 were comparatively similar to the Pakistan reported IBV Mass 41 genotype sequence (KY588135) and China reported IBV sequences (Muneer *et al.*, 2000). IBV-KPK-7 was shown closed similarity with the India reported IBV Mass41 genotype sequence (KR902510). Molecular study was based on detection of M-41 strain of IBV while HVRs for genotyping and phylogenetic analysis were not recorded in their study (Khera[1] *et al.*, 2022). Hence concluded that IBV-KPK-7, IBV-KPK-8 and IBV-KPK-10 samples amino acid sequences were 93-100% similar to the KY588135 amino acid sequence while in case of amino acid alignment of sample sequences with the KU145467 sequence, only IBV-KPK-5 was shown to be 99.16% similar.

Neither phylogenetic analysis was recorded in NCBI data while submission was directed. In previous study, mass type of IBV isolates in Pakistan and submitted IBV S gene sequence (KY588135) in Gene bank of NCBI (Umar *et al.*, 2017). We used two sequences (KY588135 and KU145467) for alignment purposes with our own sequences. However, this revealed that our 4 samples were closely related (68-90%) to Pakistan reported IBV sequences. This condition shows that there is a lot of point mutation in our sample sequences while our 4 sequences give you an idea about minimal mutation and therefore have a high similarity index. These findings emphasize the need for new control strategies of IBV in Pakistan. Consequently, for phylogenetic analysis isolates of IBV were more

similar to China reported sequences than Pakistan and India. Phylogenetic analysis and sequences alignment results proved that S1 gene HVRs are highly variable Samples sequences are highly variable from each other when they are aligned; similarity percentage was 53.55% in case of amino acid alignment. Sample sequences are away from each other in a phylogenetic tree. Diversity in sample sequences showed huge mutation property of virus due to its RNA dependent RNA polymerase enzyme which has lack proof reading activity (Mase *et al.*, 2021). Their study indicated that N-glycosylation of all positive samples was done.

CONCLUSION: It is concluded from our study that sequence analysis of the IBV isolation of Pakistan was identical to the isolates of India, China, and Pakistan which were detected through Reverse-transcriptase PCR (RT-PCR). Moreover, results of sequencing revealed that the hypervariable region of the S1 gene was highly diverse in our IBV strains. Phylogenetic analysis of most of the native isolates was mostly different from each other. IBV isolates investigated in the current study revealed an identity of 60% with the isolates reported from China, 30% identity with the isolates reported in Pakistan, and 10% identity with the isolates reported from India.

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