Research Article

Molecular Diagnosis of Persistently Very Virulent Infectious Bursal Disease Virus at Sharkia Governorate, Egypt

Hala Mohamed Nabil Tolba[1], Naglaa Fathy Saeed Awad[1]*, Gamelat Kotb Farag Kotb[2], Amany Adel[3]

[1]Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt; [2]Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt; [3]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza 12618, Egypt.

Abstract | Severe outbreaks of Infectious bursal disease virus (IBDV) were reported in Egypt despite vaccination. Therefore, this study was conducted to characterize infectious bursal disease (IBD) viruses circulating in Egypt during the period of 2017 - 2018. Sixteen pooled bursal tissue samples were collected from IBD suspected (9 Egyptian balady and 7 broiler) chicken flocks located in different localities at Sharkia governorate, Egypt. These samples were subjected for direct detection of a 620 bp hypervariable region in the VP2 gene using Reverse transcriptase polymerase chain reaction (RT-PCR). The nucleotide and deduced amino acid sequences for VP2 hypervariable region of selected five IBDV field isolates were determined and compared to well characterized reference and vaccine strains worldwide. The IBD virus was detected in 9 out of 16 (56.25 %) investigated chicken flocks. Sequence analysis revealed that the analyzed Egyptian isolates identified as very virulent Infectious bursal disease virus (vvIBDV). The identity between these Egyptian isolates and the vaccine strains is ranged from 89.5%-95.6% and 88.1%-97.8% -at the nucleotide and amino acid sequence levels, respectively. The IBDV-Egy1 isolate is related to the Egyptian vvIBDV strains but with some deviations in the amino acids (259V, 263F, 290I and 302N). There were dramatic differences in the predictive antigenic determinants between these vvIBDV isolates and the classic vaccine strain (Bursin plus). These findings could explain the persistence of vvIBDV circulation in the Egyptian environment in spite of vaccination with classical vaccine strains.

Received | May 04, 2019; **Accepted** | May 30, 2019; **Published** | June 25, 2019
***Correspondence** | Naglaa Fathy Saeed Awad, Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, Hai-Moubarak, El-Emam ElShafey street No.44, 44519, Zagazig, Sharkia, Egypt; **Email:** NF2731982@gmail.com
DOI | http://dx.doi.org/10.17582/journal.hv/2019/6.3.42.49
Citation | Tolba, H.M.N., N.F.S. Awad, G.K.F. Kotb and A. Adel. 2019. Molecular Diagnosis of Persistently Very Virulent Infectious Bursal Disease Virus at Sharkia Governorate, Egypt. *Hosts and Viruses*, 6(3): 42-49.
Keywords: vvIBDV, Chickens, HVR-VP2, RT-PCR, Antigenic determinants

Introduction

Infectious bursal disease (IBD) is one of the most economically important contagious diseases of young chickens (3-6 weeks-old) caused by infectious bursal disease virus (IBDV) and characterized mainly by hemorrhagic syndrome, severe damage in bursa of fabricious, immunosuppression and high mortalities (Eterradossi and Saif, 2013). The IBDV is a member of genus Avibirnavirus and family Birnaviridae (Lukert and Saif, 2003).

In Egypt, IBD was diagnosed for the first time on the basis of its characteristic pathological lesions at 1974 (El-Sergany et al., 1974). The interest with antigenic characterization of IBDV was triggered by the appearance of very virulent infectious bursal disease virus (vvIBDV) strains in vaccinated Egyptian flocks (Khafagy et al., 1991). These vvIBDV strains are antigenetically very similar to the classical ones with a marked increase in virulence and breakthrough high levels of maternal antibodies leading to great economic losses in chicken farms (Xiumiao et al.,

2012). Presently, the presence of both very virulent and variant IBDV strains in Egypt were reported in several studies and become serious problems circulating in chickens vaccinated with the classical strain vaccines (Metwally et al., 2009; Mohamed et al., 2014; Sara et al., 2014).

The IBDV genome consists of two segments of double stranded RNA, included segments A (3.4 kb) and B (2.8 kb). The capsid protein VP2 is the major host immunogenic component and encoded by segment A (Snyder et al., 1992). The VP2 hypervariable region (VP2-HVR) includes the amino acid sequence ranging from positions 206 to 350, is of key importance in serotype 1 for genotyping of IBDV strains and contains the most informative genetic data regarding strain variability. Therefore, it was chosen for sequence analysis to characterize IBDV molecularly and allowing variations analysis that occur in different strains (Jackwood and Sommer, 2005).

The amino acids A222, I256 and I294 were reported to be unique to all known vvIBDV strains. In addition, the amino acids I242, I256, I294 and S299 were reported to be highly conserved among VVIBDV strains. These molecular markers can be used to differentiate vvIBDV strains from classical and variant ones (Banda and Villegas, 2004). Therefore, amplifying the VP2-HVR in combination with DNA sequencing of the PCR product potentially allow for more rapid, sensitive and specific detection and differentiation of IBDV classic, very virulent and variant strains (Islam et al., 2012). This information helps us gain a better understanding of the current landscape of IBD in Egypt and provides additional scientific data to support selection of the most effective vaccination strategies and products to prevent the disease. Therefore, the current study aimed to determine molecular characteristics of the currently circulating IBDV field isolates responsible for recent outbreaks in five different localities (Zagazig, Hehia, Abu-Hammad, Faqous and Belbies) at Sharkia Governorate, Egypt during 2017–2018.

Materials and Methods

Field investigation and samples collection

In this study, 16 pooled tissue samples (5 bursas /each flock/pool) were aseptically collected from sixteen (9 Egyptian balady and 7 broiler) IBD suspected chicken flocks from 3 to 8 weeks - old located in different localities at Sharkia governorate, Egypt, during the period from December 2017 to December 2018. These examined flocks were vaccinated at 11 and 22 days -old with a live attenuated IBD vaccine (classical strain) via drinking water. Clinical signs of the morbid birds were observed. Postmortem examinations of either sacrificed or freshly dead birds were also carried out. The collected tissue pools were kept at -20 ˚C till use.

Genomic RNA extraction

The viral RNAs were extracted directly from bursa of fabricius pooled samples using a QiaAmp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. The extracted RNAs were stored at -20˚C for further investigations.

PCR Amplification of VP2 gene

Reverse transcriptase- Polymerase chain reaction (RT-PCR) was conducted on the extracted RNA using an AgPath-ID™ One-Step RT-PCR Reagents Kit (Applied Biosystems). Primers F/AUS GU: 5-TCA CCG TCC TCA GCTTAC CCA CAT C-3 and Primer R/AUS GL: 5-GGA TTT GGG ATC AGCTCG AAG TTG C-3 were used to amplify a 620 bp including the hypervariable region of the VP2 gene (Metwally et al., 2009). The cycling conditions for amplification of PCR product included 1 reverse transcription cycle of 48 ˚C for 30 min followed by 95 ˚C for 10 min then 40 PCR cycles of 95 ˚C for 30 sec, 59 ˚C for 1min and 72 ˚C for 1.5 min with a final extension cycle at 72 ˚C for 10 min. The reaction mixture consisted of 12.5 µL 2X RT-PCR Buffer, 2 µl of each primer with concentration 20 pmol, 1 µl of RT enzyme, 2.5µl PCR grade water and 5µl of extracted RNA. Known positive (IBD vaccine strain) as well as negative (Bursa sample collected from apparently healthy unvaccinated chicken) controls were included in each run of PCR reactions. After amplification, 5µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5µg/ml at 95 V for 30 minutes in 1x TBE buffer, against GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

Gene sequencing of VP2 gene

Five IBDV field isolates were selected to represent 5 different localities involved in this study. Gel containing DNA band of the expected size (620bp) was removed and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced directly

using 2 µl of Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA), 1 µl of each primer specific for VP2 gene as previously shown in amplification of VP2 gene by RT-PCR. The sequence cycling reaction was done as follows: one cycle at 96 ˚C for 1 min, 25 repeated cycles of 96 ˚C for 10 Sec, 50 ˚C for 5 Sec and 60 ˚C for 2 min. Then, the sequencing reactions were purified using a spin column Centrisep® kit (Applied Biosystems, USA) to remove the extra free dNTPs bases and followed by loading the purified reactions in a sequencer plate of ABI (Applied Biosystems 3130 genetic analyzers, USA).

Genetic and phylogenetic analysis of VP2 gene

The alignment of nucleotide and amino acid sequences of the field isolates included in this study were done by BioEdit software (version 7.1) (Hall, 1999). The phylogenetic analysis was carried out by using Neighbor-Joining (NJ) tree method in MEGA 6 software (Tamura et al., 2013) with 1000 replicates of bootstrap analysis. The phylogenetic trees were edited and displayed by Fig Tree software. *In silico* prediction for the antigenic determinants were applied by Kolaskar and Tongaonkar antigenicity scale (Kolaskar and Tongaonkar, 1990) at IEDB Analysis Resource website (http://tools.immuneepitope.org/bcell/). Five partial sequences of VP2 gene were submitted to the Gene Bank under Accession Numbers from MK333519 to MK333523.

Results and Discussion

Clinical and postmortem findings

Atypical characteristic curve of IBD mortality pattern (sharp death curve and rapid recovery) was observed in all investigated flocks. The clinical signs of the examined chickens included dullness, depression, decreased feed and water intake, ruffled feathers, inability to move followed by death. There was profuse yellowish white, greenish yellow, bloody or brownish diarrhea in most of the affected birds. Moreover, respiratory signs were also observed in 3 examined flocks. Postmortem findings of either freshly dead or sacrificed birds included ecchymotic hemorrhages on thigh and breast muscles, enlarged, edematous and hyperemic bursa with bloody or mucoid contents or atrophic in chronic cases. In some of the examined birds, hemorrhages at the junction between proventriculus and gizzard were also observed. Additionally, Nephrosis and ureters filled with urates were recorded (Figure 1).

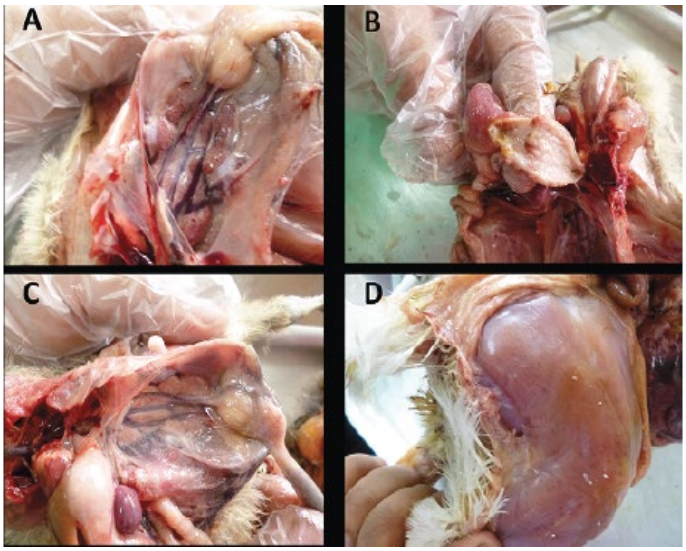

Figure 1: *PM Lesion of chickens suspected to be affected by IBD (AandC) Enlarged bursa and nehprosis (B) Hemorrhages at the junction between proventriculus and gizzard (D) Hemorrhages on thigh muscles. IBD: Infectious bursal disease; PM: postmortem.*

Detection of IBDV by RT-PCR

The PCR amplification of positive samples and control yielded a single specific band of 620 bp without any amplification in the apparently healthy unvaccinated bursal sample. The IBDV was detected in 9 out of 16 (56.25 %) investigated chicken flocks by RT-PCR assay. From which five isolates were from broiler chicken flocks and the other four were from Egyptian balady ones with a percentage of 31.25% (5/16) and 25% (4/16), respectively.

Alignment and phylogenetic analysis of HVR-VP2 gene

The nucleotide and deduced amino acid sequences of the HVR-VP2 were determined for 5 selected Egyptian IBDV clinical isolates representative to different localities included in this study. Phylogenetic analysis of the nucleotide sequences showed that the examined isolates were genetically included in the genotype 3 of the new classification of IBDV that contains the vvIBDV strains from different countries (Figures 2).

The observed identity percentages of these examined isolates with the UK661 strain (the reference strain of vvIBDV strains) were 94 to 97% and 94.6 to 100% at the nucleotide and amino acid sequence levels, respectively. Moreover, the identity % between these five Egyptian isolates is ranged from 93.9- 97.4% at the nucleotide sequence and 93.5-95.6% at the amino acid sequence. Meanwhile, the identity between these Egyptian isolates and the vaccine strains is ranged from 89.5-95.6% at the nucleotide sequence and 88.1-97.8% at the amino acid sequence. Interestingly,

IBDV-Egy1 showed the lowest identity to different viruses in comparison to other Egyptian field isolates.

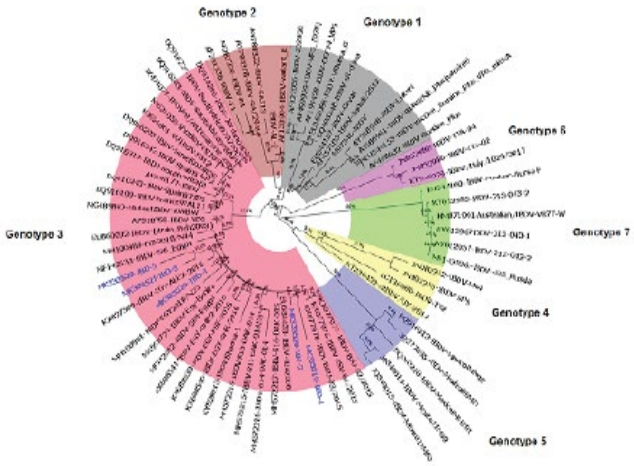

Figure 2: *Phylogenetic analysis Nucleotide sequence of the hyper variable region of VP2 gene for 5 Egyptian field isolates labeled with blue font. The tree was applied by MEGA X software with the neighbor-joining method with 1000 bootstrap replicates, this tree illustrates that the Egyptian isolates of our study are genetically included in the genotype 3 of the new classification of IBDV (with red shadow) that contains the vvIBDV strains from different countries.*

At the scale of amino acid variability, all of the examined isolates revealed the characteristic of vvIBDV amino acid substitutions at residues 222A, 242I, 256I, 294I, and 299S beside amino acid 220F that specific for the Egyptian strains (Table 1). The predictive antigenic determinants were identified by Kolaskar and Tongaonkar antigenicity scale for the HVR-VP2 of our field Egyptian isolates, UK661 and one of the commercially available vaccines (Bursin) (Classical strain). This analysis revealed changes in the predictive structure of the antigenic determinants among different strains where these isolates were closely similar to each other and to UK661 strain with minor deviations in some area. Meanwhile the classic strain (Bursin) showed strong deviation in the structure of the predictive determinants in comparison to these vvIBDV strains (Figure 3).

One of the major problems observed in the Egyptian field is the frequent outbreaks of IBD in spite of the intensive vaccination policy using the available IBD vaccines. This vaccination failure could be due to changes in VP2 (which is major protective antigen of IBDV) that may result from genetic reassortment between more than one strains or immunological pressure (Hon et al., 2006). Moreover, little information is available about the molecular characterization and phylogenetic analysis of IBDV strains especially in Sharkia governorate. Therefore, this study was aimed to direct molecular detection as well as sequence analysis of IBDV field isolates from infected Egyptian balady and broiler chicken flocks in different localities at Sharkia Governotare, Egypt.

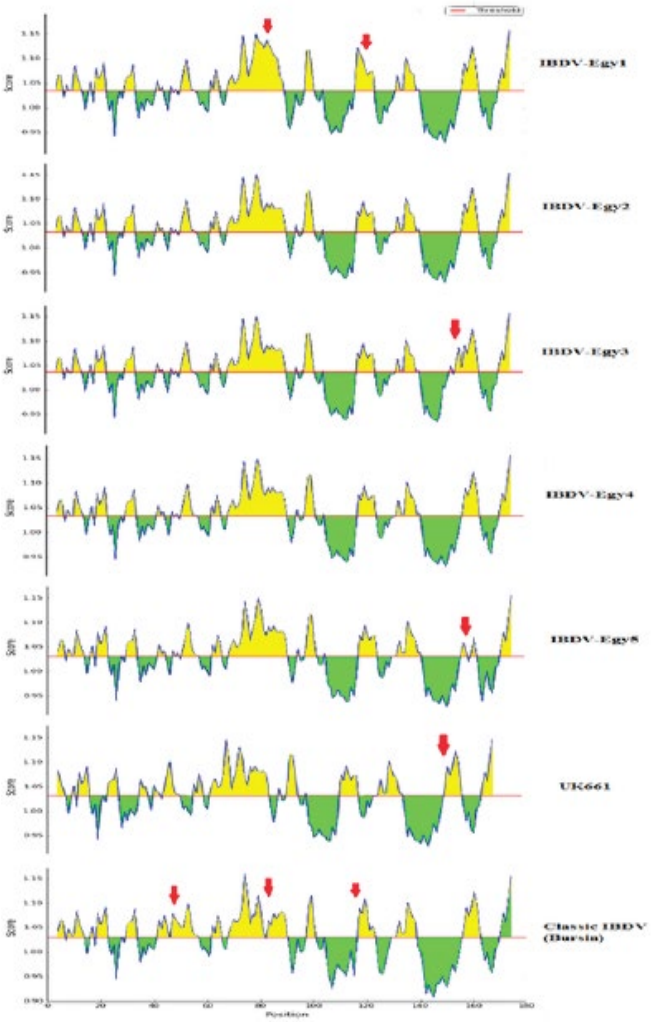

Figure 3: *The predictive antigenic determinants were identified by Kolaskar and Tongaonkar antigenicity scale for the HVR VP2 of the 5 selected isolates of our study and UK661 (vvIBDV Reference strain) and Bursin (Classical strain). This analysis revealed changes in the predictive structure of the antigenic determinants among the different strains along the HVR VP2, the vvIBDV field isolates were closely similar to each other and to UK661 the reference strain of very virulent strains with minor deviations in some area while the classic strain (Bursin) showed strong deviation in the structure of the predictive determinants in comparison to the vvIBDV. The sites of alterations were pointed with red arrows.*

In our study, the recorded clinical and postmortem findings in addition to mortality pattern confirmed the suspicion of IBDV infection as previously mentioned by various researchers (El-Bagoury et al., 2015; Alkhalefa et al., 2018). The observed Greenish yellow, bloody and brownish diarrheas were attributed to concurrent parasitic and bacterial infections.

Table 1: *The amino acid changes in the antigenic regions of P-loop domain of the HVR-VP2 protein.*

	IBDV strains	Major hydrophilic antigenic peak A-P_{BC} (aa 210–225)	Major hydrophilic antigenic peak B-P_{HI} (aa 312 to 325)	Minor hydrophilic peak 1- P_{DE} (aa 248–254)	Minor hydrophilic peak 2- P_{FG} (aa 279–290)
VVIBD strains (genotype 3)	IBDV-Giza2008	F220 in all Egyptian strains , A222	A321	I242, Q249,Q253, I256, T269	D279, A284, I294, S299
	IBDV-Egy1				
	IBDV-Egy2				
	IBDV-Egy3				
	IBDV-Egy4				
	IBDV-Egy5				
	IBDV-EGY-LAY-ALEX-016				
	IBDV-IBDV-S13-DAK-014				
	IBDV-Jordan04E				
	IBDV-Israel99ISR30				
	IBDV-SouthAfrica97SA2				
	IBDV-Spain97SP2				
	IBDV-France97AL11				
Classic IBDV strains (vaccine strains)	IBDV-IBDV78/AB-ICvacc	Y220, P/L222	A321	I242, Q249, Q253, G254, A/V256, T269, T270	D279, A284, I286, 1294, N299
	IBDV-vacc-Bursine Plus				
	IBDV-Cevac-IBD-L				
	IBDV-Lukert				
Variant IBDV strains	IBDV-variant E	Y220, T222	N317, A321	V242, H/Q249, Q253, V256, T269	N279, A284, V294, N299
	IBDV-AL-2				

The IBD virus was detected in the investigated chicken flocks by partial amplification of VP2 gene using RT-PCR. This result goes in parallel with that previously recorded in another recent study conducted on Asyut Province, Egypt, where 10 out of 15 (66.67%) examined bursal samples were positive (Mohamed et al., 2014). Other researches conducted in Egypt also recorded (37% and 38%) isolation rates of IBDV from chickens from different Egyptian Governorates (Abdel-Alem et al., 2003; Sara et al., 2014). On another hand, a higher IBDV recovery rate was also recorded in a recent study in Egypt, where RT-PCR revealed successful detection of IBDV in 17 out of 20 examined samples (85%) (Alkhalefa et al., 2018).

The VP2 is the major capsid protein in IBDV particle that possess pathogenic and antigenic determinants that specific for neutralizing antibodies (Saugar et al., 2005). The VP2 is folded into three domains designated base (B), shell (S) and projection (P) (Coulibaly et al., 2005; Lee et al., 2006). The P-loop domain is the hyper variable region of VP2 (aa 206 to 350) with high mutation rate that was found to play a critical role in the virus antigenicity (Letzel et al., 2007). Based on the nucleotide sequence of the hypervariable region of VP2 gene, IBD viruses were classified genetically into three main genogroups that were identified as: genogroup 1 that including the classical form of the IBDV and it is considered the major genotype; genogroup 2 that represents the variant viruses and genogroup 3 which includes the vvIBDV pathotype or vvIBDV reassortant (Van den Berg et al., 2004). Consequently, the phylogenetic tree of our study illustrated that all the Egyptian analyzed isolates were genetically related to the vvIBDV. However, according to the most recent study on the HVR-VP2 (Michel and Jackwood, 2017), the worldwide IBDV strains were classified phylogenetically into 7 genotypes, the Egyptian isolates were including in the genotype 3 with other Middle East and Eurasian circulating vvIBDV strains. Similar findings were reported previously in another study carried out in Egypt (Sara et al., 2014).

The amino acid residue 222 has a critical role in the antigenicity of the virus (Brown et al., 1994), also it is a characteristic for each genotype because it

was specifically identified as A222 in the vvIBDV Egyptian strains (Michel and Jackwood, 2017) and S222 in Indonesia strains (Parede et al., 2003) while it was recorded as P/L222 in classic genogroup virus (Michel and Jackwood, 2017). However, the alteration of this amino acid residue P222T in classical strains was found to be shifted antigenically and caused vaccinal failure in USA during 1980s (Brown et al., 1994). In addition to the presence of the amino acid residues Q249 and Q253 in antigenic site 2 and D279 and A284 in the antigenic site 3 along the P- loop domain of the VP2 gene of Egyptian viruses and similar to those of the vaccine strains that are used in Egypt like Bursin plus and CEVAC-IBD-L. The existence of combination of the 2 amino acid alterations H253Q and T284A was found to be enhancer for the virulence of vvIBDV (Li et al., 2015).

Accordingly, IBDV-Egy1 is related to the Egyptian vvIBDV but with some deviations in the amino acids in comparison to the other Egyptian strains; A259V, L263F and T269N substitutions were located in adjacent to the antigenic site 1 and 3, in addition to M290I and I302N substitutions that were near to the antigenic site 4. Consequently, these mutations lead to alteration in the structure of the predictive antigenic determinants in the HVR-VP2; as it was illustrated in Figure 3. These mutations in IBDV-Egy1 strain could be induced due to the massive using of vaccination in improper way (Mawgod et al., 2014). Also, it was observable that there were dramatic differences in the predictive antigenic determinants between the vvIBDV Egyptian isolates and the classic vaccine strain (Bursin plus) that may explain the failure of vaccination in protection.

In conclusion, the present study concluded that the vvIBDV strains still circulate and causing economic losses in the Egyptian field. There are dramatic differences in the predictive antigenic determinants between the vvIBDV strains and the classic vaccine strain that can explain the vaccination failure and reemergence of the disease in Sharkia governate, Egypt. Therefore, we recommend reviewing the vaccination programs and establishing new vaccination strategies to prevent the current IBDV problems.

Author's Contributions

Hala Mohamed Nabil Tolba and Gamelat Kotb Farag Kotb collected and prepared the field samples and clinical data from the examined birds. Naglaa Fathy Saeed Awad contributed in acquisition of data, analysis and interpretation of the results, writing the paper and revising it critically for important intellectual contents. Amany Adel helped in molecular analysis and revised the paper. All the authors have approved the final article version to be submitted.

References

Abdel-Alem, G.A., M.H.H. Awaad and Y.M. Saif. 2003. Characterization of Egyptian field strains of infectious bursal disease virus. Avian Dis. 47: 1452-1457. https://doi.org/10.1637/7032

Alkhalefa, N., M. El-Abasy, S. Kasem and E. Abu El-Naga. 2018. Molecular characterization of infectious bursal disease virus (IBDV) isolated from commercial broiler chickens in Nile Delta, Egypt. Bulg. J. Vet. Med. ISSN 1311-1477.

Banda, A., P. Villegas. 2004. Genetic characterization of very virulent infectious bursal disease viruses from Latin America. Avian Dis. 48(3):540–549. https://doi.org/10.1637/7157-12304R

Brown, M.D., P. Green, M.A. Skinner. 1994. VP2 sequences of recent European very virulent isolates of infectious bursal disease virus are closely related to each other but are distinct from those of 'classical' strains. J. Gen. Virol. 75 (Pt 3): 675-680. https://doi.org/10.1099/0022-1317-75-3-675

Coulibaly, F., C. Chevalier, I. Gutsche, J. Pous, J. Navaza, S. Bressanelli, B. Delmas, F.A. Rey. 2005. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell. 120 (6): 761-772. https://doi.org/10.1016/j.cell.2005.01.009

El-Bagoury, G.F., A.F. Nada, A. El-Habbaa and A.A. Abu-Zied. 2015. Molecular characterization of IBD virus isolated from Giza governorate, Egypt, 2014. Benha Vet. Med. J. 28: 223-234. https://doi.org/10.21608/bvmj.2015.32750

El-Sergany, H.A., A. Moursi, M.S. Saber and M.A. Mohamed. 1974. A preliminary investigation on the occurrence of gunmboro disease in Egypt. Egypt. J. Vet. Sci. 11: 185-208.

Eterradossi, N. and Y.M. Saif. 2013. Infectious bursal disease. In: diseases of poultry, Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez, D.L., Nair, V.L. (eds.): 13th Edn., pp: 219-46. Wiley-Blackwell, Hoboken, New Jersey, USA. https://doi.

org/10.1002/9781119421481.ch7

Xiumiao, H., G. Dingming, W. Ping Y. Xiuving, W. Guijun, Q. Aijian. 2012. Molecular epidemiology of infectious bursal disease viruses isolated from Southern China during the years 2000–2010. Virus Gen. 45: 246–255. https://doi.org/10.1007/s11262-012-0764-3

Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

Hon, C.C., T.Y. Lam, A. Drummond, A. Rambaut, Y.F. Lee, C.W. Yip, F. Zeng, P.Y. Lam, P.T. Ng, F.C. Leung. 2006. Phylogenetic analysis reveals a correlation between the expansion of very virulent infectious bursal disease virus and reassortment of its genome segment B. J. Virol. 80(17): 8503-8509. https://doi.org/10.1128/JVI.00585-06

Islam, M.R., S. Rahman, M. Noor, E.H. Chowdhury and H. Müller. 2012. Differentiation of infectious bursal disease virus (IBDV) genome segment B of very virulent and classical lineage by RT-PCR amplification and restriction enzyme analysis. Arch. Virol. 157(2): 333-336. https://doi.org/10.1007/s00705-011-1159-9

Jackwood, D.J. and S.E. Sommer. 2005. Molecular studies on suspect very virulent infectious bursal disease virus genomic RNA samples. Avian Dis. 49(2): 246-251. https://doi.org/10.1637/7294-102604R

Kataria, R.S., A.K. Tiwari, S.K. Bandyopadhyay, J.M. Kataria and G. Butchaiah. 1998. Detection of infectious bursal disease virus of poultry in clinical samples by RT-PCR. Biochem. Mol. Biol. Int. 45 (2): 315-322. https://doi.org/10.1080/15216549800202682

Khafagy, A.K., A. El-Sawy, B. Kouwenhoven, E. Vieltitz, I.M. Ismail, A.A. Amer, H. Sultan, A.E. El-Gohary. 1991. Very virulent infectious bursal disease. Vet. Med. J. Giza. 39(2): 299-317.

Kolaskar, A.S. and P.C. Tongaonkar. 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276 (1-2): 172- 174. https://doi.org/10.1016/0014-5793(90)80535-Q

Lee, C.C., T.P. Ko, C.C. Chou, M. Yoshimura, S.R. Doong, M.Y. Wang and A.H. Wang. 2006. Crystal structure of infectious bursal disease virus VP2 subviral particle at 2.6A resolution: implications in virion assembly and immunogenicity. J. Struct. Biol. 155(1):74-86. https://doi.org/10.1016/j.jsb.2006.02.014

Letzel, T., F. Coulibaly, F.A. Rey, B. Delmas, E. Jagt, A.A.M.W. van Loon and E. Mundt. 2007. Molecular and Structural Bases for the Antigenicity of VP2 of Infectious Bursal Disease Virus. J. Virol. 81 (23): 12827–12835. https://doi.org/10.1128/JVI.01501-07

Li, Z., X. Qi, X. Ren, L. Cui, X. Wang and P. Zhu. 2015. Molecular characteristics and evolutionary analysis of a very virulent infectious bursal disease virus. Sci. China Life Sci. 58(8): 731-738. https://doi.org/10.1007/s11427-015-4900-x

Lukert, P.D. and Y.M. Saif. 2003. Infectious bursal disease. In: Diseases of poultry, Saif Y.M., Barnes H.J., Fadly A.M., Glisson J.R., McDougald L.R., Swayne D.E., editors. (ed) 11th edition, pp.161–179. Iowa State Press, Ames, Iowa.

Metwally, A.M., A.A. Yousif, I.B. Shaheed, W.A. Mohammed, A.M. Samy, I.M. Reda. 2009. Re-emergence of very virulent IBDV in Egypt. Int. J. Virol. 5 (1): 1–17. https://doi.org/10.3923/ijv.2009.1.17

Michel, L.O. and D.J. Jackwood. 2017. Classification of infectious bursal disease virus into genogroups. Arch. Virol. 162 (12): 3661-3670. https://doi.org/10.1007/s00705-017-3500-4

Mohamed, M.A., K.E.S. Elzanaty, B.M. Bakhit and M.M. Safwat. 2014. Genetic characterization of infectious bursal disease viruses associated with gumboro outbreaks in commercial broilers from asyut Province, Egypt. ISRN Veterinary Science, Volume. ID 916412. https://doi.org/10.1155/2014/916412

Mawgod, S.A., A.S. Arafa and A. Hussein. 2014. Hussein molecular genotyping of the infectious bursal disease virus (IBDV) isolated from broiler flocks in Egypt. Int. J. Vet. Sci. Med. 2(1): 46–52. https://doi.org/10.1016/j.ijvsm.2014.02.004

Parede, L.H., S. Sapats, G. Gould, M. Rudd, S. Lowther, J. Ignjatovic. 2003. Characterization of infectious bursal disease virus isolates from Indonesia indicates the existence of very virulent strains with unique genetic changes. Avian Pathol. 32(5):511-518.

Sara, A.M., Abdel, S.A. and H. A. Hussein. 2014. Molecular genotyping of the infectious bursal disease virus (IBDV) isolated from Broiler Flocks in Egypt. Int. J. Vet. Sci. Med. 2(1): 46–52.

Saugar, I., D. Luque, A. Oña, J.F. Rodríguez, J.L. Carrascosa, B.L. Trus, J.R. Castón. 2005. Structural polymorphism of the major capsid protein of a double-stranded RNA virus: an amphipathic alpha helix as a molecular switch. Structure. 13 (7): 1007-1017. https://doi.org/10.1016/j.str.2005.04.012

Snyder, D.B., V.N. Vakharia and P.K. Savage. 1992. Naturally occurring neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. Arch. Virol. 127(1-4): 89–101. https://doi.org/10.1007/BF01309577

Tamura, K., G. Stecher, D. Peterson, A. Filipski, S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12): 2725-2729. https://doi.org/10.1093/molbev/mst197

Van den Berg, T.P., D. Morales, N. Eterradossi, G. Rivallan, D. Toquin, R. Raue, K. Zierenberg, M.F. Zhang, Y.P. Zhu, C.Q. Wang, H.J. Zheng, X. Wang, G.C. Chen, B.L. Lim and H. Müller. 2004. Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. Avian Pathol. 33 (5): 470-476. https://doi.org/10.1080/03079450400003650