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Abstract

Infectious bursal disease virus (IBDV) is a ubiquitous immunosuppressive pathogen causing severe burden to the poultry industry. Due to frequent mutation and reassortment events, its double-stranded, bi-segmented RNA genome displays a considerable heterogeneity, which in turn produces profound differences in antigenicity and pathogenicity.

In recent years, the investigation of IBDV epidemiology has greatly benefited from the proposal of multiple phylogenetic classification systems which prompted the execution of molecular surveys all over the world, producing a wealth of standardized and easily sharable data.

The present epidemiological survey was conducted in six Near East and Persian Gulf countries to characterize the field IBDVs circulating in the region. Ninety-three broiler flocks, immunized with different vaccination protocols, were investigated over a one-year period by collecting eight individual bursal samples at a time. Using molecular assays targeting both genome segments, field IBDVs belonging to four genotypes were detected from 42 flocks across all investigated countries. While traditional very virulent IBDVs (genotype A3B2) represented a minority, multiple atypical IBDV genotypes (A3B1, A4B1 and A6B1) were found to circulate in most of the region. Many of the detected strains were related to sequences from other continents, supporting the involvement of the Near and Middle East in complex networks of viral spreading. The obtained results, combined with the available literature, allowed to update the epidemiological picture, hopefully favoring the identification of epidemiological threats and the planning of effective monitoring and control strategies.

Research highlights

- Different field IBDVs were found to circulate in the Near and Middle East.
- Multiple atypical genotypes (A3B1, A4B1, A6B1) were found to circulate extensively.
- Traditional very virulent IBDVs (A3B2) were a minority of the detected strains.

- Viral exchanges can be hypothesized between the region and different continents.

Abbreviations

BIC: Bayesian information criterion; IBD: infectious bursal disease; IBDV: infectious bursal disease virus; ICX: immune complex; KSA: Kingdom of Saudi Arabia; UAE: United Arab Emirates; VP: viral protein.

Keywords

Infectious bursal disease virus; molecular epidemiology; vaccination; Near East; Middle East; Persian Gulf

Introduction

Infectious bursal disease (IBD), also known as Gumboro disease, is a highly contagious viral disease responsible for major losses for the poultry production worldwide. It mostly occurs in chickens between 2 and 6 weeks of age, when the bursa of Fabricius, its primary target, reaches full development. IBD may manifest itself in different ways, ranging from acute forms, with non-specific clinical signs and a variable mortality rate, to a subclinical disease, characterized by immunosuppression, which in turn leads to secondary infections, worse production performance, and vaccine failures (Eterradossi and Saif, 2020). IBD control is pursued by maintaining high biosecurity standards and through routine vaccination, whose efficacy is however hampered by the interference of maternal antibodies (Block et al., 2007). To overcome this crucial issue, different vaccine types have been made available, each with different features and requiring appropriate application strategies.

The aetiological agent of IBD is a member of the genus *Avibirnavirus*, family *Birnaviridae* known as infectious bursal disease virus (IBDV). It is a highly stable, horizontally transmitted, non-enveloped virus with a double-stranded RNA genome made of two segments. The 3.2 kb-long segment A encodes four viral proteins (VPs), namely capsid protein (VP2), scaffold protein (VP3), protease (VP4) and non-structural protein (VP5). On the other hand, the 2.9 kb-long segment B codes only for the RNA-dependent RNA-polymerase, referred to as VP1 (Maraver et al., 2003). The bisegmented nature of its genome makes the virus prone to reassortment events, which, together with the remarkable mutation rate of both segments (Gao et al., 2007; Pikula et al., 2021), has determined the emergence of numerous genetic variants with profound functional differences. Despite the existence of just two IBDV serotypes (with serotype 2 being non-pathogenic), a high antigenic diversity is observed between different IBDVs, mostly sustained by the accumulation of point mutations within the VP2 (Jackwood and Sommer-Wagner, 2011; Cubas-Gaona et al., 2023). Pathogenicity, on the other hand, is determined by changes in both the VP2 and VP1 (Brandt et al., 2001; Escaffre et al., 2013; Wang, W. et al., 2021), and consequently also by reassortment between the two genomic segments (Mató et al., 2020; Soubies et al., 2017).

A full understanding of IBDV heterogeneity should therefore be considered essential to better diagnose and control the disease, and the steady advancements in molecular biology offer a powerful and convenient tool to pursue it. Compared to classical virology, molecular assays allow for easier data sharing and standardization of laboratory procedures. Moreover, their sampling requirements are less stringent and more compliant with border regulations, meaning that they represent the most convenient (and sometimes the only) way to import and analyze specimens collected in a different country (Jackwood and Sommer-Wagner, 2007). In recent years, this approach has been further enhanced by the proposal of multiple classification systems relying on phylogeny, which provided robust criteria to characterize IBDV strains based on partial VP2 and VP1 sequencing. In particular, Islam et al. (2021) proposed a classification into nine VP2 and five VP1 genogroups, respectively numbered from A0 to A8 and from B1 to B5. Following the discovery of a novel type of reassortants (Wang, Y. et al., 2021) and an additional VP2 genogroup (A9) (Legnardi et al., 2022), the total number of known genotypes, corresponding to the documented combinations of the two genomic segments (i.e., A1B1, A1B2, A2B1, etc.) has been updated to seventeen.

Although pathogenicity and antigenicity determination are still essential for a full characterization, molecular surveys have greatly contributed to a better understanding of IBDV global epidemiology. In particular, they allowed to overcome some persisting misconceptions linked to the traditional way of classifying IBDVs in classical, very virulent and variant viruses, which was becoming less and less adequate to describe the variability observed between different strains. As a matter of fact, atypical IBDVs, often associated with subclinical disease forms, were reported with increasing frequency in many parts of the world (Hernández et al., 2015; Wang et al., 2022; Legnardi et al., 2023; Nwagbo et al., 2016), posing novel challenges to IBDV detection, impact assessment and control.

Compared to other geographical contexts, the epidemiological situation in the Near and Middle East is not entirely clear. Despite the availability of several studies, most were conducted in a limited number of countries, mainly Egypt (Abdel-Alim et al., 2003; Mawgod et al., 2014; Shehata et al., 2017), the Islamic Republic of Iran (Bahmaninejad et al., 2008; Razmyar & Peighambari, 2008; Ghaneie & Peighambari, 2014) and Iraq

(Amin and Jackwood, 2014; Al-Azzawi et al., 2021; Saeed, 2021), with little to no information on the rest of the region. In addition, the absence of classification guidelines at the time when most of these studies were conducted led to a lack of harmonization, limiting their value outside of the respective country of detection. Aiming to address these knowledge gaps, the present study describes a molecular survey conducted in Near East and Persian Gulf countries, whose results, integrated with the existing literature, allowed to update the knowledge about field IBDVs circulating in the area and define potential epidemiological networks at both regional and global scales.

Materials and methods

Samples. The study was conducted on samples sent to the Laboratory of Microbiology and Infectious Diseases of the MAPS Department of the University of Padua (Legnaro, Italy) from Near East and Persian Gulf countries. During a one-year period going from February 2022 to February 2023, 93 broiler flocks were investigated for monitoring purposes by collecting 8 individual bursae at a time and imprinting them onto FTA™ cards (GE Healthcare UK Limited, Amersham, UK). The age at sampling ranged from 21 to 41 days (mean: 28.7 days). Samples were conferred from six different countries, namely Iraq, Jordan, Kuwait, Lebanon, Oman and the United Arab Emirates (UAE). Sixteen of the surveyed flocks were immunized with conventional live vaccines (17.2%), 11 with vector vaccines (11.8%), 60 with immune complex (ICX) vaccines (64.5%), 1 was dually vaccinated with a vector vaccine in the hatchery and a live vaccine in the field (1.1%) and 3 received ICX vaccines in the hatchery and live vaccines at the farm (3.2%). No IBD vaccines were administered to the remaining two flocks (2.2%).

Sample processing and RNA extraction. A piece of approximately 4x4 mm^2 was cut from each FTA™ card circle imprinted with a single bursa, individually placed into a tube with 1.5 mL of 1× PBS solution and eluted by vortexing for 30 s. Nucleic acid extraction was then performed on 200 µL of each eluate using the High

Pure Nucleic Acids kit (Roche™, Basel, Switzerland) according to the manufacturer's instructions. Samples were stored at −80 °C until the analyses and thereafter for archiving purposes.

Molecular analysis and phylogenetic analysis. All individual samples were first analyzed with a one-step RT-PCR assay targeting a 743 nt-long portion of the VP2 gene (nucleotides 737-1479 of segment A), using the primers 743-1 (5′-GCCCAGAGTCTACACCAT-3') and 743-2 (5′-CCCGGATTATGTCTTTGA-3') designed by Jackwood and Sommer-Wagner (2005). The positive ones were then sequenced and subjected to preliminary phylogenetic analyses, which allowed to divide the investigated flocks into four categories: VACCINE, when all the obtained sequences were of vaccine origin; FIELD, when only field strains were detected; MIXED, when both field and vaccine sequences were obtained from the same flock; NEGATIVE, in case all samples tested negative. The ratio of vaccine-positive, field-positive and negative results within each flock was also calculated.

After the assessment of the identity between the field sequences detected in each FIELD and MIXED flock, one field-positive sample was selected from each of them to amplify and sequence a 751 nt-long portion of the VP1 gene (nucleotides 319-1070 of segment B) using the primer B-Univ-F (5'-AATGAGGAGTATGAGACCGA-3'), designed by Islam et al. (2012), and the newly designed primer VP1-shortR (5'-TGGAAACAAAAGCCCGCATG-3'). All molecular assays were performed with the SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase kit (Invitrogen™, USA). Sanger sequencing was conducted at Macrogen Spain (Madrid, Spain) with the same primers used in the respective RT-PCR assay. The obtained chromatograms were visually inspected and trimmed in 4peaks (Nucleobytes B.V., Aalsmer, the Netherlands) and consensus sequences were assembled with ChromasPro (Technelysium Pty Ltd, Helensvale, QLD, Australia).

The partial VP2 and VP1 sequences of all field IBDVs were included in two final datasets (one for each segment), together with reference sequences, and classified through phylogenetic analysis as proposed by

Islam et al. (2021). The analyses were conducted in MEGA X (Kumar et al., 2018) considering 366 nt-long and 431 nt-long segments of the VP2 and the VP1, respectively. Using the built-in functions, sequences were first aligned with the MUSCLE method, then the relative phylogenetic tree was generated with the Maximum Likelihood Method (1000 bootstraps), adopting the most appropriate substitution model selected by calculating the Bayesian information criterion (BIC).

To further investigate the phylogenetic relationships of the detected field strains, multiple BLAST queries were performed to retrieve the VP2 and VP1 sequences with the highest homology with each cluster. Separate subsets were thus generated and additional phylogenetic analyses were conducted to identify the closest sequences.

Lastly, available IBDV sequences from neighboring countries were downloaded from GenBank and characterized phylogenetically to establish which genotypes have been historically detected in the region and better contextualize the obtained results.

Statistical analysis. The efficacy of different vaccination protocols in preventing field IBDV infection was assessed using the Chi-square test. To this purpose, flocks were categorized depending on whether field IBDVs were detected (flocks falling in the FIELD and MIXED categories as described previously) or not (flocks in the VACCINE and NEGATIVE categories), whereas their vaccination status was categorized depending on the administered vaccine type (i.e., live, ICX, vector vaccines, and their combinations). Potential differences in the within-flock prevalence of vaccine strains (considering all vaccinated flocks) and field IBDVs (considering only flocks infected with field IBDVs) based on vaccination protocol were investigated with the Kruskal-Wallis test, followed by post-hoc Mann-Whitney U pairwise tests with Bonferroni correction. The analysis and the descriptive graphs to explore the dataset were performed in R (R Core Team, 2021). For all analyses, the significance level was set at $p<0.05$.

Results and discussion

Based on preliminary phylogenetic analyses, the sequences detected in 23 out of the 93 investigated broiler flocks (24.7%) were of vaccine origin. Only field strains were retrieved from 32 flocks (34.4%), while both vaccine and field strains were found in 10 flocks (10.8%). The remaining 28 flocks tested negative (30.1%). A breakdown of the results divided by country and vaccination protocol is provided in Table 1, whereas information on within-flock prevalences of vaccine and field IBDVs can be found in Supplementary Table 1.

All the samples from one of the two unvaccinated flocks tested positive for the Winterfield 2512 vaccine strain. This finding can be attributed to vaccine persistence, as a Winterfield-based vaccine had previously been used in previous cycles.

When the efficacy of protocols based on live, ICX and vector vaccines in preventing infection by field strains was compared, the difference proved non-significant ($p = 0.122$). Similarly, no significant differences could be found between the three vaccine technologies in terms of within-flock prevalence of vaccine strains ($p = 0.624$) and field IBDVs ($p = 0.132$) (Figure 1). In both cases, the combinations of different vaccine types and the sole unvaccinated flock were not considered due to data paucity.

Considering the number of field IBDV-positive flocks, the IBDV epidemiological pressure appears high regardless of the vaccination protocol. However, the equal efficacy of the compared vaccine types in preventing bursal colonization by field IBDV can hardly be supported. The implemented convenience sampling entails that some of the surveyed countries were over or underrepresented, potentially producing biases due to differences in vaccination strategy and consistence, administration procedures and IBDV epidemiological pressure in different productive contexts. These limitations, together with the unstandardized age of the chickens and the possible implications of phenotypic variability among the detected IBDV genotypes, appear therefore not ideal to confidently assess vaccine coverage and effectiveness.

From each of the 42 field IBDV-infected flocks, a field strain was successfully sequenced both at VP2 and VP1 level. In detail, 37 VP2 sequences belonged to genogroup A3 (Very virulent), 3 to genogroup A4 (distinct

IBDV) and 2 to genogroup A6 (Italian). At VP1 level, 40 sequences fell within genogroup B1 (Classical) and the remaining 2 in genogroup B2 (Very virulent). When considering both segments together, the represented genotypes were thus A3B1 (35 strains), A3B2 (2 strains), A4B1 (3 strains) and A6B1 (2 strains) (Figure 2). Partial VP2 and VP1 sequences were deposited in GenBank under the accession numbers OR211751-OR211792 and OR211793-OR211834, respectively (Supplementary Table 2).

To better understand the local epidemiological scenario, each of the detected genotypes was investigated separately. The most frequently retrieved genotype, and the only one found in Iraq, Jordan and Lebanon, was A3B1. However, since the vast majority of the samples came from the latter two countries, this does not necessarily support a higher prevalence in the entire region compared to other genotypes. Based on phylogenetic analyses, Jordanian, Iraqi and Lebanese strains clustered together, and additional sequences having similar features retrieved from Genbank came from Jordan, Iraq, Iran, Russia, Kazakhstan and Italy (Figure 3). Despite exhibiting a classical-like VP1, these strains form a monophyletic clade within genogroup B1, and, albeit they may be suspected to be reassortants, no plausible ancestors having the same segment B profile are known. Recent studies have also pointed out that, despite a relatively recent origin (Legnardi et al., 2023), continuous evolution may lead this clade to eventually become a separate genotype (Islam et al., 2021). This, together with the difficulty in finding the link between seemingly distant epidemiological realities, and the still undetermined pathogenicity of these strains, demands further epidemiological studies to shed light on their evolutionary history and extent of circulation, which in turn may help to better understand some underlying aspects of the global IBDV epidemiology.

A second small group of A3B1 strains, detected in Iraq, Kuwait and the USA (Abbas et al., 2019; Michel and Jackwood, 2017; Michel et al., 2019), was found to be relatively similar based on segment B (percentage of identity: 93.0-95.1%) but more distantly related at segment A level (percentage of identity: 89.6-94.5%). A separate clade of A3B1 IBDVs seems therefore to have been circulating in the surveyed region in recent years, albeit no strains with such genetic features were detected in the present study.

The two field strains retrieved in Oman were classified as A3B2 strains. This genotype shows genetic features previously associated with high pathogenicity both at VP2 and VP1 level, grouping traditional very virulent IBDVs identified in most continents since the 1980s (Islam et al., 2021). At VP2 level, the Omani IBDVs belonged to a clade including sequences detected in Iran (with which they displayed the highest similarity), India and Pakistan (overall percentage of identity: 96.4-98.9%), suggesting they may have originated in South Asia (Figure 4). On the other hand, the phylogenetic analyses conducted on segment B proved less informative, as the two strains clustered with a large number of sequences retrieved in Europe, Africa, Asia and South America with no discernible patterns (data not shown). The fact that these strains, which are still considered the main threat of the Old World by many non-experts, were only found twice and just in a single country is a clear evidence of the complexity of IBDV epidemiology in the Near and Middle East, and of the usefulness of molecular methods to update and objectively evaluate our knowledge on current IBDV field threats.

The relevance of atypical strains presence in the region was testified by the detection of three strains, all from the UAE, belonging to genotype A4B1, which groups the so-called distinct IBDVs (dIBDVs). These strains have been associated to subclinical infections with marked bursal atrophy (Tomás et al., 2019) and are antigenically different from classical, variant and very virulent IBDVs, whereas a relatedness to other atypical IBDV types was revealed by antigenic cartography (Cubas-Gaona et al., 2023). Over the years, dIBDVs have been reported in South America, North America, Eastern and Central Europe, East Asia and the Middle East (Domanska et al., 2004; Hernández et al., 2015; Hou et al., 2022; Mató et al., 2022; Ojkic et al., 2007), making them one IBDV types with the broadest geographical distribution (Figure 5). According to a recent study, the origin of this genotype was estimated to have occurred in Eastern Europe around the 1930s. Over the decades, it may have then spread to different continents through different migration routes (Tomás et al., 2020). Unsurprisingly, the three UAE strains found in the present study showed the highest similarity (96.7-98.6%) with another VP2 sequence detected in the same country in 2016, whose corresponding VP1 sequence is unfortunately unavailable (Michel and Jackwood, 2017). The UAE strains clustered with Brazilian strains both at segment A and B level, and were distinct from the two other VP2 sequences available from the region,

detected in the KSA in 2014 by Mohamed et al. (2017), which were phylogenetically closer to Canadian viruses. Despite the scarcity of available A4B1 sequences from the Near and Middle East, this allows us to theorize the occurrence of multiple spreading events involving different parts of the world.

The last genotype detected in this epidemiological survey was A6B1, which groups the so-called Italian strains. As implied by their name, these strains were first described in Italy by Lupini et al. (2016) and have been associated to subclinical infections with bursal damage (Lupini et al., 2019). However, VP2 sequences clustering within genogroup A6 were then found in the Kingdom of Saudi Arabia (KSA) as well (Michel & Jackwood, 2017). The closest strain to the two identical A6B1 sequences found in Kuwait, exhibiting 96.2% and 98.1% identity at VP2 and VP1 level, was initially reported in an unspecified country of the Middle East in 2015 and recently characterized by Cubas-Gaona et al. (2023). Lastly, another sequence in the A6 genogroup belongs to a Russian IBDV from 1994 (Shcherbakova et al., 1998) (Figure 6). Albeit sporadic and far back in time, such a report supports the existence of epidemiological ties between these three areas, adding to the detection of the same A3B1 strains in Italy and the Middle East.

Despite all the detected genotypes had been previously reported at least once in the Near and Middle East, the obtained results represent their first detection in many of the considered countries. The comparison with all available VP2 and VP1 sequences ever detected in the region allowed for a clearer description of the local epidemiological scenario, revealing that the sequences retrieved in each country mostly belonged to the same genotype. The only two exceptions were represented by Kuwait and the KSA, where the presence of two different genotypes has been documented (Figure 7).

Aside from Oman, traditional very virulent strains belonging to genotype A3B2 have also been reported to circulate extensively at the northern (Turkey), eastern (the Islamic Republic of Iran) and western (Egypt, Israel) boundaries of the region. As for more atypical IBDVs, A3B1 strains seem to circulate in an area encompassing Jordan, Lebanon, Iraq and Kuwait, whereas A6B1 strains are reported in Kuwait and the KSA, and A4B1 in the UAE and KSA. However, as detailed before, all these genotypes are also found in other continents,

supporting the existence of multiple networks of viral spread linking the Near and Middle East to other parts of the world.

In conclusion, the present results provide useful information on IBDV epidemiology in the Near and Middle East, which is characterized by the transboundary circulation of multiple genotypes and viral exchanges with different continents. Nonetheless, even if the overall epidemiological picture depicted by this study appears coherent with the available literature, many interrogatives remain. Due to the absence of up-to-date data from many parts of the region, the understanding of the circulation extent of some genotypes is still incomplete. Moreover, the actual health and economic significance of some atypical IBDV types will require further research on their pathogenicity and protection conferred by the implemented vaccination protocols. By identifying the main epidemiological threats, this study will hopefully prompt and inform such future studies, thus helping to devise better monitoring and control strategies not only in the region, but also in other contexts with similar IBDV situations.

Ethical statement

The considered samples were collected within the context of routine diagnostic activities and not for experimental purposes. No ethical approval was therefore required.

Disclosure statement

The authors report there are no competing interests to declare.

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Tables with captions

Table 1. Strain types detected in each investigated flock, divided according to the respective country and administered IBDV vaccine(s). The results are divided into the following categories: VACCINE, if only vaccine strains were detected; FIELD, if only field strains were detected; MIXED, if both vaccine and field strains were present; NEGATIVE, if all the samples from a given flock tested negative.

		Vaccine	**Field**	**Mixed**	**Negative**	**Total**
Country	Iraq	-	1	-	-	1
	Jordan	11	16	8	11	46
	Kuwait	-	2	-	3	5
	Lebanon	8	9	1	11	29
	Oman	4	2	-	-	6
	UAE	-	2	1	3	6
Vaccination protocol	Live	-	7	4	5	16
	Vector	3	4	1	3	11
	ICX	19	20	4	17	60
	Vector + live	-	-	-	1	1
	ICX + live	-	1	1	1	3
	No vaccination	1	-	-	1	2
Total		23	32	10	28	93

Figure captions

Figure 1. Within-flock prevalence of vaccine (left) and field (right) strains. All flocks vaccinated with either live, immune complex or vector vaccines were considered to estimate vaccine prevalence, while field strains prevalence was calculated only among flocks infected by field IBDVs.

Figure 2. Phylogenetic trees of VP2 (a) and VP1 (b) field IBDV sequences, color-coded according to the country of detection. The evolutionary history was inferred with the Maximum Likelihood Method (1000 bootstraps). Node support values are shown only when higher than 70. For the VP2 tree, the substitution model used was GTR+G+I (Nei & Kumar, 2000) and a 366 nt-long segment was considered, whereas the VP1 tree was inferred based on a 431 nt-long segment using the K2+G+I model (Kimura, 1980).

Figure 3. VP2 (a) and VP1 (b) phylogenetic trees of the A3B1 strains detected in the present study (marked with black circles, ●), along with relevant A3B1 IBDVs additionally retrieved from GenBank (marked with white squares, □) and reference sequences of all genogroups. Both trees were inferred with the Maximum Likelihood method (1000 bootstraps) using the K2+G+I substitution model (Kimura, 1980), considering 366 nt and 431 nt-long segments of the VP2 and VP1, respectively. Node support values are shown only when higher than 70.

Figure 4. Phylogenetic tree of partial VP2 sequences of the A3B2 strains detected in the present survey (marked with black circles, ●) and other relevant viruses retrieved from GenBank (marked with white squares, □), together with reference sequences of other genogroups. The tree was inferred with the Maximum Likelihood Method (1000 bootstraps) using the K2+G substitution model (Kimura, 1980) and considering a 366-nt long genome segment. Node support values lower than 70 were hidden.

Figure 5. Phylogenetic trees of A4B1 strains and reference sequences of other genogroups at VP2 (a) and VP1 (b) level. The three A4B1 strains detected during the present study are marked with a circle (●), whereas those additionally retrieved from GenBank are marked with a square (■). Each symbol was color-coded according to the continent of detection. Both trees were inferred with the Maximum Likelihood Method (1000 bootstraps) using the GTR+G+I substitution model (Nei & Kumar, 2000) for the VP2 tree, for which a 366 nt-long segment was considered, and the K2+G+I model (Kimura, 1980) for the VP1 tree, which is based on a 431 nt-long genome portion. Node support values are shown only when higher than 70.

Figure 6. VP2 (a) and VP1 (b) phylogenetic trees of A6B1 strains and reference sequences of other genogroups. The two A4B1 strains detected during the present study are marked with a black circle (●) and those additionally retrieved from GenBank are marked with a white square (□). Both trees were inferred with the Maximum Likelihood Method (1000 bootstraps) using the K2+G substitution model (Kimura, 1980), considering 366 nt and 431 nt-long segments of the VP2 and VP1, respectively. Node support values are shown only when higher than 70.

Figure 7. Distribution of IBDV field genotypes reported in the Near and Middle East based on the results of the present study, conducted in the countries with black borders, and available sequences extracted from GenBank. The map was prepared using QGIS 3.24.