Journal of Infection and Public Health 12 (2019) 410–418

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Phylogenetic analysis of Infectious Bursal Disease viruses according to newly proposed model of classification into geno-groups

Rai S. Ali Khan[a,b], Mudasser Habib[b], Waqas Ali[b], Muhammad Salah Ud Din Shah[b], Asma Ashraf[c], Zahid Ali Tahir[d], Zeinab H. Helal[a,e], Mazhar I. Khan[a], Shahid Mahboob[c,f,*], Khalid A-Al-Ghanim[f], Fahad Al-Misned[f]

[a] University of Connecticut, Department of Pathobiology and Veterinary Science, Storrs, CT, USA
[b] Animal Science Division, Nuclear Institute for Agriculture & Biology (NIAB) Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan
[c] Department of Zoology, Government College University Faislabad, Faisalabad, Pakistan
[d] Veterinary Officer (V.O), Poultry Diagnostic Laboratory, Kamalia, Toba Tek Singh, Pakistan
[e] Microbiology and Immunology Department Faculty of Pharmacy, Alazhar University, Cairo, Egypt
[f] Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 22 October 2018
Received in revised form 9 December 2018
Accepted 23 December 2018

Keywords:
IBDV
Classification
Geno-groups
Pak-Asian region
Phylogenetic analysis

ABSTRACT

Background: Infectious bursal disease virus (**IBDV**) is the causative agent of Infectious Bursal Disease (**IBD**), the disease causes immunosuppression which leads to secondary infections among rearing poultry flocks. Characterization of the virus is important for its control and eradication. The circulating IBDVs are classified on the basis of their antigenic and pathogenic properties. The virus is categorised as classical, variant and very virulent IBDV (**vvIBDV**). IBDV is a non-envelop, icosahedral double stranded virus. Viral protein 2 (**VP2**) is the major structural protein of capsid that determines the host-pathogen relationship. The aim of this study was to characterise the IBD virus of Pak-Asian region.
Methodology: IBDV suspected flocks were examined in Punjab, Pakistan from 2014–2018. Two hundred and fifty samples were collected with complete history of the disease. The suspected samples were collected from broiler, layer and rural poultry farms. RNA was extracted and hyper-variable region of VP2 gene was amplified using specific primers. Nucleotide sequence of the VP2 gene was determined and its Amino Acid sequence was deduced. Moreover, phylogenetic analysis was also performed.
Results: The current classifications based in a hyper-variable region of the capsid protein VP2 (**hvVP2**), classification of IBDVs is split into newly proposed geno-groups according to Jackwood group. Among these prevailing, some IBDVs are limited geographically whereas, others are reported cosmopolitan. Genetic alterations are continuously playing role in evolution of new strains of the virus.
Conclusion: During this study it was found that isolates of IBDV fall in first three geno-groups. Most of the geno-groups are prevalent around the world, whereas the mutated and re-assorted ones are confined in particular areas of the globe.

Introduction

Infectious Bursal Disease is one of the dominant cause of immunosuppression in chickens [1]. The etiological agent, Infectious bursal disease virus (IBDV) belongs to family Birnaviridae, it is non-enveloped, icosahedral in shape with double stranded RNA genome [2]. It is structurally bi-segmented. Segment A encodes for (VP2, VP3, VP4 and VP5) viral proteins that represents the major antigenic region [3] of IBDV, whereas segment B encodes for single viral protein (**VP1**) which is responsible for replication of virus through the RNA-dependent RNA polymerase (**RdRp**) [4]. IBDV has serotypes I and II, serotype I is pathogenic in nature while serotype II is limited to turkeys [5]. There are various levels of virulence in serotype I and different stages of bursal cells damage depending upon replication efficiency of IBDV [6]. Vaccination is the only effective tool to control financial losses of poultry farmers. Although, in

* Corresponding author at: Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
E-mail addresses: mazhar.khan@uconn.edu, mushahid@ksu.edu.sa (S. Mahboob).

https://doi.org/10.1016/j.jiph.2018.12.012

the presence of strict biosecurity measures and intensive immunisation programmes, still IBD outbreaks occurs globally [7]. IBD controlling measures are complex due to frequent genetic changes, genomic recombination and reassortment of viral genome which leads to vaccine failure [8]. Eradication strategy is not possible from infected farms due to resistant characteristics of IBDV against chemical and thermal exposures [9,10]. In late 1980s there was emergence of variant strains in different geographical regions of the world [11].

It was investigated that vaccines from classical strains did not provide protective immunity against variant strains and resulted in outbreaks with severe immunosuppression [12]. Immunosuppression caused by IBD increases the financial losses of poultry industry due to poor productive performance of rearing flocks [6]. In 2016, Zachar et al. investigated, in Canada, that variant strains had not only increase the rates of mortality, but also increase the bursal size atrophy and lower the rate of feed conversion ratio which result in reduction in meat production. In Saskatchewan, Canada it was investigated from 2007–2011, poultry industry losses of 3.9 million kilogram (broiler meat) per annum [13].

The research facilities and standards in developed countries are advance than the developing ones [14]. The aerosol and vectors play important role in viral infections spread [15,16]. IBD is present universally and its causative agent had been isolated from different vectors that range from water (penguin), soil (insects, rodents) and air (wild birds) [17–22]. The IBDV classification based is mainly on antigenicity/virulence; variant and classical virulent IBDVs **(cvIBDV**). The classical subtype is further divided into three pathotypes; mild, very virulent, and attenuated IBD viruses [23,24]. The classification of prevalent IBD viruses on such basis is complicated. Phylogenetic analysis provides information about genetic differentiation of circulating IBDVs. the genetic differentiation from phylogenetic tree provides summarised information [25]. The researchers categorized the clades emerging from vvIBDVs into subgroups defined as these main three streams vvIBDV-1, vvIBDV-2, vvIBDV-3, atypical etc [24] that makes the classification more complex. So there is need to introduce new basis for better understanding of emerging strains of IBDV. In 2017, Jackwood and Linda introduced new classification of IBDV on the basis of its genetic information which translate the genetic data in better way than the previous division [26].

The aim of this study was to investigate genetic characterization of circulating isolates from Pak-Asian origin and the results were interpreted in terms of geno-groups according to newly proposed model for classification against IBDVs.

Materials and methods

Source and collection of samples

IBDV suspected flocks were examined in Punjab, Pakistan from 2014–2018. Two hundred and fifty samples were collected with complete history of IBD. The selected samples were collected from broiler, layer and rural poultry farms. The samples were supplied as routinely conducted post mortem in the diagnostic labs under the control of Livestock and Dairy Development Department, Punjab, Pakistan. The bursae were stored at −80 °C [27]. The cold chain was maintained during the transportation of the samples till processing. In extreme environmental conditions, samples from far areas were carried in falcon tubes containing phenol-chloroform (5:1), pH 4.3 ± 0.2 in order to inactivate and preserve the viral genome for further amplification [28]. The isolates collected were assigned identities (**IDs**) showing year of collection, geographic regions and type of birds. Samples containing bursal tissues were grinded in sterile pestle and mortar with sterile Phosphate-buffered saline (**PBS**) then centrifuged and supernatant was collected [29].

RNA extraction

RNA was extracted with viral nucleic acid extraction kit (FavorPrep®, Taiwan) according to manufacturer's protocol.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Complimentary DNA (**cDNA**) was synthesized by using Thermo Scientific™ RevertAid™ First Strand cDNA, synthesis kit according to the manufacturer's instructions. Briefly, extracted RNA was heated for 5 min at 65 °C with 1 μl Random hexamer primer (0.2 μg/μl) and 6 μl nuclease free water in RNase free PCR tubes. Then PCR tubes were chilled in ice and added 8 μl reaction mixture containing 4 μl of 5X reaction buffer, 1 μl RNase inhibitor, 1 μl f Moloney-murine Leukaemia Virus (M-MuLV) Reverse Transcriptase (200 U/μl) and 2 μl of 10 mM dNTPs mix. Then all the mixture was mixed and short spin in centrifuge machine. Initially incubate at 25 °C for 10 min and then 42 °C for an hour in thermocycler machine. The reaction was terminated by heating at 70 °C for five minutes [30].

Primers were designed from the flanking regions of VP2 gene of IBDV; forward primer 5′-AGCCAACATCAACGACAAA-3′ and reverse primer 5′-CAAGACGGTCCCTCTCACT-3′. Estimated size of the PCR product was 782 bp (444–1225 nt).

RT-PCR was performed for the detection and amplification of hyper-variable region of VP2 gene of IBDV. Synthesized cDNA was used as template for polymerase chain reaction (PCR) using a Thermo Fisher Scientific kit, according to manufacturer's instructions. Briefly, 50 μl reaction mixture containing 36 μl double distilled water, 5 μl PCR reaction buffer (10X), 2 μl $MgCl_2$ (25 mM), 1 μl dNTP mix (10 mM each), 3 μl cDNA template, DMSO 0.8 μl, 1 μl forward and 1 μl reverse primers (10 p moles/μl) in PCR tubes. The PCR mixture was heated at boiling temperature for 2–3 min. Then 0.2 μl Taq DNA polymerase (5 U/μl) was added and PCR tube was placed in a thermal cycler (Techne Touchgene Gradient PCR Thermal Cycler, Lab Recyclers, Inc.,). Thermal cycling conditions were as follows: Initial denaturation at 98 °C for 3 min, 35 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s and final elongation at 72 °C for 10 min. The amplified PCR product was visualized under UV light following electrophoresis in the 1.5% agarose gel stained with Ethidium Bromide [31].

Sequencing of the hyper variable region (HVR) of VP2 gene

Twenty samples were selected representing different areas of the region. PCR products were purified using gel purification kit (Biobasic, USA). Dideoxy termination method was used with the aid of BigDye Terminator sequencing kit's protocol [32]. For assembling and editing of sequences for direct sequencing in both directions SeqMan Pro software (DNAStar; Lasergene 7.1.0,Madison, USA) was used [33]. The National Center for Biotechnology Information (**NCBI)** BLAST (http://www.ncbi.nlm.nih.gov/blast) was utilized for confirmation hypervariable region. Finally, edited sequences in this study were submitted to GenBank and their accession numbers were registered (Figs. 1 and 2).

Phylogenetic analysis of VP2-HVR

For phylogenetic analysis nucleotide sequence data was analysed using the software BioEdit, Lasergene DNASTAR and MegAlign. The hyper-variable region of VP2 gene was used for phylogenetic studies [24]. Nucleotide sequence alignment was done by ClustalW method with BioEdit v7.0.5 [24]. Phylogenetic and

Table 1
Details of different IBDV isolates used for construction of phylogenetic tree.

S. #	Geno-group	Previous classification	Country	Accession #	Strain/isolate
1	**G-1**	**Classical**	**Taiwan**	**AF457104.1**	**Int/228E**
2	G-1	Classical	Mexico	DQ916210.1	04M101
3	G-1	Classical	Indian	AJ621158.1	UP Indian
4	G-1	Classical	Oman	MF988099.1	Halban1-15
5	**G-1**	**Classical**	**Canada**	**D00499.1**	**STC**
6	G-1	Classical	Pakistan	KY523069	129
7	G-1	Classical	Pakistan	MF521671	154
8	**G-1**	**Classical**	**Germany**	**AY321953.1**	**F52-70**
9	**G-1**	**Classical**	**USA**	**AY918948.1**	**Lukert**
10	G-1	Classical	Egypt	MF142543.1	509
11	G-1	Classical	Taiwan	MH329181	2512
12	G-1	Classical	Thailand	AY907014.1	Thai4 Classic
13	G-1	Classical	Argentina	DQ916165.1	05A24
14	G-1	Classical	Egypt	MF142540.1	497
15	G-1	Classical	Brazil	KY556558.1	213-047-2
16	G-1	Classical	Vietnam	AY841900.1	GSG16
17	**G-1**	**Classical**	**USA**	**MH329180**	**D78**
18	G-1	Classical	Bolivia	DQ916172	05B62
19	**G-2**	**Antigenic variant**	**USA**	**MH329179**	**Variant E (DelE)**
20	**G-2**	**Antigenic variant**	**USA**	**JF736011.1**	**AL-2**
21	**G-2**	**Antigenic variant**	**USA**	**AF281238.1**	**T1**
22	G-2	Antigenic variant	Ecuador	MF142513.1	220
23	G-3	vvIBDV	Pakistan	KY484079	7
24	G-3	vvIBDV	Pakistan	KY484078	30
25	G-3	vvIBDV	Pakistan	KY484080	38
26	G-3	vvIBDV	Pakistan	KY484081	44
27	G-3	vvIBDV	Pakistan	KY484082	45
28	G-3	vvIBDV	Pakistan	KY484083	46
29	G-3	vvIBDV	Pakistan	KY484085	126
30	G-3	vvIBDV	Pakistan	KY523068	11
31	G-3	vvIBDV	Pakistan	KY000833	37
32	G-3	vvIBDV	Pakistan	KY412848	9
33	G-3	vvIBDV	Pakistan	KY973953	50
34	G-3	vvIBDV	Pakistan	KY973954	62
35	G-3	vvIBDV	Pakistan	KY973955	83
36	G-3	vvIBDV	Pakistan	MF521667	134
37	G-3	vvIBDV	Pakistan	KT281984.1	PK-1
38	G-3	vvIBDV	Pakistan	KY523067	75
39	G-3	vvIBDV	Iran	DQ785171.1	JRMP24IR
40	G-3	vvIBDV	India	MF142548.1	568
41	G-3	vvIBDV	India	MF142522.1	316
42	G-3	vvIBDV	Pakistan	KU321594.1	MM127
43	G-3	vvIBDV	India	KJ621050.1	VRDC/FS1
44	G-3	vvIBDV	India	KY020408.1	HYA15/ABT/MVC
45	**G-3**	**vvIBDV**	**China**	**KT884486.1**	**HN (Henan)**
46	G-3	vvIBDV	Kuwait	MF142502.1	101
47	G-3	vvIBDV	Egypt	MF142533.1	420
48	G-3	vvIBDV	Iraq	MF142534.1	422
49	G-3	vvIBDV	Iraq	MF142535.1	423
50	G-3	vvIBDV	Egypt	MF142542.1	500
51	G-3	vvIBDV	Egypt	MF142544.1	510
52	G-3	vvIBDV	Egypt	MF142545.1	512
53	G-3	vvIBDV	Indonesia	MF142551.1	616
54	G-3	vvIBDV	Malaysia	MF142568.1	739
55	G-3	vvIBDV	Jordan	MF142517.1	276
56	G-3	vvIBDV	Jordan	MF142560.1	710
57	G-3	vvIBDV	Indonesia	MF142570.1	748
58	G-3	vvIBDV	Indonesia	MF142521.1	304
59	**G-3**	**vvIBDV**	**China**	**AF092943.1**	**HK46**
60	**G-3**	**vvIBDV**	**UK**	**NC_004178.1**	**UK661**
61	**G-4**	**dIBDV**	**Japan**	**LC136880.1**	**TY2**
62	G-4	dIBDV	Argentina	AM084695.1	B641A34
63	G-4	dIBDV	UAE	MF142569.1	741
64	**G-4**	**dIBDV**	**Uruguay**	**KT336459.1**	**dIBDV/2202**
65	**G-4**	**dIBDV**	**Brazil**	**JN982252.1**	**MG4**
66	G-4	dIBDV	Canada	EF138954.1	06-51480L7
67	G-5	Variant/classical recombinant	USA	JQ277695.1	06M11
68	G-5	Variant/classical recombinant	USA	AF498627.1	C-278
69	**G-5**	**Variant/classical recombinant**	**USA**	**DQ916210.1**	**04M101**
70	**G-6**	**ITA**	**Italy**	**JN852986.1**	**ITA-02**
71	G-6	ITA	Russia	Z97002.1	RF-5
72	G-6	ITA	Italy	KY930929.1	1829
73	G-7	Australian	Brazil	KY612967.1	213-043-1
74	G-7	Australian	Australia	HM071991.1	V877-W
75	G-7	Australian	Russia	MF142536.1	429_Russia
76	**G-7**	**Australian**	**Brazil**	**KY612971.1**	**211-177-4**

The bold rows indicate the reference isolates (26) for the construction of Phylogenetic tree

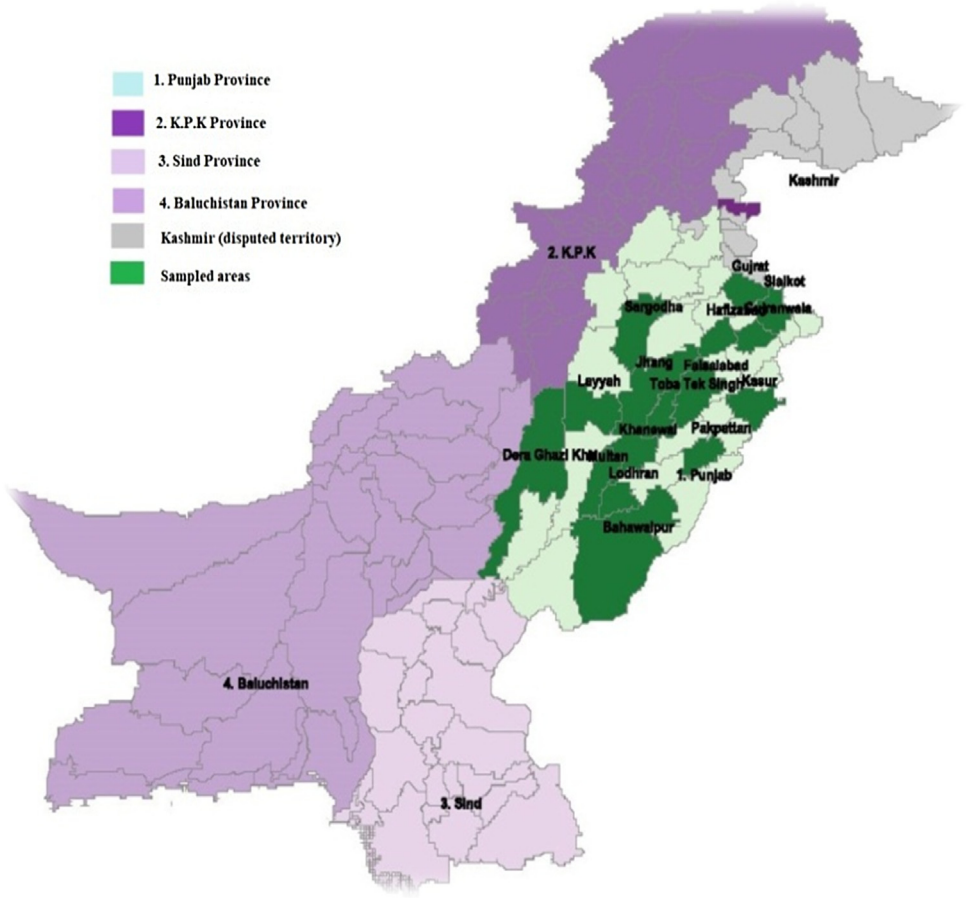

Fig. 1. Regions of Pakistan showing areas of sample collection.

Table 2
Genetic markers and their descriptions in different geno-groups.

Geno-group	222[a]	242[a]	256[a]	279[a]	294[a]	299[a]
G-1	A/P/S	V/2	V/I/A	D/N	I/L	N
G-2	T/Q	V	V	N	L	N/S
G-3	A/S	I	I	D/N	I	S/N
G-4	S	V	V	N	L	H/S/N
G-5	T	V	V	N	L	N
G-6	Q	V	K/I	D	L	S
G-7	P	V	V	G	L	S

[a] Position of amino acid in the VP2 gene of IBDV.

molecular evolutionary analyses were conducted using molecular evolutionary genetics analysis (**MEGA**) version 6 software using the neighbour joining method with the Kimura three-parameter evolutionary model [34,35]. The topological reliability of the trees was inferred by the bootstrap method with 1000 replicates. Phylogenetic analysis relay on arrangement of partial nucleotide VP2 sequences from 628–117 covering the hyper variable region at the nucleotide 845–1126 [33]. All the aligned sequences were analysed with reference isolates mentioned in Table 1. Sequences of the deduced amino acid 210–391 were investigated for genetic markers Table 2. The incorporated sequences of isolates were grouped into seven geno-groups using circular option for branches of phylogenetic tree. Each geno group was separated from main phylogenetic for detailed study 1000 bootstrapping replicates were selected to estimate the robustness of tree branches [36,37].

For comprehensive global studies sequenced data from Pak-Asian and other regions was selected to evaluate the genetic information on the basis of neighbour joining method. The profile of deduced amino acids sequence present in the hydrophilic regions was evaluated to investigate the mutations at amino acid level [45,46].

Results and discussion

Molecular detection of IBDV isolates

Two hundred and fifty samples were collected from suspected flocks having 13–41 days of age. Morbidity rate was 10%–90% while 0.2%–70% mortality rate was observed. Birds up to six weeks of age were mostly affected by IBDV. For screening of samples, RT-PCR was done using VP2 specific primers that flanks the hyper variable region of VP2 gene of IBD virus. The detail of genetically explored sequences that were originated from Pak-Asian countries is listed in Table 1. The GenBank accession numbers from Pakistan (this study) were KY484079, KY523068, KY523069, MF521671, KY484078, KY000833, KY484080, KY484081, KY484082, KY484083, KY973953, KY412848, KY523068, KY973954, KY973955, MF521667 and KY484085 whereas the accession numbers from other regions were MH329179, MH329180 and MH329181.

Sequence analysis of hyper variable region of VP2 of geno groups

All of the selected sequences were aligned for nucleotide at positions 628–1173 and amino acid at the positioned 210–391 for detailed analysis. For this purpose phylogenetic tree was constructed by neighbour joining method illustrated in Fig. 3 [37]. On the basis of similarities data displayed into seven geno groups. In

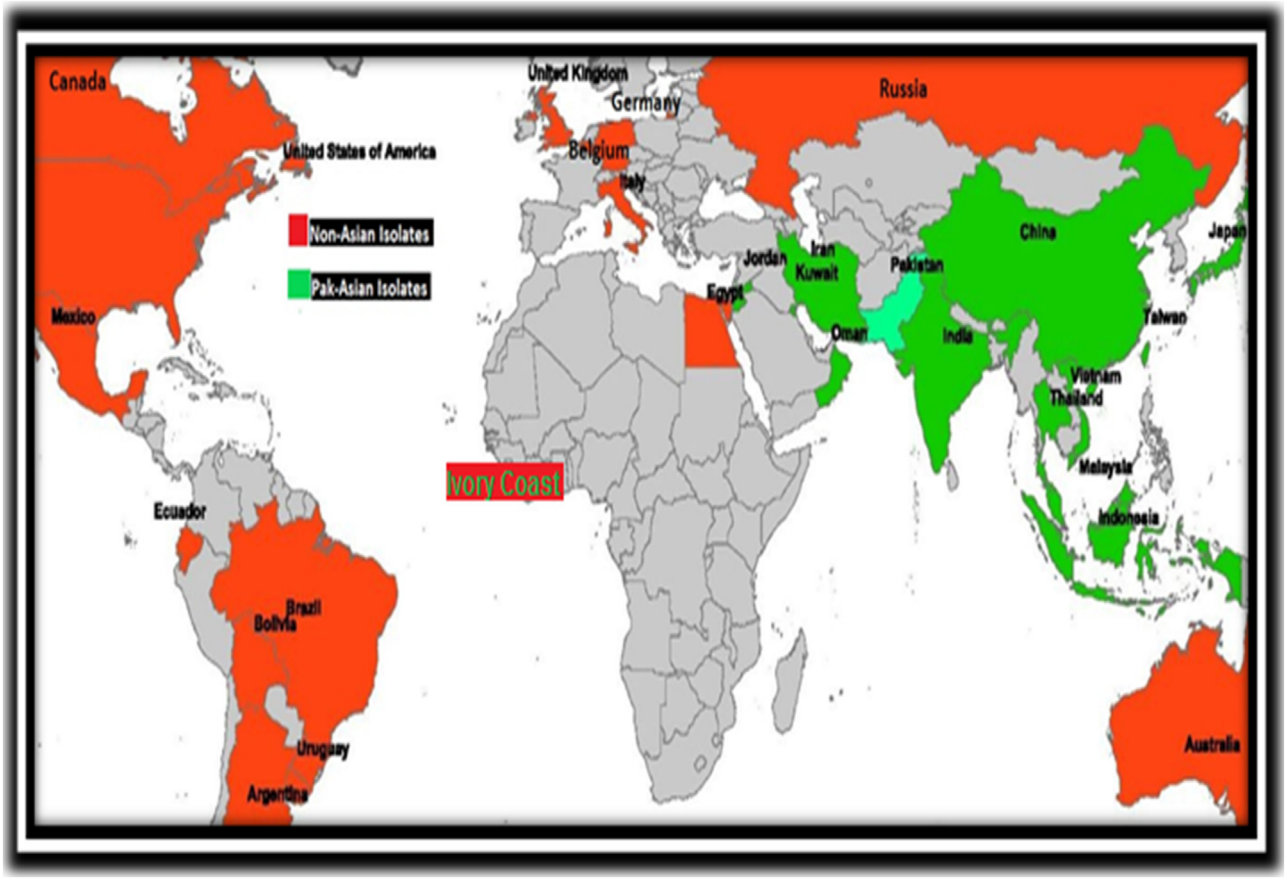

Fig. 2. Map showing the geographical regions of the selected sequences around the world.

conventional classification it was related to its pathogenicity and serotype. All the reference isolates displayed in phylogenetic tree incorporated in the same group as reported by Michel and Jackwood in December, 2017 [26].

In the present study 17 isolates fall in geno-group 1, which were diversified and reported globally [26]. In this group at location 222, amino acid (**aa**) the genetic hallmark, more than 80% of isolates had Proline (**P**) and others shared S/A in the variable region. At position 242 majority of isolates had V whereas I was located in two sequences. The amino acid positioned at 256 were valine (**V**)/Isoleucine (**I**)/Alanine (**A**). Eighty eight percent sequences contributed V at this location; F52-70-Germany and Luckert-USA. In minor hydrophilic region 2, at position 279 majority of specimen had Aspartate (**D**) and 12% sited Asparagine (**N**). The genetic marker 294 had I/Leucine (**L**). In all isolates of geno-group 1, Asparagine (N) was found at position 299 unanimously.

In geno-group 2, the amino acids located in the genetically conserve locations 222 were Threonine (**T**), 242V, 256V, 279N, 296L and 299N. In current study, only single sequence matched with the reference strains is 220-Ecudor. This group is limited in Non- Asian countries. The reference isolates were from USA and the remaining specimen belonged to South America. According to our knowledge, this group is still conserved in the South and North America. The sequence of 220-Equador has similar aa at position 242V, 256V, 279N and 296L with the referred strains but it disagrees at location T222Q and N299S. Previously the classification of IBDV members prevailing in genogroup2 was antigenic variant. This group was confused with Variant/classical recombinant (Gengroup5). Now, for the first time it is categorised into separate group clearly.

The prevalence of isolates of genogroup3 is universal. Typical genetic hallmark of genogroup3 is similar at 222A, 242I, 256I, 294I and 299 Serine (**S**). The two specimens from Jordon (276-Jordon & 210-Jordon) mutated from D279N whereas Indonesian isolate (616-Indonesia-Asian strain) was heterologous in major hydrophilic peaks A and B at amino acid positions A222S/S299N. This isolate displayed nine mutations in amino acids located in different hydrophilic regions (N212D, A222S, I264V, G281) Arginine (**R**), S294N, E300A, I305V and A321 Glutamate (**E**). The non-Asian isolates of Egypt (420-Egypt, 500-Egypt, 510-Egypt and 512-Egypt) showed variation at aa N212D, Tyrosine (**Y**)220 Phenylalanine (**F**) and Glycine (**G**)254S, first two changes occur in major hydrophilic region A while the last take place just next to minor hydrophilic region 1. The same single amino acid change (G254S) is noted in 83-Pakistan. There are four common changes in amino acid profiles of 739-Malaysia and 616-Indonasia (N212D, D213N, I305V and A321E) in the Asian-Isolates. The Malaysian sequence (739-Malaysia) mutates at G254D that is common to the Jordan strains (276-Jordon & 210-Jordon). In major hydrophilic region B three unique changes are also reported at amino acid S315T, S317R and D323N. It is interesting to note that these Asian border sharing country's isolates (739-Malaysia & 616-Indonesia) have shown heterologous record in all the hyper variable regions of IBDVs from the other Asian-isolates. In genogroup3, three Pakistani viral isolates (75-Pakistan [this study], MM127-Pakistan & 50-Pakistan [this study]) showed high or low variations from routine in hydrophilic region A. The sequence of 50-Pakitan isolate was mutated at amino acid G224A whereas MM127-Pakistan showed variation at positions N212D and Glutamine (**Q**) 221H. The isolate 75-Pakistan sights variation just in the beginning of Baylsis hyper-variable region (deduced amino acids 206–350) [38] at locations A210G and N212 Lysine (**K**).

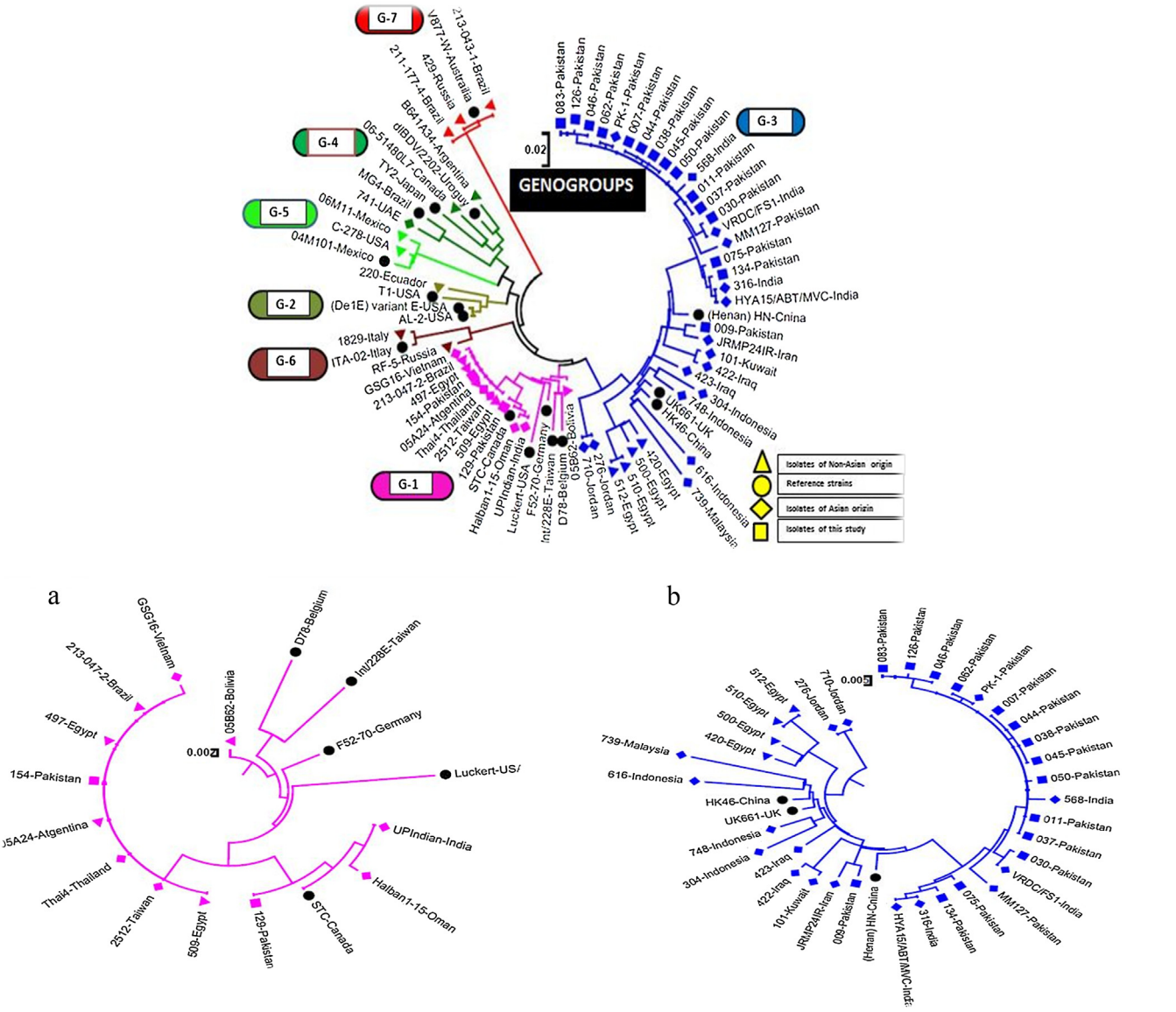

Fig. 3. Phylogenetic tree showing relationship of different IBDV isolates:.
The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 1.12774365 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The analysis involved 75 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 386 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The circle indicates reference strains, diamond shape indicates isolates from Asian geographic origin, triangle indicates isolates from non-Asian countries and square shows the isolates of this study. Fig. 3a: presents the split tree of geno-group1. Fig. 3b: presents the split tree of geno-group 3.

In genogroup4, there are common amino acids at genetic markers 222S, 242V, 256V, 279N and 294L while amino acids at position 299 displays N/S/Histidine (**H**). The amino acid profiles of all studied isolates in this group is quite diversified and showed more similarities with the neighbouring group (genogroup5). The more mismatch region within this group was presented between minor hydrophilic region1 and 2. The strains of this group spreads all over the world.

In the genogroup5, all studied isolates were shared the same genetic hallmark (222T, 242V, 256V, 279N, 294L and 299N). This group is not reported in Pak-Asian region to date.

The genoroup6 has five common genetic references at location 222Q, 242V, 279D, 294L and 299S whereas isolate RF-5-Russia differs at aa K256I within group. This group is prevailing in Non-Asian countries especially in upper region of Asian and Middle-East countries.

All the members of genogroup7 have same genetic markers at position 222 Proline (**P**), 242V, 256V, 279G, 294L and 299S. It is restricted in non-Asian regions. All the selected isolates show nearly 100% amino acid similarity except one specimen from Brazil (211-177-4-Brazil) reveals single mutation at location D213N in the major hydrophilic peak A.

Phylogenetic studies

Phylogenetic tree was developed from all the aligned sequences. The tree revealed that incorporated sequences clustered into seven separated geno-groups. The genogroup1, genogroup3 and genogroup4 shared the genetic information globally. All other groups were preserved in a particular region. In genogroup1 the classical strains of the circulating IBDVs were segregated whereas in genogroup3 the vvIBDVs were grouped. Most of universally

existing strains fall in these two groups. The antigenic variant viruses clustered into genogroup2. Most of the economic losses in present farming are due to this group. The genogroup4 represented the distinctive class of IBDVs (dIBDVs) which was maximally present in the non-Asian countries previously but a single sample (741-UAE) from United Arab Emirates matches with this lineage and needs to be investigated further. Genogroup5 gathered the IBDVs from recombinant origins. These viruses shared the common amino acid profile from variant and classical viruses [26]. The genetic profile of IBDVs in genogrop6 was reported initially in the Italy [39] which is Non-Asian in origin whereas in recent reports its existence is noted in Middle East of Asian region and Russia [26,40]. In geno group7, its isolates were mainly in Australian territory but its prevalence in Russia was also reported [26]. The strains of genogroup7 were non-Asian in origin.

After Newcastle Disease (ND), Infectious Bursal Disease is the second most important disease among viral diseases of poultry [41]. It infects the rearing birds but immunosuppressive wave enhances the complex disease continuously which adds economic losses worldwide. In this viral infection the humoral and cellular immune responses are compromised due to excessive destruction of B cells or its stem cells in early growing birds that resulted in lower growth rate/feed conversion ratio [42]. The most economical method to control the infectious agent is vaccination. However, it is investigated that most of outbreaks are observed in vaccinated flocks but the role of non-vaccinated infected flocks is also well documented in introducing new viruses [43]. The single point mutation (as amino acid change) may alters the protection levels [23].

The large populations of circulating IBD viruses fall in first three genogroups. Classical strains of viruses were grouped in genogroup1. The vaccination is the only economical way to minimise the losses from viral diseases. It was found that most of classical vaccinal strains (D78, strain 2512, Lukert and 228E) were present in this group. Typical amino acid profile of vaccine strains in the region from 249-258 is QTSVHGLVLG and from 279 to 286 amino acid arrangement is NNGLTTGT. The aligned data showed that these two regions were not 100 percent matched among D78, Lukert and 228 strains. The extensive vaccination at different intervals in the rearing flocks mutated the field IBDVs. The vaccination procedures, quality and quantity of vaccines are also questionable in this scenario. In routine the amino acids 222P, 249Q, 286T and 318G are consider as genetic hallmark [31]. In-spite of variations in the genetic markers of Pak-Asian and other isolates; amino acid at position N299 was conserved in all isolates of this geno-group. Major amino acid alterations were seen between first and second minor hydrophilic peaks of hypervariable region.

The genogroup2 showed different genetic information in loop; P_{BC} (amino acid 219–224), P_{DE} (amino acid 249–254), P_{FG} (amino acid 283–286) and P_{HI} (amino acid 316–324) so it was categorised in a separate group. Usually variant shares common amino acids at position 222T, 242 V, 249 K, 256 V, 279N, 286I, 294L, 299N and 318D as genetic markers [31]. Most of the economic losses are reported due to variant strains. There are no pathognomonic post-mortem lesions due to variant strains that lead to late or mis diagnosis and immunosuppression predisposes to secondary infections [47].

In majority of outbreaks, members of genogroup3 were responsible globally. Typical genetic marker (amino acids A222, I242, I256, D279, I294 and S299) was present in this group. The pin point mutations in the epitopes of viruses alter antigenicity. The HVR region of VP2 gene has four loop structures; PBC, PDE, PFG, and PHI that have been linked to alteration in IBD virus virulence and escape from vaccine sequence [48]. The isolate from Indonesia (616-Indonesia) showed mutations in all hydrophilic regions except minor hydrophilic region I; amino acids at positions N211D, D212N, **A222S,** G281R, **S299N**, E300A, I305V and A321E respectively. The amino acid change at position 299 and 300 is very critical as it is most conserve area in the HVR due to presence of β-hairpin [48]. The European and Asian genogroup3 (vvIBDV) has aa SE marker at position 299 and 300 whereas it showed NA amino acids which is not reported in any major regional markers. In Pak-Asian genogroup3 the regional marker is SE but it in Malaysia (739-Malaysia) there is mutation at β-hairpin area due to high vaccine pressure to NQ, regional marker of Ivory Coast [49]. The Malaysia and Indonesia are present at the lower region of Asian continent whereas Ivory Coast located on outer boarder of African Continent, comprehensive study is needed to investigate these changes. Fig. 3.The genogroups provided ample information that may lead to sub-groups within these major groups in future classification.

In genogroup4 the hallmark was common in S222, V242, V256, T272, N279, P289, I290, L294, and F296 amino acids [26]. The 299 revealed different aa at 299H/S/N. The brief investigations of HVR demonstrated that dIBDV/2202-Uroguy had 88.9 to 92.1% similarities in amino acid sequences with D78 (Nobilis D78) and Winterfield 2512 (Cevac IBD L) [vaccinal isolates] that are mostly used in South America [50]. Its members may be spread through vaccination in Pak-Asian and Non-Asian regions. Majority of dIBD viruses isolated from non-Asian countries.

The genogroup5 is restricted in non-Asian parts and have common genetic markers with genogroup2; T222, V242, V256, N279, L296 and N299 where as it differs from variants at S251N and S254N [31]. It has been observed that there is mutation at aa T270K in between L_{BE} and L_{FG} from the genogroup1 and genogroup2. Moreover two important aa substitutions were noted in major hydrophilic region B; S317K and A321P. The aa change at position G318D is particular in Mexico IBDVs belonging to Variant stains. The presence of both Variant and Classical sequences at antigenic epitopes in the single IBDV provides solid ground for genogroup5 [31].

IBD virus belong to geno-group 6 were found in other regions of the world. These are circulating in the Italian territory. There were no typical clinical signs noted in affected vaccinated flocks. The pathogenic studies conducted in an isolated conditions but its sequenced data could be shared worldwide without restriction. Phylogenetic analysis revealed that ITA strains grouped independently from reference isolates of IBDVs, either part of classical or/and very virulent strains, reported in Italy. Adding more ITA were different from vaccine strains [39]. In major hydrophilic region A, amino acid altered at position 220H and 222Q whereas aa 321A change was observed in major hydrophilic region B. Other important genetic markers include 253E and 256K amino acids [51]. The sequence RF-5 showed more 95.8% similarity with 1829 and 95.3% matched with ITA-02. The common genetic hallmarks among all sequences were 222Q, 242V, 279D, 296D and 299S [51]. This sequence branched in the same cluster of geno-group 6.

In the last geno-group, mostly Australian IBD viral strains are present in the phylogenetic profile. All the sequences of this group are nearly 100 percent similar so all the genetic markers are conserved. This group is limited in the specific region. The classical and antigenic variant vaccines are prepared from these Australian isolates [52]. At present, epidemiologically vaccinal strains is backbone for newly emerging IBD viruses with different stages of pathogenicity globally. Trade plays major role in spread of viruses throughout world [53].

In Pakistan before this study, sequences of only eight IBDV isolates had been submitted in Genbank. Isolates were collected from all over the major growing areas of Pakistan during this study. Before 1980s classical strains were dominated in the poultry industry in Pak-Asian region. The amino acid at position 222 might mutate from Proline (P) to Throline (T) contributed in mutation from classical to variant geno-group [26,54–57]. These mutations may be the result of high vaccination pressure because these two

strains were used universally for immunising the rearing flocks [58]. The changes at 222 and 321 amino acid play role in neutralising maternal antibodies so these variations may have critical importance for antigenicity of circulating IBDVs [59].

Conclusion

During this study it was found that isolates of IBDV fall in first three geno-groups. Most of the geno-groups are prevalent around the world, whereas the mutated and re-assorted ones are confined in particular areas of the globe.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgements

The authors would like to express their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. Prolific Research Group No. 1436-011.

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