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RESEARCH ARTICLE

Isolation and Molecular Characterization of Infectious Bronchitis Virus (IBV) Variants Circulating in Commercial Poultry in Pakistan

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ABSTRACT

Avian infectious bronchitis is a contagious viral disease of chicken. It is present everywhere in the world where poultry is reared. This study was designed to detect, isolate and sequence the circulating Infectious bronchitis viruses (IBVs) in poultry in Pakistan. Out of 905 clinical specimens tested, 358 were found positive for IBV, having serotype distribution of Mass (43%), 4/91 (51%), and different variants of IBV (5%), respectively. One of the variants recovered from broiler-breeder (BB) flock and designated as Pak-973, was further propagated and characterized on molecular basis. Virus neutralization test did not neutralize it with the reference antisera IBV serotypes M41, 4/91, D274, D1466 and IT-02, identifying it as a distinct serotype. Furthermore, Haemagglutination Inhibition test (HI) has proven useful in differentiating various serotype of IBV. In addition to this, partial Spike 1 (S1) gene was amplified by reverse transcriptase-PCR (RT-PCR) and subjected to sequencing for determining its genetic diversity. Sequenced data was submitted in the GenBank and the IBV isolate named as KX013102_NARC/973_Pakistan_2015. The phylogenetic analysis of Pak-973 showed a maximum homology of 93% with the Indian IBV variant KF360983_23/B/2008_India. However, an overall nucleotide homology with some other Indian isolates is between 82-93%. This study revealed the presence of IBV variant isolates in the field in Pakistan during 2015.

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INTRODUCTION

Infectious bronchitis (IB) is a contagious disease, it is caused by infectious bronchitis virus (IBV), belongs to genus Gamma-coronavirus and family Coronaviridae. IBV is basically an enveloped positive sense RNA virus causing IB disease (Valastro *et al*., 2016), a highly infectious upper-respiratory tract disease in chickens. Epithelial cells in the respiratory tract are the primary target of IB virus, thus IB is characterized by respiratory signs including coughing, sneezing, tracheal rales and gasping. Currently, IBV exists ubiquitously and different serotypes and emerging variants have been established globally. New virus classification is based on rapid recombination, deletion, insertion or point mutation events (Simmonds, 2015).

IBV viral genome is about 27.6 kb and contains 5' and 3' un-translated regions (Abro *et al*., 2012a), and has three major virus-encoded structural proteins, one of them is spike (S) glycoprotein that is highly variable, it is translated as a precursor protein that is later cleaved into the N-terminal S1 and C-terminal S2 glycopolypeptides (Farsang *et al*., 2002). Furthermore, the S1 part of the spike glycoprotein contains serotype specific virus neutralizing epitopes and is responsible for the hemagglutinating activity and for infectivity (Abro *et al*., 2012b). In addition to this, due to this variability in nucleotide sequences, the cross protection between serotypes is low. Moreover, changes as little as 5% in the S1 sub-unit have been able to alter the protection ability of a vaccine (Casais *et al*., 2003). The spike glycoprotein of IBV is involved in induction of protective immunity, neutralization and attachment to the host cell (Johnson *et al*., 2003).

The emergence of new variants and serotypes of IBV along with outbreaks of IB, which are often found to be a distinct serotype from the vaccine type, are not uncommon. Testing should be requiring in countries importing poultry genetic material before accepting such birds because IB is a notifiable disease (OIE, 2018).

There must be an effective surveillance system that is based on the characterization of the virus type causing disease (Jackwood and de Wit, 2013). Around 50 different IBV genotypes have been recognized some with restricted geographical distribution (Jackwood, 2012) while others worldwide distribution.

Pakistan has huge presence of commercial poultry to meet the animal protein need of its 190 million populations. However, apart from many other infectious diseases, IBV infection brings major economic losses while raising commercial flocks. Although IBV is prevalent all over the world including Pakistan (Ahmed *et al*., 2007), less work is reported in this country regarding the characterization of various IBV serotypes and/or its variants. However, some of the studies have earlier reported the circulation of classical IBV strains of Massachusetts type, and some of the European variants (Ahmed *et al*., 2007). On the other hand, repeated failure of multiple IBV vaccinations including Mass-41, 4/91, D-274 and D-1466 strains locally, indicates the presence of some of the un-identified IBV variants in the field. So, to better understand the failure of current IBV vaccination programs it would be important to monitor and characterize the circulating IBV isolates from the field. The present work focuses on the isolation and molecular characterization of one of the field isolates of IBV recovered from commercial poultry showing the condition of Infectious bronchitis.

MATERIALS AND METHODS

Experimental design: During the surveillance carried out for Infectious Bronchitis virus infections among commercial poultry, clinical specimens (Organ samples, cloacal and tracheal swabs) were received at the National Reference Lab for Poultry Diseases (NRLPD) located in Islamabad. The samples were processed and subjected to virological and molecular evaluation accordingly. The nested RT-PCR positive samples were further grouped into M-41, 4/91 and D-274 like IBV serotypes. These isolates were subjected to biological characterization through in-ovo inoculation. One of the isolates producing distinct lesions of dwarfism, curling, and haemorrhages was selected for further characterization. This isolate was first typed by virus neutralization (VN) and Haemagglutination Inhibition (HI) tests followed by its processing for spike gene sequencing.

Viral extraction and reverse transcriptase-PCR (RT-PCR): Viral RNA was extracted from the suspected tissues and swabs, extraction was done using QIAGEN kit (QIAGEN, Valencia, California, USA) following the manufacturers protocol. A nested RT-PCR was used for IBV serotype M41, 4/91 and D-274 identification using Superscript^{Tm} One step RT-PCR kit on Veriti thermal cycler (ABI, USA) according to the earlier designed primers in Table 1 (Roussan *et al.*, 2008; Poorbaghi *et al.*, 2012).

In-ovo virus propagation and identification: The suspected specimens including swabs and tissues were inoculated into 9-13 days old specific pathogen free (SPF) eggs (Senne, 2008; Jackwood and Wit, 2013; OIE, 2017). Briefly, virus is propagated by inoculating via the allantoic cavity in 10-day-old SPF embryonated eggs for more than four passages after homogenized the tissue pool of kidneys and trachea. For IBV related lesions, the embryos after 24 hours up to 7 days post-inoculation were scored. The allantoic fluid from these eggs was further tested by virus neutralization assay to determine IBV serotype.

Virus cross-neutralization: To confirm the identity of the recovered IBV-isolate, monospecific antisera against the IBV serotypes, M41, 4/91, D274, D1466 were provided by GD Animal Health Lab, Netherland. Briefly to run the test for determining IBV presence, the recovered IBV isolate reacted with reference antisera at a dilution of 1:20 and incubated for 30-60 min at 4ºC. The mixture was inoculated into the allantoic cavity of each of four embryonated eggs and incubated for 7 days. Upon completion of incubation the eggs were examined for IBV related lesions on the embryo. The living embryo without any lesion indicated complete neutralization of the virus with known antisera, thereby recognizing the serotype of the recovered IBVs (Jackwood and Wit, 2013; OIE, 2017).

Table 1: Oligonucleotides sequence and position used for RT-PCRs

Primer	Sequence	Position	Gene
XCE2-	CCTCTATAAACACCCTTGCA	1170 to 1193	S
XCE2+	CACTGGTAATTTTTCAGATGG	729 to 749	S
XCE3-	CAGATTGCTTACAACCACC	1093 to 1111	S
BCE1+	AGTAGTTTTGTGTATAAACCA	958 to 978	S
DCE1+	ATACAATTATATCAAACCAGC	895 to 915	S
MCE1+	AATACTACTTTTACGTTACAC	817 to 837	S

Hyper immune serum preparation: Antiserum against the new IBV isolate was prepared using the protocol of (OIE, 2013). For this purpose, 10 ml of the virus was inactivated using 0.2% formaldehyde for one week at 10ºC. The material was blended in Montanide 70 emulsion according to the procedure recommended by the manufacturer (SEPPEC-France). A group of 2-week chickens was given this vaccine @ 0.5 ml per bird, subcutaneously. The birds were bled 3-weeks later and the sera were collected accordingly.

Haemagglutination inhibition test for serological identification: To conduct Haemagglutination Inhibition (HI) test, 96-well U-shaped plates were used by following the recommended procedure (Villegas, 2008; OIE, 2017). For running HI, the test antigen of IBV was treated with Phospholipase C following the protocol of Ahmed *et al*. (2007). For running the test, 25µl of phosphate buffer saline (PBS) pH 7.2 was dispensed into all wells, 25µl of test sera was added into first well of row-A. It was serially diluted after mixing in first well. To this equal quantity (25µl) of treated IBV, containing 4HA unit of the virus was added from column-2 to 12 of each micro-titer plate which was incubated at room temperature (25±3ºC) for 30 min. To each well 50µl of 0.5% chicken RBCs suspension was added. After another incubation of 30 min, the results were recorded.

Spike gene (S) sequencing and analysis: To undertake sequence analysis of new isolate, the spike gene (S) was sequenced using the specific primers, following the steps including primary amplification, post-PCR product purification, cycle sequencing and post-cycle sequencing

purification. The sequencing plate was run in the genetic analyzer (3130 ABI). The consensus sequence of the Pak-973 isolate was generated using the DNASTAR software version 7.1. The sequences of the S genes were compared with other IBVs using the MegAlign program of the DNASTAR software. Using the MEGA 6.0 software, deduced amino acid (aa) sequences were aligned and phylogenetic tree was constructed (Tamura *et al*., 2013). The maximum likelihood method was used with 1000 bootstrap replicates.

RESULTS

Virus detection and typing: A total of 905 specimens were tested by RT-PCR from which 358 were positive for IBV. Out of this, 183 were grouped as serotype 4/91, 154 as serotype M41 and 21 samples remained unclassified (Table 2 and Fig. 1). Through in-ovo inoculation of 21 unclassified isolates, one of the isolate gave overt clinical signs. Furthermore, through cross neutralization of one of the unique IBV isolate, major lesions post *in-ovo* inoculation revealed dwarfism, curling, and haemorrhages (Fig. 2). The virus was labeled as Pak-973 isolate (KX013102_Ck/Rwp/NARC-973/2015_Pakistan). In addition to this, the Pak-973 didn't get neutralized by HI test using the known reference antisera (M41, 4/91, D274 & D-1466). However, it offered complete neutralization with its own antiserum (Table 3). This virus was investigated through molecular testing.

Phylogenetic analysis of IBV sequences: The Pak-973 isolate (KX013102_Ck/Rwp/NARC-973/2015_Pakistan) was partially sequenced from nucleotide 1-678. It included two hypervariable regions (HVR). The phylogenetic tree of Pak-973 showed the genetic relationship (Fig. 3), when compared with the few IBV lineages and most similar blast sequences, maximum homology with two Indian strains; was approximately 93% with PDRC/Pune/India/9/99 (AY091551) and 23/B/2008 (KF360983), 92% sequence homology with number of Indian IBV strains including IBV415 (KF809790), HBL/IB/13/10 (AB861538), V55 (KF757462) and Ind/TN/168/06 (JX966396), while 91% sequence similarity with IBV470 (KF809795). Around 77% sequence homology was also recorded with number of Chinese variants including ck/CH/LSD/110115 (JQ739339), ck/CH/LSD/090401 (HM194689) and so on. This isolate is distantly related to the M41 and 4/91 strains, that basically comprising the most common vaccine strains in Pakistan.

Sequence analysis: S1 gene partially sequenced, the nucleotide and amino acid sequence similarities between isolate Pak-973 and other IBV strains were not more than 93%. Isolate Pak-973 showed the highest identity to virus 23/B/2008 (KF360983) was 93% at nucleotide level. The nucleotide and amino acid identities of S1 gene of isolate Pak-973 and M41 vaccine strain didn't exceed 72%, while with 4/91 vaccine strain didn't exceed 70% (Table 4). The S1 gene showed multiple mutations as shown in Table 5 in comparison with the most similar Indian variants and in use IBV vaccines. Moreover, we had much higher genetic diversity where they were 13 amino acid substitution in comparison to 4/91 and M41.

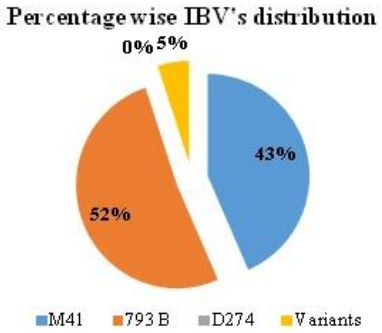

Fig. 1: Percentage wise detection of different serotypes of IBV's during 2015.

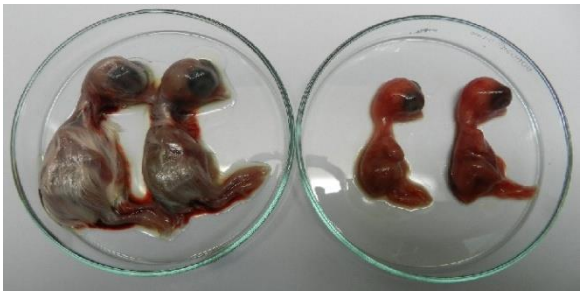

Fig. 2: *In-ovo* propagation of IBV; Left side has control group and right side has IBV infected group.

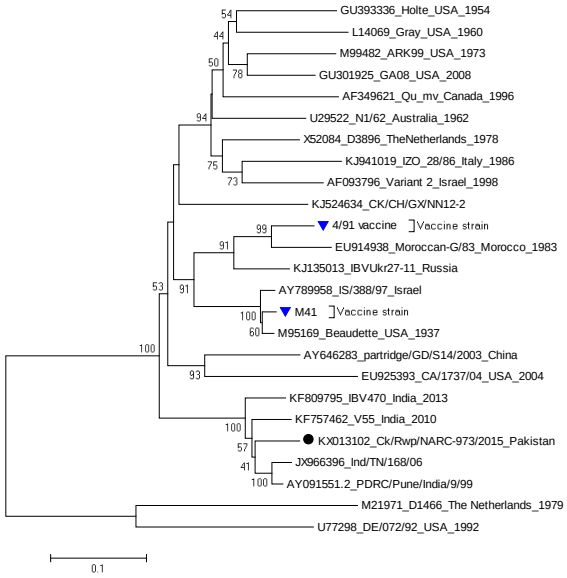

Fig. 3: Phylogenetic tree showing spike glycoprotein and inter-relationship of IBV isolates. The Pakistani isolate are highlighted in black square and vaccines strains highlighted in blue triangle.

Table 2: Percentage wise distribution of different serotypes of IBV during 2015

Total samples	Positive samples	Serotype diversity			
		M41	4/91	D274	Variants
905	358	154	183	00	21
Percentage (%) wise		43%	51%	0%	5%

Table 3: Virus neutralization assay to determine the serotype of Pak-973

Antigens	Antisera					
	Pak-973	M41	4/91	D274	D1466	IT-02
Pak-973	1046					
M41	64	215				
4/91	64		215			
D274	16			215		
D1466	>16				215	
IT-02	>32					215

Table 4: Percentage similarity between deduced nucleotide and amino acid of Pak-973 with reference IBV genotypes

Amino acid Similarity in Percentage %	Nucleotide Similarity in Percentage %																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
1		91	92	92	91	75	73	70	73	70	72	72	66	68	64	68	67	66	70	64	65	35	1 Pak-15N-973
2	87		92	92	90	76	72	71	76	70	75	75	68	65	66	68	67	67	71	66	67	32	2 V55 India
3	89	89		97	92	75	73	73	73	69	71	71	69	66	66	70	66	69	72	63	67	34	3 Ind/TN/168/06
4	90	91	94		93	75	73	73	74	71	73	73	71	66	67	71	66	69	72	64	68	36	4 PDRC/Pune/India/9/99
5	87	86	87	90		75	72	72	72	69	71	72	70	68	68	72	66	69	73	64	68	34	5 IBV470 India
6	73	73	73	74	73		73	73	87	88	85	85	66	67	69	73	82	71	74	72	70	35	6 IBVUkr27-11 Russia
7	76	71	72	75	74	76		73	76	67	75	76	70	68	69	73	68	71	72	65	72	32	7 Ck/CH/GX/NN12-2
8	70	69	67	68	70	70	72		73	70	73	73	67	65	71	73	68	67	71	74	71	30	8 Partr/GD/S14/2003China
9	72	72	68	70	68	84	74	70		74	97	97	72	71	70	75	73	71	76	72	74	33	9 IS/388/97_Israel
10	65	63	63	63	65	81	69	68	67		74	74	66	67	71	72	89	70	73	68	71	28	10 4/91 Vaccine
11	68	68	65	66	65	80	72	69	94	67		97	73	71	70	75	73	72	76	72	74	33	11 M41
12	70	68	65	68	65	82	73	70	95	67	94		73	71	69	75	73	71	77	72	74	33	12 Beaudette_USA_1937
13	59	60	60	61	61	64	63	64	65	64	66	67		78	76	76	65	72	73	60	81	26	13 Holte_USA_1954
14	67	63	63	64	64	70	66	63	70	70	70	70	74		75	77	65	72	73	61	79	28	14 Gray_USA_1960
15	62	61	64	65	65	70	70	68	69	70	68	70	76	75		78	70	73	75	61	84	36	15 ARK99_USA_1973
16	68	63	65	66	69	72	72	70	73	74	72	73	78	80	82		70	77	84	65	79	29	16 D3896_Netherlands_1978
17	65	60	59	61	62	74	68	66	68	84	68	68	62	68	70	73		69	72	64	70	28	17 G/83_Morocco_1983
18	65	64	68	69	69	72	73	64	70	68	71	71	73	74	76	80	66		81	61	75	23	18 IZO 28/86_Italy_1986
19	71	67	70	72	73	71	76	70	71	72	70	70	73	74	78	86	72	81		67	75	29	19 Variant 2_Israel_1998
20	62	60	57	59	58	65	64	75	68	62	65	68	56	60	59	63	62	59	63		62	25	20 CA/1737/04_USA_2004
21	64	61	64	65	66	70	69	68	70	70	72	72	79	80	87	85	71	80	79	58		29	21 GA08_USA_2008
22	24	23	24	23	24	27	28	26	26	17	24	24	12	18	23	19	19	18	27	23	17		22 D1466_Netherlands_1979
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	

Table 5: Amino acid substitution mutations in partial S1 gene of IBV

Virus nomenclature	Unique/Common Amino acid positions in S gene												
	HVR1 (38-67)					HVR2 (91-141)							
	2	4	24	56	63	76	92	96	117	120	124	187	189
Pak-973	S	P	R	R	G	T	T	M	A	A	T	H	I
V55 India	L	T	S	G	A	S	A	T	V	P	P	Y	T
Ind/TN/168/06	W	R	L	E	A	S	G	T	V	P	P	H	T
PDRC/Pune/India/9/99	L	K	D	E	A	S	G	T	V	H	P	Y	T
IBV470 India	L	ND	G	E	A	S	G	M	V	S	P	Y	T
4/91 Vaccine	L	K	ND	G	V	N	S	A	K	Q	P	E	T
M41	L	T	S	E	S	V	A	S	K	ND	P	E	T

HVR: Hyper variable region, S: Serine, P: Proline, R: Arginine, T: Threonine, M: Metheonine, A: Alanine, H: Histidine, I: Isoleusine, L: Leusine, W: Tryptophan, G: Glysine, V: Valine, Y: Tyrosine, K: Lysine, N: Asparagine, E: Glutamic acid, ND: Not detected.

DISCUSSION

IB is a highly infectious disease of chickens that continues to emerge in the field despite the use of number of vaccines both in the form of live attenuated and inactivated vaccines. The study was designed to characterize the isolated field IBVs from the cases where both conventional vaccines of Mass serotype and 4/91 variant were used in this country.

The present report mentions detection of different serotypes of IBVs during one-year study. Here 43% of the isolates belonged to Mass serotype and 51% were of 4/91 type, however, only 5% belonged to different un-typed IBV variants. Interestingly, no IBV isolates belonged to D-274 serotype. In some previous serological studies in this country, significant levels of antibody to Mass, D-274, D-1466 and 4/91 have been reported in flocks not previously vaccinated against these strains (Ahmed *et al.*, 2007).

When IBV Mass serotype live-attenuated and inactivated vaccines were first introduced they were effective at controlling IB among poultry flocks (Cook *et al.*, 2001). However, later isolation of new IBV strains highlighting the phenomenon of emergence of IBV variants, evolved from the vaccinated flocks to evade the immune defenses induced by the vaccines in use. Unfortunately, in many parts of the world no mechanism of regular IBV monitoring has been operating and furthermore almost all the vaccines used in commercial poultry are supplied by a few multinational companies, using only those vaccine strains registered in the developed countries. This lack of information of the circulating strains of IBV, mismatch of vaccine strain and field virus along with the inability of local agencies in the developing countries to produce vaccines of the variant viruses at local level, the use of available vaccines usually result in poor efficacy or complete failure to control Infectious bronchitis in many parts of the world, including Pakistan. This justified the need to undertake further investigations about emerging variants of IBV in this country.

In this study the isolates Pak-973 was analyzed in detail on the basis of biological and molecular characteristics to reveal that this virus was not neutralized properly by using the reference antisera, however, it reflected better neutralization capabilities once tested with its own antisera. On the other hand, while analyzing the sequencing data of Pak-973 it was found that around thirteen amino acid substitutions were observed in the sequenced S1 region. These mutations at amino acid position 02, 04, 24, 56, 63, 76, 92, 96, 117, 120, 124, 187 and 189 leads into synonymous and non-synonymous mutations. Out of 13 amino acid mutations, 5 were found in HVR1 and 6 were found in HVR2 region. In S1 gene three hyperactive regions are reported to be located within amino acids 38-67, 91-141 and 274-387 (Moore *et al.*, 1997). Specific IBV serotypes molecular signatures are present in HVR1 and HVR2as well as serotype specific

neutralizing epitopes (Kant *et al*., 1992). 20 to 25% difference in S1 usually resulted into the new IBV serotype (Kingham *et al*., 2000) but some serotypes differ around 2% in S1 gene (Cavanagh *et al*., 1992). Here in isolate Pak-973, the HVR1 and HVR2 contained the unique mutations at amino acid level when compared with the most related isolates and vaccine strains (Table 5). Briefly, Pak-973 contained different mutations when compared with 4/91 and Mass vaccine strains. It possesses serine at position 2 instead of leucine, proline at position 4 instead of lysine and threonine, arginine at position 24 instead of serine, arginine at position 56 instead of glycine and glutamic acid, glycine at position 63 instead of valine and serine, threonine at position 76 instead of asparagine and valine, threonine at position 92 instead of serine at alanine, methionine at position 96 instead of alanine and serine, alanine at position 117 instead of lysine, alanine at position 120 instead of glutamine, histidine at position 187 instead of glutamic acid and isoleucine at position 189 instead of threonine (Table 5). Overall the sequenced isolate Pak-973 differed 7% from the rest of serotypes and variants. These distinct substitutions or mutations in HVR1 and HVR2 ensured that this isolate is quite distinct from the vaccinal strains used in vaccines.

In some earlier studies, the possible region between aa 123-152 has been involved in the differing pathogenicity of non-virulent JMK and Graystrains (Kwon and Jackwood, 1995) whereas Pak-973 isolate have one unique substitution and few deletions in the same region. Here isolate Pak-973, when compared with the most related isolates it possesses different amino acid; threonine at position 124 instead proline. In one of the report some IBV serotypes may differ in S1 by as little as 10 amino acids (Kwon *et al*., 1993). So, the present study indicates that this mutation may have enabled the variant isolate Pak-973 to show lesser neutralizing ability in VN test carried out here. Furthermore, the recovery of isolate Pak-973 from the breeder flock showing signs of IB despite its multiple vaccination with both classical (Mass) and a variant strain (4/91), indicates that such vaccines were not able to neutralize the isolate Pak-973 despite the fact it has only shown low level of sequence variability in S1 region. This further shows the significance of vaccine matching approach prior to the selection of any strain as vaccine seed for introducing any new IBV vaccine strain in a country.

In a previous study, Shane (1997) suggested the multiple inoculation of breeding flocks with live and killed IBV vaccines, major factor contributing to the incidence of IB in chickens are multiple strains of the contagion, poor correlation of humoral neutralizating antibody titre to protection against re-infection with IBV, alteration in the antigenic characteristics and emergence of new and antigenically distinct IBV strains.

From the findings of present investigations, it is concluded that the circulating IBV in commercial flocks, have a sequence data closer to variant, indicating independent evolution of IBV in Pakistan and persistence of divergent strains currently circulating in the field. This study revealed the new variant of IBV in the field, despite the regular use of available vaccines, suggesting a regular mechanism of vaccine matching prior to the selection of vaccines for proper control of Infectious Bronchitis in the commercial poultry in this country.

Conclusions: Infectious bronchitis virus is a highly infectious viral disease of chicken and despite the use of various IB vaccines the disease appears in such vaccinated flocks in commercial poultry locally. This study first time reports the circulation of IBV variants in the field in Pakistan which are not covered under the existing IB vaccines and highlights the need for adopting vaccine matching approach for selecting an effective vaccine prior to its use in this country.

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Authors contribution: SR and KN conceived this study; SR and AS conducted virus propagation. SR conducted virus neutralization and sequencing. KN, NS, AAS and MA helped in data interpretation. All the authors contributed in manuscript preparation and its final approval.

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