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The Role of Genetic Variation of IBD on the Severity of AI

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THIS WORK IS DEDICATED TO …….

My Parent,

Brothers,

Sister,

Wife

&

My sons Rayan &Ali

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aa	Amino acid
Ab	Antibody
AI	Avian influenza
AIV	Avian influenza virus
ALV	avian leukosis virus
atIBDV	Attenuated infectious bursal disease virus
avIBDV	Antigenic variant infectious bursal disease virus
BF	bursa of Fabricius
bp	Base pair
CAM	Chorioallantoic membrane
CAV	chicken anemia virus
CEF	chick embryo fibroblast cells
CRCs	Chicken red blood cells
cvIBDV	classical virulent Infectious bursal disease virus
DNA	Deoxy ribonucleic acid
dsRNA	Double strand Ribonucleic acid
ECE	embryonated chicken eggs
ELISA	Enzyme linked immunosorbent Assay
GMT	geometric mean titer
HA	Hemgglutination
HI	Haemagglutination inhibition
HPAI	High pathogenic avian influenza
IBA	Infectious bursal agent
IBD	Infectious bursal disease

IBDV	Infectious bursal disease virus
IL	interleukin
MDA	maternally derived antibody
MDV	Marek's disease virus
mRNA	Messenger ribonucleic acid
NDV	Newcastle disease virus
nt	nucleotide
OIE	World Organisation for Animal Health
ORF	Opening reading frames
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
RE	reticuloendothelial
RLQP	reference Laboratory for Veterinary Quality control on poultry production
RNA	Ribonucleic acid
rRT-PCR	Real-time Reverse Transcriptase polymerase chain reaction
RT-PCR	Reverse Transcriptase polymerase chain reaction
SPF	Specific pathogen free
ssRNA	single-stranded ribonucleic acid
TIR	Toll-interleukin-1 receptor
TLRs	Toll-like receptors
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3

VP4	Viral Protein 4
VP5	Viral Protein 5
vv IBDV	Very Virulent infectious bursal disease virus
WHO	World health organisation

INTRODUCTION

I. INTRODUCTION

Citrus is one of the most important fruit crops in the world and ranked first among fruit crops in Egypt. The cultivated area with citrus in Egypt has enormously increased through the last decades with **429778.6** fed. fruiting area and Producing about **4388325** tons with average of **10.21** tons/ fed. Tangerine (*Citrus reticulata*, Blanco) is one of the most important citrus species. Total fruiting areas of mandarin and tangerine varieties occupy **109609.5** fed. producing about **1038753** tons with average of **9.48** tons/fed., representing **25.5%** of total citrus production, (**FAO statistics, 2017).**

Murcott is believed to be produced by the USA department of agriculture citrus breeding program in Florida around 1916, which is a cross between a tangerine and sweet orange. Murcott trees are moderate in size and vigor with a upright growth habit. The fruit tends to be borne near the outside of the tree. The fruit is medium-sized when the tree is carrying a moderate fruit load. The peel is yellowish-orange and the flesh is a deep orange at maturity. The rind is thin, smooth, and peels moderately well. Seeds per fruit about 10-20 and commercial harvest season is in January – March **(Futch and Jackson, 2003).** Alternate bearing often occurs in this variety. In years of large crops, the nutritional inputs particularly nitrogen and potassium must also be increased due to the greater nutrient demand extending into the fall of the year (**Tucker *et al.,* 1998**). Nutrition adequate of nitrogen for citrus trees is essential for optimum vegetative growth and top fruit yield and quality. Nitrogen is very

important element in nutrition not only because of its high requirement by plants, but also because it has an extreme importance in plants as a constituent of proteins, nucleic acids, vitamins, hormones, chlorophyll pigments and many other organic compounds, meaning that it is structurally involved in most catalytic molecules **(Sakakibara *et al.*, 2006 and Garnica *et al.*, 2010)**. Also, nitrogen affects the absorption and distribution of all other elements, and it is particularly important to the tree during flowering and fruit set. **(Obreza, 2001, Zekri and Obreza, 2002 and Obreza *et al.*, 2008).**

Studies in this respect, revealed that 900 - 1300 g N/ tree/year was optimum for Navel orange in South Africa **(De Villiers, 1969),** Australia **(Mungomery *et al.*, 1978)** and Spain **(Legaz *et al.*, 1981).** Increasing nitrogen rate over the optimum dose encourages excessive vegetative growth and may cause ground water contamination when leached with excess irrigation water **(Davies and Albergo, 1994 and Schuman *et al.*, 2003).**

Mineral fertilizers are expensive in Egypt, as well as the various disadvantages of it and danger to human health. Therefore, some natural organic materials were used to achieve great improvement in soil fertility and productivity of fruit trees.

Organic fertilization is used as a substitute for mineral N fertilization. Application of organic manure has numerous merits such as reducing soil pH, increasing the availability of all nutrients, reducing soil salinity, enhancing soil fertility, water retention, soil organic matter, as well as increasing biological activity of microflora, soil cation exchange, natural hormones and antibiotics (**Nijjar, 1985**). Application of organic fertilizers

is a production system avoids or largely excludes the use of synthetic chemical fertilizers **(Abdelaal *et al.*, 2010).** Chicken manure is relatively rich in content of nitrogen and has a low cost per pound of nitrogen **(Granatstein, 2003)**. Application of poultry manure can improve chemical, biological and physical quality for soil and plant growth **(Canali *et al.*, 2004 and Hilimire *et al*., 2012).** Chicken manure contains both organic and inorganic forms of the plant nutrients. Nutrients present in the inorganic form can readily available to plants whereas organic nutrients become available as the manure decomposes, but may not be available until the next season **(McCall, 1980).**

Bio-fertilization based on altering the rhizosphere flora by seed or soil inoculation with certain organisms capable of inducing beneficial effects on a compatible host **(El-Haddad *et al.*, 1993)**.Bio-fertilizers are biological preparation containing life or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing or cellulolytic microorganisms which accelerate certain microbial process to augment the extent of the availability of nutrients in a form can be easily assimilated by plants **(Subba-Rao, 1993)**. Several processes other than nitrogen fixation could account for these positive effects, including production of growth regulators, protection from root pathogens and modification of nutrient uptake by the plant **(Techan, 1988).** The use of bacteria in combination with organic fertilizers results in encouraging yield, particularly in new reclaimed soils, through overcoming drought, salt and some pathogens stresses as well as decreasing the applied fertilizers and increasing the availability of most macro and microelements. Inoculation with N bio-

fertilizers could save half the normal field rate of N chemical fertilizers and at the same time promote plant production **(Ishac, 1989).**

Recently, bio fertilization is considered an important tool to enhance yield and fruit quality of citrus trees and becomes a positive alternative to chemical fertilizers. They are safe for human, animal and environmental conditions. Its use was accompanied with reducing the great pollution occurred on our environment as well as for producing organic foods for export **(Abdelaal *et al.*, 2010).** Biofertilizers have been developed to enhance nutrient uptake and satisfy requirements of several composts for fruit trees. Hence, several beneficial micro-organisms can be effectively used as alternative to chemical fertilizers to minimize the environmental pollution. N-fixing bacteria like *Azotobacter* spp. have been developed in several laboratories in Egypt **(Fawzi *et al.*, 2010).** Nowadays, clean agriculture has received more attentions by application of different compost sorts and biofertilizers to minimize environmental problems, as well as improving structure and fertility in different soil types. **(Shahain *et al.*, 2007).**

Effective microorganisms (EM1) is a biofertilizer, created in the University of Ryukyus in Okinawa in Japan over 25 years ago and marketed as EMRO (EM Research Organization). The basic purpose of EM1 is the restoration of healthy ecosystem in both soil and water by using three major genera of microorganisms which are found in nature: phototrophic bacteria (*Rhodopseudomonas*), lactic acid bacteria (*Lactobacillus*) and yeast (*Saccharomyces*). EM contains *Lactobacillus plantarum*,

L. casei, *L. fermentum*, *L. delbrueckii*, *Saccharomyces cerevisiae* and *Rhodopseudomonas palustris* **(Abd-Rabou, 2006 and Higa, 2010)**.

Higher yield, greater juice contents and thinner peels in the EM treatments, can be correlated with improved soil chemical and physical conditions, determined by the use of effective microorganisms at the time the citrus plants were in bloom and fruits were forming in late winter **(Paschool *et al.*, 1999)**. The positive action of bio and organic fertilizer on Balady mandarin trees could be mainly due to their effect in supplying the trees with their adequate requirements of various nutrients at relatively longer time, as well as, reduce nitrite pollution and produce organic fruits with higher quality **(Tawfiek & Gamal, 2000, Ahmed *et al.*, 2013 and Mostafa and Abdel-Rahman, 2015).**

The main objective of this experiment is to evaluate the effect of replacement mineral N fertilization by organic and biofertilizers on vegetative growth, yield, fruit quality, leaf mineral content, as well as avoiding the contamination of the Murcott tangerine fruits as a one of newly major exportal fruit crop.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

History

Infectious bursal disease (IBD) was firstly recognized as a distinct clinical entity in 1957 **and** initially was described as "avian nephrosis" on account of the tubular degenerative lesions found in the kidneys of infected broiler chickens. The syndrome adopted the name "Gumboro disease" since the first outbreaks occurred in and around the area of Gumboro, Delaware, USA. Predominant signs of illness included trembling, ruffled feathers, watery diarrhea, anorexia, depression, severe prostration, and death. In addition, hemorrhages in the thigh and leg muscles, increased mucus in the intestine, renal damage, and enlargement of the bursa of Fabricius were lesions commonly observed at necropsy **(Cosgrove, 1962)**.

Early studies suggested that the causative agent was a nephropathogenic strain of infectious bronchitis virus due to similar gross changes observed in the kidney and after successful isolation of IBA in embryonated chicken eggs (**Winterfield and Hitchner, 1962)**.

Hitchner (1970) proposed that the disease be termed "infectious bursal disease" due to its pathognomonic bursa lesions.

The immunosuppressive effects of IBD virus (IBDV) infections were first disclosed by **Allen *et al.*, (1972)**. Subsequent studies by **Winterfield, *et al.* (1978),** however,

revealed that IBV immunized birds could still be infected with the "infectious bursal agent" (IBA) and develop changes in their cloacal bursa specific for the disease.

In 1980, a second serotype was reported **McFerran, *et al*. (1980).** These factors, along with the high tendency for IBDV infections to recur in successive flocks, emphasized the need for stringent measures of prevention and control. Prior to 1984, spread of both the clinical and subclinical forms of the disease was satisfactorily controlled by vaccination programs. However, in 1984 and 1985, a significant increase in mortality, condemnations, and vaccine failures were reported in the Delmarva Peninsula broiler growing area (**Saif, 1984).**

These newly emergent viruses were capable of breaking through maternal immunity against classic strains of IBDV (Rosenberger **and Cloud, 1986).** *In vivo* reciprocal cross-challenge tests showed that unlike classic or standard strains of IBDV, the field isolates caused rapid atrophy and minimal inflammation of the cloacal bursa when inoculated into susceptible Specific pathogen free (SPF) leghorns chicks (**Rosenberger, *et al.*, 1987).** Studies suggested that a major antigenic shift in serotype I viruses had occurred in the field **(Snyder, *et al.*, 1992).** The IBDV field isolates were characterized as antigenic "variants" of serotype 1 IBDV, while the older serotype 1 viruses discovered prior to these newly emergent viruses were called classic strains of IBDV (**Rosenberger and Cloud 1986).** For more than decade in the United States, clinical cases are rarely reported and these variant

strains are the predominant viruses circulating in the field **(Eterradossi and Saif 2008).**

Outbreaks of very virulent IBDV (vvIBDV) were first reported in Europe in 1987-1988 **(Eterradossi *et al.*, 1992).** These vvIBDV infections are characterized by a peracute onset of severe clinical disease and high mortality **(Tsukamoto *et al.*, (1992).**

IBD was first reported in Egyptian flocks in the early seventies **(El-Sergany *et al.*, 1974)** however interest in IBDV antigenic characterization was triggered by the appearance of the very virulent IBD in vaccinated Egyptian flocks **(El-Butrawi and El Kady 1990 and Khafagy *et al.*, 1991).** Several reports have classified the Egyptian IBDV isolates as classical IBDV **(Khafagy *et al.*, 1991 and Bakhit, 1996).** On the other hand, some reports have provided partial evidence of the presence of antigenically variant IBD strains in Egyptian flocks **(El-Sonousi *et al.*, 1994 and Sultan, 1995).** Presently, evidence of circulating variant IBDV strains were isolated from flocks vaccinated using classical IBDV vaccines **(El-Khiat, 2003; Hussein *et al*., 2003 and Metwally *et al*., 2003).**

Several reports classified the Egyptian IBDV isolates as classical IBDV **(Ibrahim, 2000)**. Presently, evidence of circulating variant IBDV strains was isolated from flocks vaccinated using classical IBDV vaccines **(Metwally *et al.*, 2009)**

A molecular and antigenic study of numerous previously isolated Egyptian IBDV strains based only in the hypervariable region have demonstrated their close similarity to vvIBDV strains that circulated earlier in Europe, Asia, and Africa **(Abdel-Alim *et al.*, 2003, Abdel-Mawgod *et al.*, 2014**, **El-Batrawi and El-Kady,1990**, **Metwally *et al*, 2015**, **Mohamed *et al.*, 2014)**

Further invisible flow involving evaluation of the efficacy of the currently used vaccines, as well as continuous genetic characterization of the circulating Egyptian IBDV strains are needed to overcome the vaccination failure problem. **(Abou ElFetouh *et al.*, 2018).**

Etiology

Infectious bursal disease virus (IBDV) infects chickens and belongs to the family Birnaviridae, genus Avibirnavirus. Infection outcomes can be lethal, clinically diseased, or immunosuppressed, depending on the host and virus virulence interactions (**Balamurugan Kataria, 2006),** but all lead to atrophy of the viral target organ, the bursa of Fabricius. The form of acute disease is most prominent in the age between 3-6 weeks**.**

Viruses in this family possess bi-segmented, double-stranded RNA (dsRNA) genomes, which are packaged into single-shelled, non-enveloped virions (**Müller *et al.*, 1972).** The capsid shell exhibits icosahedral symmetry composed of 32 capsomeres and a diameter ranging from 55 to 65 nm **(Ozel and Gelderblom1985).** Its structure is based on a T = 13 lattice

composed of trimeric subunits. Cryoelectron microscopy and image processing analysis showed that the outer surface of the viral capsid is made up of 260 trimeric VP2 clusters, while the inner surface is composed of 200 Y-shaped trimeric VP3 structures **(Bottcher *et al.*, 1997)**.

Infectious bursal disease virus is very stable and can persist indefinitely in poultry houses despite thorough routine cleaning and disinfection (**Lukert and Hitchner, 1984)**.

Due to the economics of commercial poultry production, which involve the re-use of litter and short time intervals between flocks, as well as the virus's resistance toward heat and several physical and chemical agents, IBDV survives in poultry houses for long periods (**Lukert, and Saif, 1997).**

Two serotypes of IBDV (1 and 2) are described, distinguished by cross-virus neutralization test **(Jackwood *et al.*, 1985).** IBDV strains of serotype 1 are pathogenic only in chickens **(Oladele *et al.*, 2008),** and further classified as classical virulent IBDV (cvIBDV), very virulent IBDV (vvIBDV), antigenic variant IBDV (avIBDV) and attenuated IBDV (atIBDV) **(Den Berg *et al.*, 2004).** Whereas, strains of serotype 2 are naturally avirulent for chickens **(Cummings *et al.*, 1986; Ismail *et al.*, 1988).**

The genome of IBDV is comprised of two segments of dsRNA as demonstrated by polyacrylamide gel electrophoresis **(Müller *et al.*, 1979).** The larger segment, A, is 3261 nucleotides long and contains two open reading frames (ORF). The first

ORF, preceding and partially overlapping ORF2, encodes a 17 kDa non-structural protein, known as VP5 **(Mundt *et al*,. 1995)**. The second ORF encodes a 110 kDa precursor polyprotein (NH3-VPX-VP4-VP3- COOH), which is co-translationally processed to yield the protein capsid precursor VPX or pVP2 (48 kDa), and proteins VP4 (28 kDa) and VP3 (32 kDa) **(Hudson, *et al*., 1986).**

The pVP2 is further processed by serial cleavages near its carboxy-terminus into mature VP2 (41 kDa) and four peptides which remain associated with the virion **(Da Costa *et al*., 2002).**

VP2 and VP3 are the major structural proteins of the virion, whereas VP4 is a virus-encoded protease **(Kibenge and Dhama, 1997).** The pVP2 initially undergoes VP4-mediated processing events to yield shorter pVP2 polypeptides **(Irigoyen *et al*., 2009)**.

Recently, **(Irigoyen *et al*., 2009)** proposed that VP2 Asp-431 is responsible for catalyzing the last pVP2 to VP2 proteolytic event that occurs during capsid maturation. Genomic segment B, which is 2827 nucleotides long, encodes VP1, a 97-kDa RNA dependent-RNA-polymerase (RdRp) **(Von Einem *et al*., 2004).**

Recently, four antigenic sites were predicted in the hypervariable region of VP2, designated site 1 (aa 211-225), site 2 (aa 245-256), site 3 (aa 277-289) and site 4 (aa 313-331). (**Islam, 2015)**

In both genomic segments, short 5' and 3' terminal sequences (79 to 111 nucleotides long) flank the coding regions **(Mundt and Müller 1995.** The 3' untranslated regions of both the A and B segment have the potential to form stem and loop secondary structures that may be essential for RNA replication **(Boot and Pritz-Verschuren, 2004.).**

The first ORF encodes the nonessential nonstructural viral protein 5 (VP5) **(Delmas *et al.*, 2005)**.The second ORF encodes a polyprotein that is cotranslationally self-cleaved by the viral protease VP4, yielding the precursor pVP2, VP4, and VP3. The resulting intermediate, pVP2, is further processed at the C-terminal region during maturation into VP2 polypeptide and several peptides that remain associated with the capsid **(Lombardo *et al.*, 2000).**

VP2 is the single structural component of the viral capsid. VP2 and VP3 are the major structural proteins, constituting 60% and 35% of the virion, respectively **(Luque *et al.*, 2009).** Segment B is monocistronic and encodes the viral RNA-dependent RNA polymerase VP1 **(VonEinem *et al.*, 2004).**

Propagation of IBDV field isolates in tissue culture requires previous adaptation involving serial virus passage, a process that invariably leads to the introduction of mutations at specific residues on the VP2 capsid polypeptide, as well as a significant reduction of virus virulence **(Cursiefen *et al.*, 1979; Lange *et al.*, 1987; Hassan *et al.*, 1996;and Yamaguchi *et al.*, 1996b).** This phenomenon constitutes a major obstacle to characterizing

the interaction of pathogenic IBDV strains with susceptible host cells.

In a recent report, **(Terasaki *et al.,* 2008)** showed that very virulent IBDV isolates can be grown directly in chicken lymphoid DT40 tumour cells infected persistently with avian leukosis virus (ALV) **(Baba *et al.,* 1985),** without the requirement for a preliminary adaptation process. Most importantly, serial passage in this cell line does not result in the incorporation of mutations at the specific VP2 residues. Additionally, DT40 cells show an extremely high frequency of homologous recombination **(Buerstedde and Takeda, 1991).** This property has been exploited widely for the generation of derivative cell lines in which selected target genes are inactivated **(Winding and Berchtold, 2001).** Accordingly, DT40 cells appear to be an excellent candidate system to undertake a systematic analysis aimed at determining the specific roles of virus and cellular proteins during the IBDV replication process.

In virions, VP1 exists as both a covalently bound protein at the 5' ends of the genomic dsRNA strands, as well as, a free polypeptide (**Kibenge and Dhama, 1997**). It is responsible for viral RNA replication following cellular infection and mRNA synthesis (**Spies *et al.,* 1987.**). It has also been reported that VP1 may play an important role in IBDV virulence (**Wei *et al.,* 2006.**). The catalytic motifs of polymerases from *birnaviruses* are arranged in a permuted order in the sequence (**Pan *et al.,* 2007**). As a result, the structure of the *birnavirus* polymerase

VP1 adopts a unique active site topology that has not been previously found with other RNA and DNA polymerases (**Pan *et al,* 2007**).

VP2 (441 amino acids [aa]) is the main capsid protein, constituting 51% of the viral proteins in serotype 1 viruses **(Dobos, 1979).** It is the host-protective antigen, as it contains serotype- and strain-specific epitopes responsible for inducing neutralizing antibodies (**Becht *et al.,* 1988.**). It has also been reported to be an apoptotic inducer in mammalian cells, but not in chicken embryo cells (**Fernandez-Arias *et al.,* 1997.**). VP2 is highly hydrophobic and folded into three distinct domains termed the base (B), shell (S), and projection (P) (**Lee *et al.,* 2006**). The B and S domains are comprised of the conserved N- and C-terminal sequences of VP2, while the P domain contains the conformation-dependent central variable region of VP2 (aa 206 to 350) (**Bayliss *et al.,* 1990**). The significance of protein conformation for VP2 interaction with monoclonal antibodies has been affirmed by several studies where denaturing conditions using Western immunoblotting prevented reactivity (**Becht *et al.,* 1988**). Within the central variable region are four stretches of hydrophilic amino acids that are more prone to antigenically significant amino acid changes (**Heine *et al.,* 1991**). These areas, known as major hydrophilic peaks A (aa 212-224) and B (aa 314-324) and minor hydrophilic peaks 1 (aa 249-254) and 2 (aa 279-290) (**Jackwood*et al.,* 2006**) reside in the most exposed parts of the P domain (**Coulibaly *et al.,* 2005**).

The VP3 protein contains both conformational-independent, group-specific epitopes (common in both serotypes) and serotype-specific epitopes (**Casañas *et al.,* 2008**) that elicit non-neutralizing and non-protective antibodies (**Becht, *et al.,* 1988**). This multifunctional protein interacts with VP1, VP2, and with genomic double-stranded RNA and plays a pivotal role in virus assembly and morphogenesis (**Kochan *et al.,* 2003.**).

VP4 is described as a minor, non-structural viral protease. Using a catalytic serine-lysine (Ser-652 and Lys-692) dyad conserved among bacterial Lon proteases, VP4 is responsible for the proteolytic processing of the precursor polyprotein (**Lejal *et al.,* 2000**). **Sanchez and Rodriguez (1999**) identified two cleavage sites, 511LAA513 and 754MAA756, that are important for the processing of the pVP2–VP4 and VP4–VP3 precursors, respectively. In addition, the self-assembling VP4 protease gives rise to specific microtubules (type II tubules), which accumulate within infected cells; however, they are not components of the mature virion (**Granzow*et al.,* 1997**).

The IBDV genome harbours three open reading frames (ORFs) encoding: (i) the RdRp; (ii) a large polyprotein containing three domains, corresponding to the capsid protein precursor (pVP2), the viral protease (VP4) and a multitasking structural polypeptide (VP3); and (iii) a 17 kDa polypeptide, VP5, that accumulates at the cell membrane **(Lombardo *et al.,* 2000).** Although VP5 plays an important role in virus dissemination and pathogenesis **(Yao *et al.,* 1998),** it is not

essential for virus replication in cell culture **(Mundt *et al.*, 1997).** Mutations affecting VP5 expression and/or functionality might reduce virus spreading and thus contribute to the selection of virus populations with reduced infectivity.

IBDV enters the host cell by binding to cellular receptors; several different membrane proteins have been shown to interact with IBDV of different virulence **(Delgui *et al.*, 2009; Luo *et al.*, 2010).**

Lin *et al.*(2007) demonstrated that chicken heat-shock protein 90 (cHsp90) is a functional component of the cellular receptor complex essential for IBDV infection. Among the cellular factors involved in the attachment of IBDV sub viral particles to chicken fibroblast DF-1 cells, cHsp90 has been identified to be a dominant factor by mass spectrometry. In addition, both Hsp90 and anti-Hsp90 can inhibit infection of DF-1 cells by IBDV. They investigated the feasibility of suppressing IBDV infection by using vector-expressed anti-cHsp90 miRNAs. The results show that anti-cHsp90a, but not anti-cHsp90b, miRNA has an inhibitory effect on IBDV infection.

Host cells use various receptors to detect viral infections by recognizing pathogen-associated molecular patterns (PAMPs) and subsequently induce an antiviral response. Prominent among these are Toll-like receptors (TLRs) **(Sang *et al.*, 2008)** Several TLRs recognize viral PAMPs: TLR3, detects double-stranded RNA (dsRNA) derived from viral replication whereas single-stranded RNA (ssRNA) are detected by TLR7 and TLR8 **(Sang**

***et al.*, 2008)**. The TLR signaling proceeds via two pathways; the myeloid differentiation factor 88 (MyD88)-mediated pathway and the Toll-interleukin-1 receptor (TIR)-domain-containing adaptor inducing IFN-b (TRIF)-mediated pathway **(Kawai and Akira 2009)** The TLR signaling pathways arise from intracytoplasmic TIR domains, which are conserved among all TLRs. The TLR7 specifically involves MyD88-dependent pathway, whereas TRIF is implicated in the TLR3-mediated MyD88-independent pathway **(Takeda and Akira, 2004).**

The number of apoptotic cells in the bursa is correlated with viral replication. VP2 and VP5 are the only two viral proteins that have been associated with apoptosis induction **(Fernandez-Arias *et al.*, 1997 and Yao *et al.*, 1998).** Apoptosis occurs at the late stage of viral replication; about 12 to 14 h post infection. Apoptosis was also suggested to be the anti-viral mechanism of the host to prevent virus spread **(Jungmann *et al.*, 2001).** VP5 deficient viruses caused earlier and greater apoptotic effects than the wild-type strain. The apoptosis was demonstrated to be caspase-dependent **(Liu and Vakharia, 2006).** This early apoptosis was accompanied by diminished production of the virus. It was suggested that VP5 acts as an anti-apoptotic regulatory protein at early stages of infection to prevent infected cells from dying before the virus completes its infection cycle and there after targets the plasma membranes to induce lysis **(Liu and Vakharia, 2006).**

Variant strains of IBDV are usually isolated from vaccinated flocks. These IBDV variants are antigenically

different from classical one as a method of prevention of IBD classic strains of IBDVas it is devoid of classical epitope(s) defined by neutralizing monoclonal antibodies **(Helal*et al.*, 2012)** Most of these epitopes are located in the VP2 hyper variable region **(Mardassi *et al.*, 2004)** Very virulent IBDV (vvIBDV) strains have now spread all over the world **(Aricibasi *et al.*, 2010)**

Transmission

Only horizontal transmission has been described, with healthy subjects being infected by the oral or respiratory pathway. Infected subjects excrete the virus in faeces as early as 48 h after infection, and may transmit the disease by contact over a sixteen-day period **(Vindevogel *et al.*, 1976).** The possibility of persistent infection in recovered animals has not been researched. The disease is transmitted by direct contact with excreting subjects, or by indirect contact with any inanimate or animate (farm staff, animals) contaminated vectors. Some researchers have suggested that insects may also act as vectors

Howie and Thorsen (1981) stated that the extreme resistance of the virus to the outside environment enhances the potential for indirect transmission. The virus can survive for four months in contaminated bedding and premises **(Benton *et al.*, 1967)**, and up to fifty-six days in lesser mealworms (Alphitobius sp.) taken from a contaminated building **(McAllister *et al.*, 1995).** In the absence of effective cleaning, disinfection and insect control, the

resistance of the virus leads to perennial contamination of infected farm buildings.

Resistance to disinfectants

The virus is sensitive to sodium hydroxide (it is totally inactivated when pH exceeds 12), but it is not affected at pH 2 **(Benton, 1967)** the iodinated and chlorinated derivatives, as well as the aldehydes (formaldehyde, glutaraldehyde) are also active **(Shirai, 1994).**

The virus is resistant to chloroform and ether treatments, and it remains unaffected after a 5 hour incubation at 56°C and pH 2, although, it can be inactivated at pH 12. Exposure to 0.5% phenol and 0.125% thimerosal for 1 hour at 30°C had no effect, while contact with 0.5% formalin for 6 hours markedly reduced virus infectivity **(Benton *et al.*, 1967).**

Landgraf *et al.*, (1967) demonstrated that IBDV is able to withstand 30 minutes at 60°C, but not 70°C and it is killed after 10 minutes in 0.5% chloramine disinfectant. In addition, invert soaps with 0.05% sodium hydroxide can inactivate or strongly inhibit the virus **(Shirai *et al.*, 1994).**

Clinical signs

The symptoms of the disease are diarrhoea, trembling, weight loss, paleness, depression, lameness, ruffled feathers and ultimately death. The course usually takes 5-7 days during which mortality rises rapidly. The primary target organ, the bursa of Fabricius, shows lesions within 24 hours after infection **(Kaufer**

and Weiss, 1980). Chickens surviving the disease have permanent immunosuppression **(van den Berg *et al.*, 2000),** which results in increased susceptibility to a variety of infectious diseases and poor or no response to vaccination **(Liu *et al.*, 1994).**

Pathogenesis

IBDV infection was also shown to cause changes in the potassium current, as a result of virus attachment or membrane penetration **(Repp *et al.*, 1998).** Potassium channels play important roles in cellular signaling processes and as transport proteins for passive potassium ion movement across membranes **(Shieh *et al.*, 2000).** The effect of IBDV infection on cellular responses was studied by **(Zheng *et al.* 2008).** IBDV infection was shown to turn off the host translational machinery for initiating its viral translation in the infected cells. The infection also caused cytoskeleton disruption, which was suggested to be a mechanism by which IBDV particles are released from infected cells. The infection also suppressed the expression of proteins involved in signal transduction, ubiquitin-mediated protein degradation, stress response, RNA processing, biosynthesis and energy metabolism.

Although IBDV persists in chickens for a short period of time, the lesions in the bursa can last for at least 10 weeks **(Winterfield *et al.*, 1972),** resulting in immunosuppression in infected chickens. The suppressed immune status can increase susceptibility to secondary bacterial or viral infection

(Rosenberger and Cloud, 1989) and reduce the immune response to vaccination against Newcastle disease, resulting in significant economic loss in the poultry industry **(Muller *et al.*, 2003)**

IBDV field isolates mainly infect and destroy actively dividing IgM-bearing B cells in the bursa of Fabricius (BF) and other locations **(Hirai *et al.*, 1981; Rodenberg and Sharma, 1994).** While recent data show that the virus also infects and replicates in macrophages **(Kim *et al.*, 1998; Khatri *et al.*, 2005; Palmquist *et al.*, 2006 and Khatri and sharma., 2008).** Additionally, IBDV can also replicate in chick embryo fibroblast cells (CEF) **(Yamaguchi *et al.*, 1996a),** Vero cells **(Kwon and Kim, 2004),** DF-1 cells (a spontaneously immortalized cell line derived from primary CEF) **(Lin *et al.*, 2007).** VvIBDVs cannot be propagated directly in tissue cultures but the virus can adapt to the tissue cultures by serial blind passages and become attenuated **(Muller *et al.*, 1986).**

The differential immune-pathogenesis of classical and variant strains of IBDV, as compared to vIBDV, cIBDV induced early bursal lesions, extensive infiltration of T cells in the bursa and induced higher expression of pro-inflammatory cytokine and mediators.

IL-6 and iNOS Further, there were differences in the expression of TLR3 and TLR7 and their adapter molecules, TRIF and MyD88, in the bursa of cIBDV and vIBDV-infected chickens. These data demonstrate the differential induction of

innate and T cell responses by cIBDV and vIBDV. Elucidation of the TLRs signaling pathway and factors leading to activation of the immune response to IBDV infection may provide new strategies for the development of cross-protective vaccines that can augment T cell responses in addition to an antibody response, in which mortality may reach as high as 100%. Infection during the first week after hatching may lead to severe defects of humoral immune response as a consequence of the early destruction of the bursa of Fabricius (BF). In contrast, older chickens with regressed bursa do not show signs of illness upon infection (**Sharma *et al.*, 2000),** thus, prevention of IBD by vaccination is critical to poultry health and well-being.

Rosenberger and Cloud (1989) stated that IBDV is resulting in immunosuppression in infected chickens. The suppressed immune status can increase susceptibility to secondary bacterial or viral infection. and reduce the immune response to vaccination against Newcastle disease, resulting in significant economic loss in the poultry industry **(Sharma *et al.*, 2000)** Furthermore, there is growing evidence that IBDV-infected chickens are better vessels than healthy chickens in adaptation of water fowl avian influenza virus (AIV) in domestic poultry, a process that can lead to pathogenic AIV generation **(Ramirez *et al.*, 2010)**.

The IBDV has been characterized molecularly on the basis of sequence analysis of the VP2 variable region. Amino acid (aa) changes might lead to variations in antigenicity, antibody recognition, immunogenicity, tissue tropism, and virulence of

IBDV strains **(Boot *et al.*, 2005).** Sequencing of the hyper variable region of the VP2gene is a preferred tool to identify the sequence characteristics of the classical, variant, or very virulent IBDV (vvIBDV) **(Van den Berg., 2000).** It causes an immunosuppressive disease of young chickens, attacking mainly the bursa of Fabricius. The consequences of IBDV immunosuppression are vaccination failure and increased susceptibility of chickens to other pathogens. Also, the IBDV-infected birds may be a good propagator for other viral agents **(Saif, 1991).**

Gross lesions

Gross lesions observed in birds that succumb to IBDV infection include dehydration of the breast and leg musculature, darkened discoloration of the pectoral muscles, occasional hemorrhages in the leg, thigh, and pectoral muscles, increased mucus in the intestine, and renal changes. The gross appearance of the kidneys may appear normal in birds that are necropsied during the course of infection. In birds that die or are in advanced stages of the disease, kidneys frequently show swelling and pallor with heavy accumulation of urates in the tubules and ureters **(Eterradossi and Saif, 2008, Singh *et al.*, 2015).**The bursa of Fabricius is the predominant lymphoid organ affected by IBDV. Infections with classic strains of IBDV cause inflammation and hypertrophy of the bursa as early as day 3 post-infection. By day 4, the bursa is double its original size and weight due to edema and hyperemia. By day 5, the bursa returns

to its normal weight, but continues to atrophy until reaching one-third or less of its original weight following day 8 post-infection.

In contrast, variant strains of IBDV typically cause a rapid atrophy, mucosal edema, and firmness of the bursa in the absence of inflammation **(Rosenberger and Cloud, 1986)** Only one variant isolate has been reported to cause bursal inflammation **(Hassan *et al.*, 1996)** By day 2 or 3 post-infection, a gelatinous yellowish transudate covers the serosal surface of the bursa and longitudinal striations become visible. The bursa's normal white color shifts to cream and then, in some cases, gray during and following the period of atrophy. In addition, necrotic foci and petechial or ecchymotic hemorrhages on the mucosal surface may be observed in infected bursas.

Moderate to severe splenomegaly with small gray foci uniformly distributed on the surface has been reported**.** Occasionally, petechial hemorrhages will occur in the mucosa at the juncture of the proventriculus and gizzard **(Eterradossi and Saif. 2008).**Compared to moderately pathogenic IBDV strains, vvIBDV strains induce similar bursal lesions, but cause more severe damages to the cecal tonsils, thymus, spleen, and bone marrow **(Tanimura *et al.*, 1995).**

Microscopic lesions

IBDV infections produce microscopic lesions primarily in the lymphoid tissues (i.e. cloacal bursa, spleen, thymus, cecal tonsils, and Hardarian gland). Pathologic observations of experimental cases were reported by Helmboldt and Garner

(Helmboldt and Garner1964). Degeneration and necrosis of B lymphocytes in the medullary region of the bursal follicles is apparent within one day of exposure. Depleted lymphocytes are quickly replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial (RE) cells. By day 3 or 4 post-infection, IBDV-associated lesions are visible within all bursal follicles. At this time, infections with classic IBDV strains have caused an inflammatory response marked by severe edema, heterophil infiltration, and hyperemia in the bursa. Inflammation diminishes by day 4 post-infection (PI), and as necrotic debris is cleared by phagocytosis, cystic cavities develop in the medullary areas of the lymphoid follicles. Necrosis and phagocytosis of heterophils and plasma cells occur within the follicle, as well as, in the interfollicular connective tissue. In addition, a fibroplasia in the interfollicular connective tissue may appear and the surface epithelium of the bursa becomes involuted and abnormal (**Naqi and Millar, 1979).** Proliferation of the bursal epithelial layer generates a glandular structure of columnar epithelial cells that contains globules of mucin. During this stage of the infection, scattered foci of repopulating lymphocytes were observed; however, these did not develop into healthy follicles **(Elankumaran *et al.*, 2002)** Microscopic lesions caused by variant strains are characterized by extensive follicular lymphoid depletion and rapid plical atrophy of the cloacal bursa in the absence of an inflammatory response.

In the early stages of infection, the spleen exhibits hyperplasia of reticuloendothelial cells surrounding the adenoid

sheath arteries. By day 3 PI, diffuse lymphoid necrosis occurs in the germinal centers and around the periarteriolar and periellipsoid lymphatic sheaths. Cell populations in the spleen rapidly recover and the germinal follicles sustain no permanent damage **(Elankumaran *et al.*, 2002)**.

During the acute phase of the infection, the thymus undergoes a marked atrophy and widespread apoptosis of cortical lymphocytes. However, within a few days of infection, the lesions are overcome and the thymus is restored to its normal state **(Inoue *et al.*, 1994)** These lesions have not been associated with virus replication in thymic cells **(Müller *et al.*, 2003),** IBDV-induced damage to the cecal tonsils may involve acute heterophilic inflammation and lymphocyte depletion with regeneration on day 5 PI.

Tanimura and Sharma (1997) reported that antigen-positive cells mainly localized to the germinal centers of the cecal tonsils.

Infection with IBDV causes severe plasma cell depletion in the Harderian gland and prevents the normal infiltration of plasma cells into the gland. This reduction is short-lived and plasma cell counts are restored in approximately 14 days PI **(Dohms *et al.*, 1981)** Lesions characterized by large casts of homogeneous material infiltrated with heterophils have been reported in the kidneys; however, they are minimal and believed to be non-specific **(Eterradossi and Saif, 2008).** Slight perivascular infiltration of monocytes may be observed in the

liver **(Peters, 1967)**. There is evidence that IBDV replication also occurs in the bone marrow **(Elankumaran *et al.*, 2002).**

Serology

In areas contaminated by IBDV, most broiler flocks have anti-IBDV antibodies when leaving the farm. Current serological tests cannot distinguish between the antibodies induced by pathogenic IBDV and those induced by attenuated vaccine viruses, so serological diagnosis is of little interest in endemic zones. Nonetheless, the quantification of IBDV-induced antibodies is important for the medical prophylaxis of the disease in young animals, in order to measure the titre of passive antibodies and determine the appropriate date for vaccination **(Muskett, 1979)** or in laying hens to verify success of vaccination **(Meulemans., 1987).** Serology is likewise essential to confirm the disease-free status of SPF flocks. Each serological analysis must include a sufficient number (at least twenty) of individual serum samples representative of the flock under study. A kinetic study requires at least two serological analyses separated by an interval of three weeks (paired sera).

The ELISA is the most rapid and sensitive method, and presents the fewest variations due to the viral strain used as an antigen (**Roney and Freund, 1988).** Considerable inter- and intra-laboratory variability can occur with certain commercial kits **(Kreider *et al.*, 1991).**

Although the correlation between results obtained using serum neutrilization and ELISA is high, ELISA remains less

sensitive, and does not detect low neutralising titres which are sufficient to block vaccine administration (residual maternal antibodies). Enzyme-linked immunosorbent assays which use a recombinant VP2 protein as the sole antigen may be better correlated with protection **(Van den Berg *et al.*, 1997).**

Infectious bursal disease virus may be detected in the bursa of Fabricius of chicks in the acute phase of infection, ideally within the first three days following the appearance of clinical signs.

Virus isolation

A filtered homogenate of the bursa of Fabricius could be inoculated in nine- to eleven-day-old SPF – or eggs originating from hens free of anti-IBDV antibodies embryonated chicken eggs (ECE). The most sensitive route of inoculation is the CAM. Isolation in embryonated eggs does not require adaptation of the virus by serial passages, and is suitable for vvIBDVs but three successive passages are necessary.

Embryo death occurs three to seven days following inoculation. The affected embryos are oedematous, congested, with a gelatinous appearance of the skin, and haemorrhages are often present in the toes or the encephalon. The embryonic membranes are not modified. The variants from the USA cause less embryonic mortality, splenomegaly and no marked lesions of hepatic necrosis. Among the different compartments of the inoculated egg, the embryo is the place where the highest titres of virus occur. The liver shows scattered petechiae and foci of

necrosis, and is the organ which is the richest in viral particles (**McFerran J.B. (1993)**

The specificity of the lesions observed must be demonstrated by neutralising the effect of the virus with a monospecific anti-IBDV serum. (**Rosenberger, 1989).**

Reverse transcription Polymerase chain reaction (Rt-PCR)

The RT-PCR allows the detection of viral RNA in homogenates of infected organs or embryos, as well as in cell cultures, irrespective of the viability of the virus present. The choice of amplified genomic zones depends on the objective. When the only objective is to detect multiple strains of the virus, primers are selected in the highly preserved zones **(Wu *et al.*, 1997).**

The amplified fragment may then be characterized by direct sequencing **(Lin *et al.*, 1993),** and the analysis of the coded amino peptide sequence.

The electrophoretic profile of the amplified fragment may also be studied after digestion with different restriction endonucleases (RT-PCR/RE) **(Liu *et al.*, 1994).** The value of the results obtained will depend on the choice of endonucleases. In a given virus, the absence of restriction sites for enzymes BstNI and Styl, located respectively at codons 222 and 253 of the gene coding for VP2, has been correlated with an atypical antigenicity, such as that found in the variant viruses from the USA (**Jackwood and Nielsen 1997).**

Reverse transcription-polymerase chain reaction (RT-PCR) allows the detection of viral RNA in homogenates of infected organs or embryos, as well as in cell cultures, irrespective of the viability of the virus present. The choice of amplified genomic zones depends on the objective. When the only objective is to detect multiple strains of the virus, primers are selected in the highly preserved zones (**Wu *et al.*, 1992; Stram *et al.*, 1994; Tham *et al.*, 1995, Wu *et al.*, 1997**). When the characterization of the amplified fragment is to allow for identification of the virus strains, the central, so-called variable portion of VP2 is generally chosen **(Lin *et al.*, 1993; Liu *et al.*, 1994).** The amplified fragment may then be characterized by direct sequencing.

The simultaneous presence of four amino acids (alanine 222, isoleucine 256, isoleucine 294 and serine 299) is considered as indicative of vvIBDV **(Yamaguchi *et al.*, 1997, Brown *et al.*, 1994, Cao *et al.*, 1998, Eterradossi *et al.*, 1999).** The electrophoretic profile of the amplified fragment may also be studied after digestion with different restriction endonucleases (RT-PCR/RE).

Control

Exclusion/eradication

The very high resistance of IBDV to physical and chemical agents **(Benton *et al.*, 1967)** accounts for persistence of the virus in the outside environment, particularly on contaminated farms, despite disinfection. Eradication in the affected countries

therefore seems unrealistic. Prevention of IBD necessitates hygiene measures and medical prophylaxis. No vaccine can solve the problem if major sanitary precautions are not taken. These precautions include 'all-in/all-out' farming methods, cleaning and disinfection of premises, and observance of a 'down time' (a period of rest between depopulation and restocking) **(Maris, 1986).**

Given the very contagious nature of the disease and the resistance of the virus, certain essential steps in the cleaning/disinfection process should be adhered to. Prior to cleaning, all insects and pests (e.g. rats and mice) must be eliminated as soon as the farm premises are empty. Old bedding and dung must be eliminated and composted. All farm equipment must be disassembled and stored in cleaning rooms located outside the farm buildings. The buildings, immediate surroundings and farm equipment must be dry-cleaned first, in order to eliminate all dust, and then washed using hot water (60°C) with a detergent, at a pressure of 80 bar to 150 bar. A second disinfection of the full premises must be performed before the introduction of the chicks. Feed silos must be emptied completely and cleaned inside and outside. Under no circumstances may feed remains from previous flocks be reused. Disinfection is to be undertaken only after all the buildings have been cleaned. All disinfectants are more active at a temperature above 20°C; however, chlorinated and iodinated disinfectants cannot be heated above 43°C. The quantity of disinfectant

solution to be used is approximately 4 litres per 15 m 2(**Meroz and Samberg ,1995).**

Vaccination

Immunization is the principle method used for the control of IBD in chickens. There are many available live vaccines based on virulence, such as intermediate virulence and highly attenuated strains, while virulent vaccine not available commercially till now. The vaccine must be safe, pure and efficient **(Mardassi *et al.*, 2004)**.

Live virus vaccines

Live virus vaccines are very widely used. These are made from strains of virus that have been attenuated by serial passages in embryonated eggs. Depending on the degree of attenuation the vaccine strains cause histological lesions of varying severity to the bursae of SPF chickens, and are classified as mild, intermediate or hot **(OIE (2000).**The hot strains induce histological lesions in SPF chickens which are comparable to those caused by pathogenic strains, the only difference being that the hot strains do not cause mortality.

The mild strains are used chiefly for the vaccination of breeder flocks. These are very sensitive to interference by homologous maternal antibodies, and are administered when these antibodies have disappeared, i.e. between the fourth and eighth week of age, depending on whether the grandparent flocks have or have not been vaccinated with an oil-emulsion inactivated vaccine before lay.

Intermediate vaccines are used for vaccinating broilers and pullets **(Mazariegos *et al.*, 1990).** These are also administered to chicks in breeder flocks which are at risk of challenge by highly pathogenic strains at an early age. Although intermediate vaccines are also sensitive to neutralisation by passive antibodies, these vaccines may be administered at day-old in order to protect a chick that may not have a sufficient level of specific antibodies. Another reason for such early vaccination is to bring about replication of the vaccine virus in the chicks, and the dissemination of the virus within the farm; this would, at least partially, provide indirect vaccination to the other chicks at a time when they become sensitive to the infection. In high-risk farms, two vaccinations are generally performed. The age at vaccination depends on the maternal antibody titers present in the chicks at hatch. Vaccines are usually administered through drinking water, although neutralisation is also possible.

Live IBDV vaccines are compatible with other avian vaccines. However, the strains that cause serious lesions to the bursa of Fabricius may also provoke immunosuppression, exacerbate the pathogenicity of other immunosuppressive viruses (Marek's disease virus [MDV] and chicken anaemia virus [CAV]) and jeopardise the immunisation of poultry against other diseases. Registration procedures for these vaccines must include tests to verify the absence of interference with other vaccinations as well as the absence of reversion to virulence in the course of serial passages in three- to six-week-old SPF chickens.

Inactivated vaccines

Inactivated vaccines are essentially used to produce high, uniform and persistent antibody titres in hens prior to lay that have been vaccinated with a live virus or have been naturally infected through exposure to the virus on the farm **(Wyeth and Cullen, 1979).**These vaccines are administered by the subcutaneous or intramuscular route at the age of sixteen to twenty weeks. Progeny of hens that have been vaccinated in this way have protective antibodies until the age of approximately thirty days **(Wyeth *et al.*, 1992).** The chicks are thus protected during the period of susceptibility to the IBDV strains that only provoke immunosuppression. However, the chicks are not protected from other highly pathogenic strains that may inflict high mortality rates at later stages **(Abd El mawgod *et al.*, 2014).**The decision to use an inactivated vaccine will thus depend on the epidemiological context, namely: presence or absence of highly pathogenic strains requiring vaccination of broilers with live virus vaccines. Where no risk of infection with vvIBDVs exists, boosting of laying hens with an inactivated vaccine just before lay is fully justified. However, the duration and uniformity of the immunity thus conferred upon chicks will, to a great extent, depend on the concentration and the antigenic specificity of the virus present in the vaccine. These vaccines are obtained either from bursal homogenates of infected chicks, or from viral cultures on embryonated eggs or fibroblasts, which are then inactivated by formaldehyde and presented as oil emulsions. Sub-unit vaccines produced in yeast **(Macreadie et**

al., 1990) or insect cell cultures **(Vakharia *et al.*, 1993)** have also been described, but are not currently in use.

Broiler breeder vaccination against IBD is usually based on the injection of at least one inactivated vaccine in oil adjuvant **(Maas *et al.*, 2001),** typically included in a combined vaccine. Priming using one or several live IBD vaccine (s) has been the most common way to immunize the breeders so far, as early protection against IBD is required by vaccination programs of the breeders.

Vector Vaccines

Many new IBDV vaccines were developed, including subunit, DNA and vector vaccines. Most of them are still experimental but some have been used commercially. The main advantage of these vaccines is their ability to overcome difficulties in managing MDA on vaccine intake **(Bublot *et al.*, 2007; Hsieh *et al.*, 2007; Rong *et al.*, 2007; Villegas *et al.*, 2008; Le Gros *et al.*, 2009 and Rojs *et al.*, 2011, Cazaban *et al.*, 2018).** Among these vaccines, a recombinant turkey herpes virus (HVT)-IBD vaccine was generated by inserting an IBDV VP2 gene expression cassette into the HVT genome.

The use of a HVT-IBD vector vaccine injected at day-old to future broiler breeders **(Bublot *et al.*, 2007)** has been investigated since the launch of this type of vaccine intended for day-old vaccination of chickens. A vaccination against Marek's Disease (MD) in future breeders requires a Rispens serotype 1

vaccine to be mixed with the HVT-IBD vector vaccine **(Lemiere *et al.*, 2001).**

Various vaccines using recombinant viruses expressing the VP2 protein of IBDV have been described, and have proven efficacy in laboratory tests. The advantages of these vaccines are the absence of residual pathogenicity, sensitivity to maternal antibodies and risk of selection of mutants, as well as the possibility of use in *ovo* and of differentiation between infected and vaccinated animals **(Tsukamoto *et al.*, 1999).**

A vaccine for *in ovo* vaccination of embryos has recently been developed. The vaccine is a mixture of virus and specific antibody, and is injected into eighteen-day-old embryos. Broiler chicks hatched from these eggs are immunized against IBDV throughout the growing period. This method avoids interference by parental antibodies **(Haddad *et al.*, 1997).**

MATERIAL AND METHODS

III. MATERIAL AND METHODS

III.1. Material

1.1. Birds

A total of forty clinically unlike broiler farms were investigated at different localities of Sharkia province from January 2013 till September 2019, after the approval of the farm owners. One hundred and twenty birds were investigated for sample collection (Table 1).

III.1.2. Tissue and blood samples

A total of five hundred and sixty four tissue samples (3 per each) were collected from the examined birds for viral isolation, pathological investigation and molecular characterization. The samples included bursae, kidneys, lungs and spleen (n= 168-324-72 for each one)**.** Twice sample collection were carried out in regular monitoring group (at each 14 and 21 days). Also a total of seventy blood samples were collected from each flock at age of 14 day and 21 days of regularly monitored farms. Additional sixty blood samples representing six flock were collected suspected IBD infection and six respiratory affected flock (n=5) once at clinical stage.

III.1.3. Embryonated chicken eggs (ECE)

A total six hundred, 9-11 day old commercial embryonated chicken eggs (ECE) were obtained from Hehia Private Hatchery and were used for isolation and propagation of virus isolates.

III.1.4. Reagents used for virus isolation

1.4.1 Antibiotic

Penstrept (penicillin 10.000U /ml / streptomycin 10.000 µg/ml), **(BioChrom-leonorenstr-Berlin)**, Lot no: 0501X

Myocostatin **(GSK. UK)** 0.5x106 IU/liter

1.4.2. Phosphate Buffer Saline (PBS), Sigma reagents (WHO Manual 2002)

1.4.3. Chicken red blood cells (CRCs) were obtained from adult healthy chicken for preparation of washed RBCs.

1.4.4. Alsever's solution for collection of erythrocytes (Sigma reagents).

III.1.5. Reference Antigens and Antisera

Reference antigens and antisera specific for: H5N1, H9N2 and NDV were supplied by reference Laboratory for Veterinary Quality control on poultry production (RLQP), Dokki, Giza, Egypt.

III.1.6. Reagents used for molecular identification of virus(s)

1.6.1 Reagents used for RNA extraction

The viral RNA was extracted using QIAamp viral RNA kit (QIAGEN, USA) according to manufacture instructions

QIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 5290

Table (1): The descriptive data for The examined broiler flocks in Sharkia Province, Egypt.

No	Locality	Age	Breed	Total No. of birds	No. of submitted birds	Mortality #	Vaccination/Age (Days)
(I) Regular Monitoring (M) *							
M1	Abu Hammad	1-28	Ross	5000	3*2	18	Hitchner+IB classic, D78 (7d), H5N2+ND (9d), D228 (14d), LaSota (19d)
M2	Abu Hammad	1-28	Ross	5000	3*2	35	Hitchner+IB classic, D78 (7d), H5N2+ND (9d), D228 (14d), LaSota (19d)
M3	Dyarb Negm	1-28	Cobb	5000	3*2	22	Hitchner+IB classic, D78 (7d), H5N1 (9d), D228 (14d), Clone 30 (18d)
M4	Abu Hammad	1-28	Arbor	6000	3*2	15	H5N2+ND (5d) ,Hitchner+IB classic,D78 (8d), H5N1 (9d), D228 (14d), Clone 30 (18d)
M5	Dyarb Negm	1-28	Cobb	20000	3*2	113	H5N1 (5d), Hitchner+IB (7d), Bursine (8d), plus (14d), LaSota (18d)
M6	Abu Hammad	1-28	Cobb	8000	3*2	37	Hitchner+IB (7d),H5N2+ND (9d), Bursine plus (14d), Clone 30 (19d)
M7	Fakous	1-28	Arbor	7000	3*2	44	Vaxxitech (1d), Hitchner+IB (7d),H5N1 (9d),Clone 30+Ma5 (15d)
II) Suspected (S) to be affected with IBDV**							
S1	Menia El-Qamh	22	Ross	5000	3	82	Hitchner+IB (8d), Gumboro intermediate (13d)
S2	Menia El-Qamh	19	Ross	4000	3	299	Bursine plus(14d)
S3	Menia El-Qamh	25	Ross	5000	3	47	D78 (7d),D78(14d)
S4	Mashtool ElSoak	21	Arbor	15000	3	255	IBD Blen(14d)
S5	Menia El-Qamh	18	Hubbard	8000	3	139	Bursine plus (14d)
S6	Belbis	20	Arbor	5000	3	147	D78 (7d),D78(14d)
S7	Abu Hammad	26	Ross	5000	3	72	D78 (7d),D78(14d)
S8	Kfr Sakr	23	Arbor	8000	3	70	Bursine plus (14d)
S9	Menia El-Qamh	17	Arbor	5000	3	302	IBD Blen(14d)
S10	Hehia	20	Arbor	10000	3	80	D78 (7d),D78(14d)

Table(1) continued

No	Locality	Age	Breed	Total No. of birds	No. of submitted birds	Mortality	Vaccination/Age (Days)
S11	Abu kabier	21	Arbor	5000	3	126	IBD Blen(14d)
S12	Fakous	15	Arbor	6000	3	57	Gumboro intermediate (14d)
S13	Abu Hammad	18	Hubbard	5000	3	52	D78 (7d),D78(14d)
S14	Dyarb Negm	20	Ross	10000	3	171	IBD Blen(14d)
S15	Mashtool ElSoak	21	Hubbard	7000	3	128	IBD Blen(14d)
S16	Belbis	17	Arbor	8000	3	71	IBD Blen(14d)
S17	Abu kabier	19	Arbor	5000	3	103	D78 (7d),D78(14d)
S18	Abu Hammad	18	Ross	7000	3	143	Bursine plus (14d)
S19	Abu kabier	21	Cobb	10000	3	144	D78 (7d),D78(14d)
S20	Fakous	20	Cobb	12000	3	156	Bursine plus (14d)
S21	Dyarb Negm	15	Cobb	6000	3	72	IBD Blen(14d)
S22	Menia El-Qamh	18	IR	5,000	3	407	D228 (14d)
S23	Belbis	15	Ross	4,000	3	237	IBD Blen-Ceva (14d)
S24	Menia El-Qamh	18	Cobb	6,000	3	122	D78 (7-14d)
S25	Menia El-Qamh	19	Ross	5,000	3	253	D228 (14d)
S26	Menia El-Qamh	16	Cobb	7,000	3	241	Bursine plus (14d)
S27	Belbis	18	Hubbard	6,000	3	212	IBD Blen-Ceva (14d)
(III) Respiratory(R) affection ***							
R1	Menia El-Qamh	19	Arbor	8,000	3	407	Vaxxitech (1d), Hitchner+IB (7d), H5N1(9d), H9N2 (11D), Clone 30 (19d)
R2	Menia El-Qamh	22	Cobb	6,000	3	237	Vaxxitech (1d) ,Clone 30+MA5 (7d),H5N1 (9d), H9N2(11d),LaSota (16d)
R3	Belbis	18	Ross	10,000	3	122	H5N1(5d),Hitchner+IB (7d), D78(8d), H9N2 (9d), D228(14d), LaSota (18d)

Table(1) continued

No	Locality	Age	Breed	Total No. of birds	No. of submitted birds	Mortality	Vaccination/Age (Days)
R4	Zagazig	25	Arbor	5,000	3	253	IB primer (1d), Hitchner+IB (7d), Bursine 2 (8d), H5N1+ND (9d), Bursinee plus (14d), LaSota (18d)
R5	Belbis	23	Hubbard	7,000	3	241	H5N1 (5d), Hitchner+IB (7d), D78(7d), H5N1+ND (9d), D228 (14d), LaSota (19d)
R6	Dyarb Negm	24	Arbor	10,000	3	212	Vaxxitech, IB primer (1d), H5N1 (5d), H9N2+ND (9d), Clone 30+MA5 (15d)

*Samples were collected from apparently healthy flocks for regular monitoring (twice at age 14-21 days)

**Samples were collected from clinically affected cases only

*** Samples were collected from birds suffering from respiratory signs

. The mortality was calculated at the visit time.

1.6.2. The primers and probes for real time rt-PCR

The oligonucleotide sequences of the specific primers and probes used in detection of IBDV, AIV and NDV were purchased from (QIAGEN, USA) According to previously designed (Table 2)

Table (2): Sets of primers and probs used in rt-PCR reaction for detection of IBDV, AIV and NDV

Virus	Gene	Primer/ probe sequence 5'-3'	References
IBD	VP2	**F: AUS GU** TCACCGTCCTCAGCTTACCCACATC	Metwally *et al.*, 2009
		R: AUS GL GGATTTGGGATCAGCTCGAAGTTGC	
	VP2	F: GAG GTG GCC GAC CTC AAC T	Moody *et al.*, 2000
		R: AGC CCG GAT TAT GTC TTT GAA G	
		Probe: (FAM)-TCC CCT GAA GAT TGC AGG AGC ATT TG-(TAMRA)-3	
	VP1	**F:** TTCTGCAGCCACGGTCTCT	LeNouën *et al.*, 2006
		R: ATGACTTGAGGTTGATTTTG	
AI	M	F: AGATGAGTCTTCTAA CCGAGGTCG	Slomka *et al.*, 2007
		R: TGCAAAAACATCTTC AAGTCTCTG	
		Probe: [FAM]TCAGGCCCC CTCAAAGCCGA [TAMRA]	
	H5	F: ACATATGACTAC CCACARTATTCA G	Lŏ ndt *et al.*, 2008
		R: AGACCAGCT AYC ATGATTGC	
		Probe: [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	
	H9	F: GGAAGAATTAATTATTATTGGTCGGTAC	Ben Shabat *et al.*, 2010
		R: GCCACCTTTTTCAGTCTGACATT	
		Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[BHQ]	
ND	Matrix	**M+4100** AGTGATGTGCTCGGACCTTC-3'	Wise *et al.*, 2004
		M-4220 CCTGAGGAGAGGCATTTGCTA-3'	
		M+4169 [FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3'	

III.1.6.3. Reagents used for sequencing of VP2 gene of IBDV

Reagent for sequencing were supplied by Elim Biopharmaceuticals, Germany

1.6.3.1. DNA Molecular weight marker Molecular weight marker gel pilot 100 bp plus ladder (QIAGEN,USA Cat.# 52904) It is composed of eleven chromatography purified individual DNA fragments in base pairs: (1500-100)

1.6.3.2. Gel Pilot 100 bp plus ladder cat.#No. 52904(QIAGEN,USA).

1.6.3.3. Reagents used for conventional RT-PCR kits one step (QIAGEN, USA) was used for amplification of VP2-gene.

1.6.3.4. Reagents used for agarose gel electrophoresis

a) Agarose.(Sigma)

b) Tris acetate EDTA bufferIt is 50x stock solution (fermontas). It was used as 1x buffer solution for preparation of agarose and for gel electrophoresis.

d) Ethidium bromide

A stock solution of ethedium bromide (Fluka) was prepared as following:

Ehtedium bromide	5mg
RNase free water	10 ml

It was used for staining the agarose gel electrophoresis DNA by adding 50 µl from stock solution to 50 ml 1.5% melted agarose to give a final concentration of 0.5 µg/ ml.

III.1.6.4. Reagents used for Purification of PCR product

The PCR products were purified from agarose gel using *QIAquick gel extraction kit* **(QIAGEN, USA)**

1.7. Material used for ELISA. (Biocheck)

ELISA kit from **(Biocheck, Netherlands)** was used for detection of IgG of IBDV in collected serum.

1.8. Reagents for histopathology (Elgomhoria-Egypt)

- 10% buffered neutral formalin solution
- Graded Alcohol and Xylol concentrations
- Paraffin
- Haematoxyline & Eiosin (H&E) stain

1.9. Equipment

1.9.1. Micro-centrifuge, 16000 rpm **(SIGMA-Sartorius 3-16P)**

1.9.2. Vortex **(MAXI MIX II)**

1.9.3. Uni and multi -channel micropipettes (100-1000), (0.5-10) and (20-200) µl **(Biohit)**

1.9.4. Forma Class II, A2 biological safety cabinet **(Thermo)**

1.9.5. Electronic digital timer **(Guest Medical)**

1.9.6. Sterile 1.5 ml micro-centrifuge eppindorf tubes

1.9.7. PCR tubes 0.2 ml capacity **(QIAGEN)**

1.9.8. Sterile filter tips. (100 µl ,1000 µl) capacity**. (BrandTech scientific)**

1.9.9. (-20˚C) Freezer **(Toshiba).**

1.9.10. Thermoblock **(Biometra).**

1.9.11. Glass ware (Calibrated cylinders, flasks and beakers) (Singla scientific industries)

1.9.12. Digital Balance **(Scaltec).**

1.9.13. Microwave **(Panasonic).**

1.9.14. Power supply **(Biometra).**

1.9.15. Gel documentation system **(Alpha Innotech)**

1.9.16. Deionizer **(Millipore).**

1.9.17. Double distillator **(Sanyo).**

1.9.18. Horizontal submarine gel electrophoresis (*VARI-GEL-MIDI SYSTEM) (Sigma, UK).*

1.9.19. ELISA Plate Readers (Sunrise, Tecan, Gr¨odig, Austria).

III.2.METHODS

III.2.1.Clinical and postmortem examination

The farms were regularly visited, and the analysis was made on the basis of the clinical findings of the disease, source of the chicks, breed, age, vaccination, observed signs, and mortalities the forty flocks were subdivided into 3 categories:

A) Regular monitoring.

B) Suspected to be affected with IBD

C) Exhibited respiratory signs.

III.2.2. Histopathological examination

Specimens from the chicken bursae, kidney and spleen were collected and immediately fixed in 10% buffered neutral formalin solution for 48 hours, dehydrated in gradual ascending ethanol cleared in xylene, and embedded in paraffin. Five-micron thick paraffin were sliced using a microtome (Leica RM 2155, England). The sections were prepared and then routinely stained with hematoxylin and eosin stains and examined microscopically (**Suvarna *et al.*, 2013**).

III.2.3. Inoculum preparation

The tissue samples were collected under aseptic condition in pools (3/each). (Kidney; n=14, Bursae; n=34,). Each separate pool was prepared 10% W/V in sterile PBS, the homogenates were centrifuged (3000 RPM) for 10 minutes at 4ºC and

antibiotic were added to supernatants (Penstrept 1000 U ; Myocostatin 1000 U/ml)

III.2.4.Virus isolation

The ECE of 9-11 days age were inoculated via CAM route with 0.2 ml of sample supernatant fluids, each sample pool was inoculated in 5 ECE, negative control ECE were supplied with each isolation trial. Embryos were candled daily for 5 days. The collected embryos were

Chilled at 4^{o}c for 4 hours or overnight to be examined. Allantoic fluids were harvested and tested for HA activity **(OIE, 2012).**

II.2.5. Hemgglutination inhibition (HI) test

The HI test was performed according to **OIE terrestrial manual (2012)**. For AIVs (H5and H9) and NDV chicken sera of the examined birds.

III.2.6. ELISA Technique: according to Biocheck instructions

A commercially available IBDV antibody test kit (BioChek CV, Gouda, and the Netherlands) was used for the detection of antibodies against IBDV in chicken serum. The kit was used according to the manufacturer's instructions. Samples were tested in duplicates. As controls; antigen, known positive and negative sera were included. Optical density values were read at 450 nm by using an ELISA microplate reader (Sunrise, Tecan, Austria).

III.2.7 .RNA extraction

The viral RNA was extracted either from 10% tissue suspension. A reference IBDV isolate and non infected CAM were used as positive and negative controls, respectively. A Pipet 560 µl of were prepared Buffer AVL containing carrier RNA (5.6µl) into a 1.5 ml micro-centrifuge tube with adding Add 140 µl of sample. Mix by pulse-vortex for 15 s. and incubated at room temperature (15-25 C) for 10 min. after that centrifuged the tube to remove drops from the inside of the lid. Added 560 µl of ethanol (96-100%) to the sample, and then mixed by pulse-vortex for 15 s. After mixing they were centrifuged. Applied 630 µl of the solution to the QIAamp Mini spin column (in a 2 ml collection tube). Close the cap, and centrifuged at (8000 rpm) for 1 min. Place the QIAamp s pin column into a clean 2 ml collection tube, and discard the tube containing the filtrate then repeat centrifugation again.

QIAamp Mini spin column were opened, and added 500 µl of Buffer AW1. Close the cap, and centrifuged at (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. With Careful opened the QIAamp Mini spin column, and added 500 µl of Buffer AW2. Close the cap, and centrifuge at full speed (14,000 rpm) for 3 min. Then Placed the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. The Centrifuged at full speed for 1 min. placed the QIAamp Mini spin column in a clean 1.5

ml micro-centrifuge tube. Discard the old collection tube containing the filtrate. Opened with care the QIAamp Mini spin column and add 60 µl of Buffer AVE equilibrated to room temperature. Closed the cap, and incubated at room temperature for 1 min. Centrifuged at (8000 rpm) for 1 min.

III.2.8. Preparation of PCR Master Mix

Master mix for PCR was prepared according to QuantiTect probe RT-PCR kit handbook (January, 2008) as shown in Table 3

Table (3): Master mix components for PCR

Component	Volume/reaction
2x QuantiTect Probe RT-PCR Master Mix	**25 *µl***
Forward primer (50 pmol)	**1 *µl***
Reverse primer (50 pmol)	**1 *µl***
Probe (30 pmol)	**0.25 *µl***
QuantiTect RT Mix	**0.25 *µl***
RNase Free Water	**8.5 *µl***
Template RNA	**14 *µl***
Total	**50 *µl***

III.2.9. cycling conditions for primers probe:

The cycling conditions for real time PCR for IBDV(VP1 and VP2),AIV(M, H5 and H9) and NDV (M) genes were summarized in Table 4.

Table (4): Oligonucleotides Real time PCR Cycling conditions of Primers and probes

Virus	Reverse transcription	Primary Denaturation	Secondary Denaturation	Annealing	Extension	No. of cycles	References
IBD VP1	50˚C 30 min.	95˚C 15 min.	94˚C 30 sec.	59˚C 40 sec.	72˚C 1 min.	35	Metwally *et al.*, 2009
IBD VP2				60˚C 40 sec.	72˚C 45 sec.		LeNouën *et al.*, 2006
AI (M)	50˚C 30 min.	95˚C 15 min.	94˚C 15 sec.	60˚C 45 sec.		40	Slomka *et al*., 2007
AI (H5)				54˚C 30 sec.	72˚C 10 sec.		Lȍ ndt *et al*., 2008
AI (H9)				60˚C 45 sec.			Ben Shabat *et al*., 2010
ND (M)				55˚C 30 sec.	72˚C 10 sec.		Wise *et al.*, 2004

Interpretation of results

Controls

- The negative control should have neither CT value not crossing point and no amplification curve.
- The positive control should have a Ct value or crossing point less than 35.0 cycles, and should be typical amplification curve.

Positive samples

If a sample has a Ct value or crossing point less than 35 cycles with a typical amplification curve, then the sample is considered positive. These results indicated that the sample contains avian influenza virus RNA of the indicated type or subtype.

Negative samples

If a sample has no Ct value (or crossing point) and no amplification curve, then the sample is considered negative for avian influenza virus of the indicated type or subtype.

III.2.10. RT-PCR and Sanger sequencing of IBDV-VP2 gene

The positive RNA samples were retested by using a specific primer flanking the VP2 gene of IBDV, yielding a 620-bp region.

The VP2 gene of IBDV strains was amplified by using Qiagen One Step RT-PCR Kit (Qiagen) following the

manufacturer's guidelines. The PCR was done in a volume of 50 µl in sterile 0.2-ml PCR tubes as described previously **(Metwally *et al.*, 2009)**

Agarose gel electrophoreses (Sambrook *et al.*, 1989) with modification

The amplified products were visualized on 1.5% agarose gels. The viral PCR products were purified by using a PCR purification kit (Qiagen).

purification of the PCR Products:

Purified DNA was sequenced separately by using the same forward and reverse primers used in RT-PCR (Elim Biopharmaceutical, Hayward,CA).

Sequencing reaction

Positive strains were subjected to Elim bio pharmaceuticals for sequencing

A purified RT-PCR product was sequenced in the forward and reverse directions on DNA Baser Sequence Assembler version 4.36 (31) was used to generate consensus sequences, which were further identified bynucleotide (nt) in The Basic Local Alignment Search Tool of theNational Center for Biotechnology Information (NCBI) and then submitted to the GenBank under accession numbers MK493456

III. 2. 11. Phylogenetic analysis

On the basis of the variable region within the VP2 gene, the phylogenetic relationship between viruses included in current study and reference IBDV strains were determined by using MEGA 6.0 software **(Tamura *et al.*, 2013).** First, sequences were aligned by using the Clustal method and then cut into equal length. Then, the nt and aa identities were estimated. The genetic pattern was assessed by constructing a neighbor-joining phylogenetic tree on the basis of the VP2 nt sequences, using the Kimura two parameter at 1000 bootstrap replicates. The deduced aa sequence analysis was performed by using DNASTAR Lasergene 7.2 software **(Burland, 2000)**

RESULTS

IV.RESULTS

Forty broiler farms at different localities in Sharkia province were investigated for clinical signs and postmortem lesions of IBD virus infection and / or accompanied agents beside immune status of the examined birds under different circumstances for the purpose of evaluation the impact of genetic variation of the isolated IBDV on the increase susceptibility of other viruses.

Clinical findings among examined birds

a) Regular monitoring

Regular weekly visit for seven broiler chicken farms with total of 56000 birds of various breeds and vaccination programs non-significant mortality rates (0.25%-0.7%), the observed clinical findings all over the broiler cycle were sporadic cases of day old chick with weakness and not in standard weight, bloody feces, brownish diarrhea, whitish diarrhea, ruffled feather, loss of weight and slightly respiratory signs.

b) Clinically suspected affected with IBDV

Examined birds from twenty seven (27/40) broiler chicken farms suspected to be infected with IBDV with a total population of 184000 birds which had increase in mortality by (2%-20%). General signs of illness (Ruffled feather, decrease feed consumption, elevated water consumption, shivering, vent picking, huddling together, perfuse watery and whitish diarrhea were the commonly recorded clinical signs among the examined chickens (Figure 1).

c) Clinically exhibited respiratory manifestation

Examined birds collected from six farms of 46000 birds showed respiratory ***manifestation*** and mortality rate up to (18.7%) as well as signs of illness, cyanosis of head, comb and wattles, edema of head and neck. Respiratory signs, included, nasal discharge, conjunctivitis, sinusitis and lacrimation. Greenish diarrhea as well as nervous signs were also observed.

Post mortem findings

a) Regular monitoring

Lesions in chickens were variable representing to different age and localities of the farm. Most of them revealed with bacterial and protozoa infection and away from clinical viral diseases.

a) Clinical suspected affected with IBD

Gross lesions in IBD-affected flocks were recorded in bursae (Figure.2), including edematous and congested bursae with the presence of gelatinous exudates and bursal hemorrhages in 82.1%. In addition, hemorrhages on the thigh muscle were seen in 21.4% and petechial hemorrhages at the junction between the proventriculus and the gizzard were observed in 17.9%. Swelling of kidneys and ureters extended with urates were frequently seen among 78.6% of examined birds.

Figure (1) clinically suspected to be infected chicks with IBD broilers at the age of 18 days ruffled feather and lying down. (S 5)

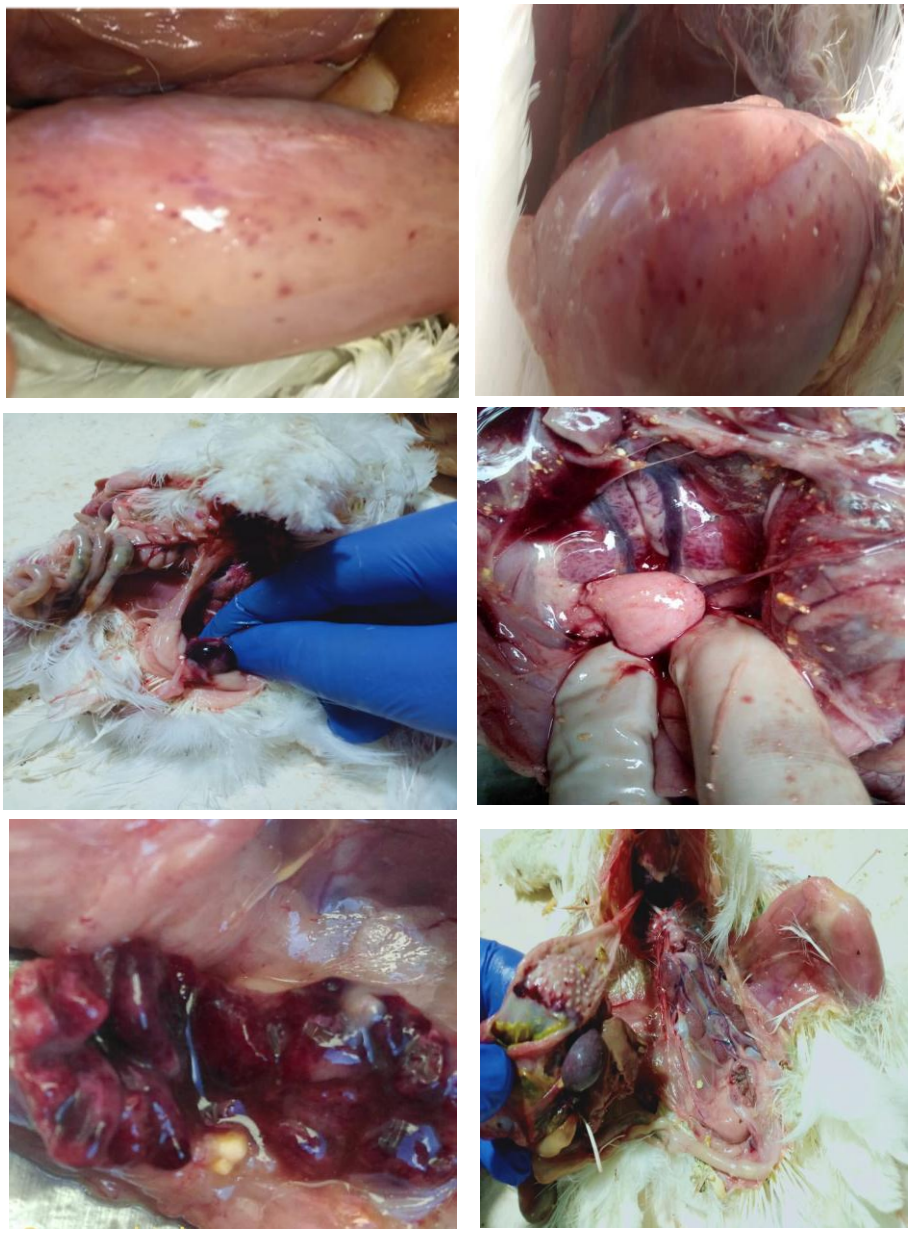

Figure (2): Gross lesions of IBD suspected chicken (a) & (b) petechial &punctate hemorrhages on thigh muscle and in chicken (c), (d), (e) & (f) glandular tips of proventriculus with - haemorrahge on bursa and congested kidney

b) Clinically exhibiting respiratory manifestation

In birds exhibiting respiratory illness, the gross examination revealed hemorrhages in the trachea with the presence of inflammatory exudates, as well as congested or hemorrhagic lungs or both. Petechial hemorrhages on the proventriculus were observed in 28.6% of examined birds. Nephritis and swelling of the kidneys were also seen.

Histopathological examination from infected bursa

Kidney, spleen and bursa of fibricious of suspected to IBDV infected birds showed interstitial hemorrhages with various degrees of degeneration in renal tubules with granular casts in lumina of kidney (Figure 3) and 6 S Case showed shrunken glomeruli with increase of Bauman's spaces diffuse degeneration of renal tubules sometimes contained albuminous casts in their lumina(Figure 4) . Chicken spleen (Case 5S) showed sub capsular splenic depletion with prominent necrotic changes on most lymphoid follicle (Figure 5). (A Case S26) showed depletion and necrotic of lymphocytes with cystic formation beside exudates in the medullary zones of bursal follicles) surrounded by inflammatory infiltrations, hemorrhages and edema (Figure 6)

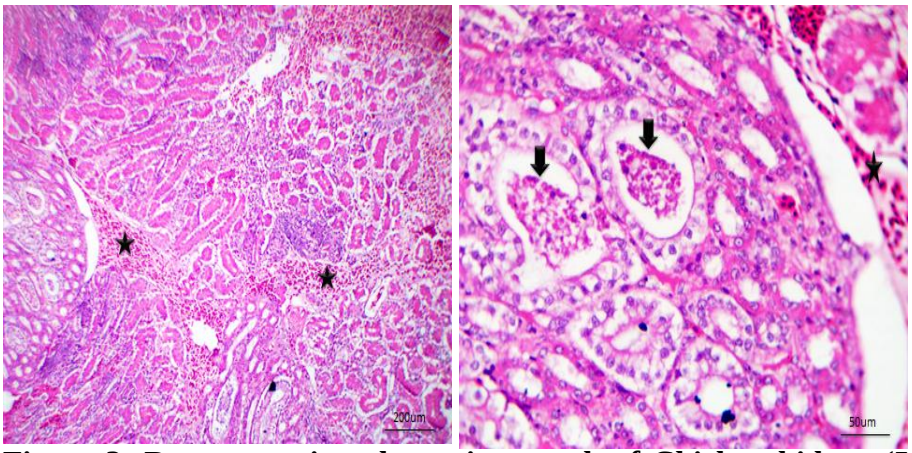

Figure 3: Representative photomicrograph of Chicken kidney (5 Case) showing interstitial hemorrhages (stars) beside various degrees of degenerated renal tubules with granular casts in lumina (arrows). Hematoxylin and eosin stain.

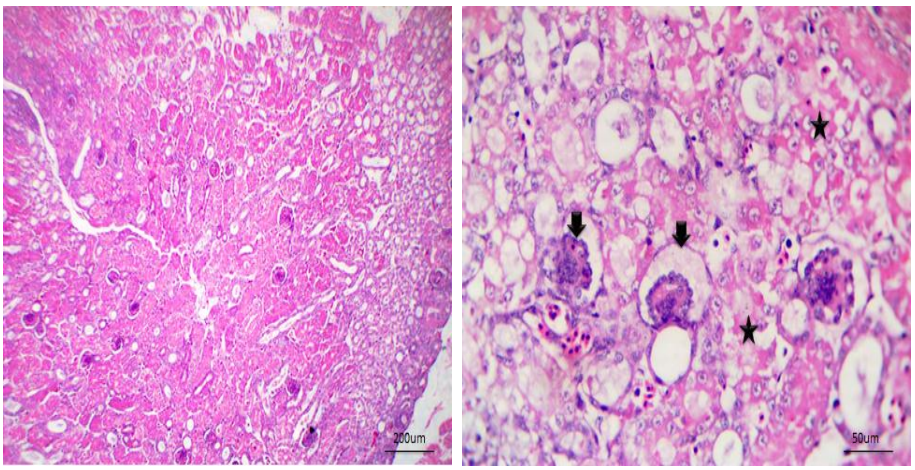

Figure. 4: Representative photomicrograph of poultry Kidney (6 Case) showing shrunken glomeruli with increase of Bauman's spaces (arrows), diffuse degeneration of renal tubules sometimes contain albuminous casts in their lumina (stars). Hematoxylin and eosin stain

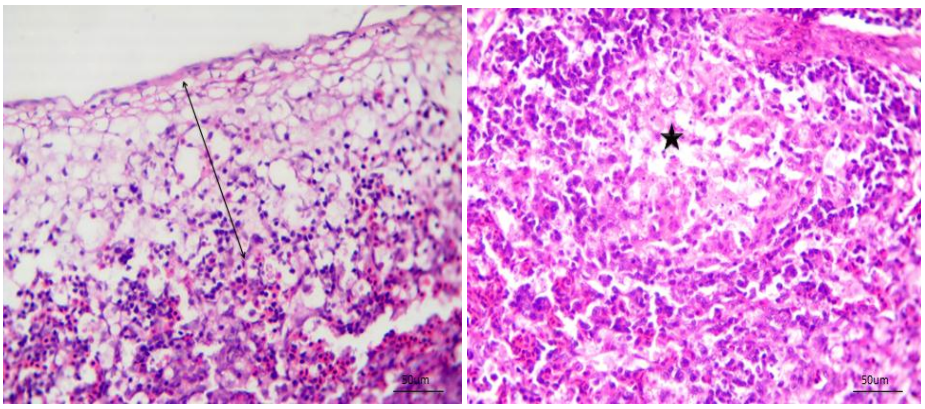

Figure. 5: Representative photomicrograph of chicken spleen (5 S Case) showing sub capsular splenic depletion (tow head arrow) with prominent necrotic changes on most lymphoid follicle (star). Hematoxylin and eosin stain.

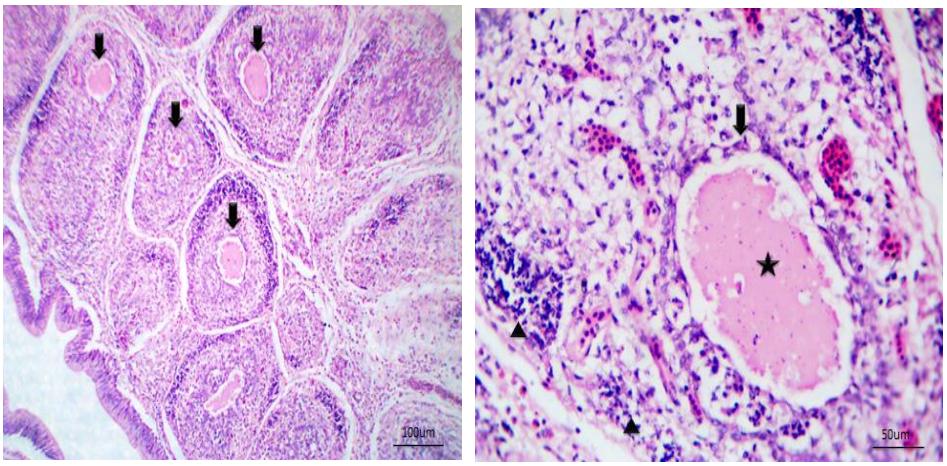

Figure. 6: Representative photomicrograph of poultry Bursa of Fabricius (3 Case) showing depletion and necrotic of lymphocytes with cystic formation (arrows) beside exudates in the medullary zones of bursal follicles (star) surrounded by inflammatory infiltrations, hemorrhages and edema (arrow heads). Hematoxylin and eosin stain

Virus isolation in embryonated chicken eggs

All samples (14 and 21 days of age) collected from regularly monitoring farms showed normal CAM and embryo without changes along three successive embryo passages for both bursa and kidney tissue pools .all harvested allantoic fluids were HA negative. While, in clinically suspected affected with infectious bursal disease showed varying degrees of congestion of CAM and embryos. Inflamed swollen kidney, enlarged gall bladder and pale liver were also observed. Embryo deaths ranged from 40% to 60% after 72 to 96 hours post inoculation.

Serological findings

Haemagglutination inhibition test (HI)

In regular monitored farms samples were collected in twice, at 14 and 21 days of age for the purpose of HI test investigation for antibodies (Abs) against, H5N1, H9N2 and ND viruses. The geometric mean titer (GMT) H5N1 Abs for 14 days collected blood samples ranged from Zero to 2.75 while those of 21 days of age ranged from $log_2$0.75 to 2.25.The H9N2 Abs were for both 14 days and 21 days (Zero to 1) however NDV testing at 14 days revealed from 1.6 to 2.6 and in 21 days from 3 to 4.25 (Table 5).

Sera of suspected flocks to be infected with IBDV flocks results were for H5N1 ranged from 1.83 to 2.50 while for H9N2 ranged from 1.67 to 2.67 and in NDV ranged from 1.5 to 2.67 in average age (15-19) days. But Those of exhibiting respiratory

manifestation, H5 flock Ab titer ranged from 2.6 to 5.6 on the other hand there is a variation on farms in H9N2 ranged from 0.8 to 4.8 then in NDV ranged from 3.2 to 4.2 (Table 6).

IV.5.2. ELISA test

In regular monitoring farms, variable results according to vaccination program and maternal immunity of the flock. At the age of 14 days two farms with some variation for IBD immune titer ranged from 425 to 1452 and after one week all samples were in normal ranged from 1215 to 2039 (Table 5).

While, in clinically suspected flocks affected with IBDV, there was a great variation for all farms, mean titer varied between (117-2543) with different level of coefficient variation which indicate flock infected with IBD. And in suspected exhibited respiratory manifestation, farms revealed normal titer for IBD mean titer ranged from (2054 to 3065), except the last 2 farms ranged over 4000 without high mortality rates and abnormal changes (Table 6).

Table (5): Results of serological evaluation of immune response in regularly monitored broiler chickens

No	Locality	G. Mean H5N1		G. Mean H9N2		G. Mean ND		ELISA IBD Mean	ELISA IBD C.V	ELISA IBD Mean	ELISA IBD C.V
Age		14 d	21 d	14 d	21 d	14 d	21 d	14 d		21 d	
M1	Abu Hammad	2.75	1.75	0.5	0.25	2	4.25	1452	70.2	2039	19.7
M2	Abu Hammad	0.75	1.5	1	0	2.6	4.5	625	17.4	1265	20.5
M3	Dyarb Negm	0	1.75	0	0.25	1.6	4.75	1025	21.23	1287	23.4
M4	Abu Hammad	0	1.25	0	0	1.6	4	425	62.4	1215	25.3
M5	Dyarb Negm	0	1	0.75	0.25	1.6	3	502	22.15	1330	26.4
M6	Abu Hammad	1.5	2.25	0.5	0	2.2	3.75	725	17.8	1324	22.5
M7	Fakous	0.75	0.75	1	0.25	1.6	4.25	625	19.4	1779	22

*G. Mean= Geometric mean

Table (6): Results of serological evaluation of immune response in suspected to be affected with IBD and respiratory affected broiler chickens

No	Locality	Age	HI H5N1	HI H9N2	HI ND	ELISA IBD	
						Mean	C.v
(II) Suspected to be affected with IBDV							
1	Menia El-Qamh	18	1.83	1.67	1.50	117	92
2	Belbis	15	2.50	2.17	1.83	2,241	43
3	Menia El-Qamh	18	2.00	1.67	2.33	1,661	99
4	Menia El-Qamh	19	2.00	2.67	2.00	2,543	44
5	Menia El-Qamh	16	2.33	2.50	2.67	1,765	97
6	Belbis	18	2.17	1.83	1.83	2,139	42
(III) Respiratory							
1	Menia El-Qamh	19	3	3.2	4.2	2644	24.65
2	Menia El-Qamh	22	3.6	3.2	3.6	2945	27.12
3	Belbis	18	3.2	4.8	3.4	3065	29.23
4	Zagazig	25	5.6	1.8	3.4	2054	26.87
5	Belbis	23	3.8	3.4	3.4	4565	42.12
6	Dyrab Negm	24	2.8	0.8	3.2	4965	45.32

Molecular identification of IBD using rRT-PCR

The positive RNA samples were retested by using a specific primer flanking the VP2 gene of IBDV, yielding a 620-bp region.

Regular monitoring

All bursal and kidney tissue pools collected in both 14 and 21 days of age (100%) from regularly monitored farms have no cut of any i.e. were negative for IBD using real time RT-PCR (Figure 7).

Clinical suspected affected with IBDV

Nineteen IBD samples 70.37% of clinically suspected infected farms were positive, using real time RT-PCR their curve cut before 19.8 cycles and 33.4% (Figures 8, 9)

Six positive VP1 samples from suspected to be infected with IBDV were submitted to examined RT-PCR for VP1 and only4 showed positive detection of VP1 gene (Figure 10)

One sample from suspected respiratory manifestation farms was positive for IBD and other 5 samples were negative (Figure 11)

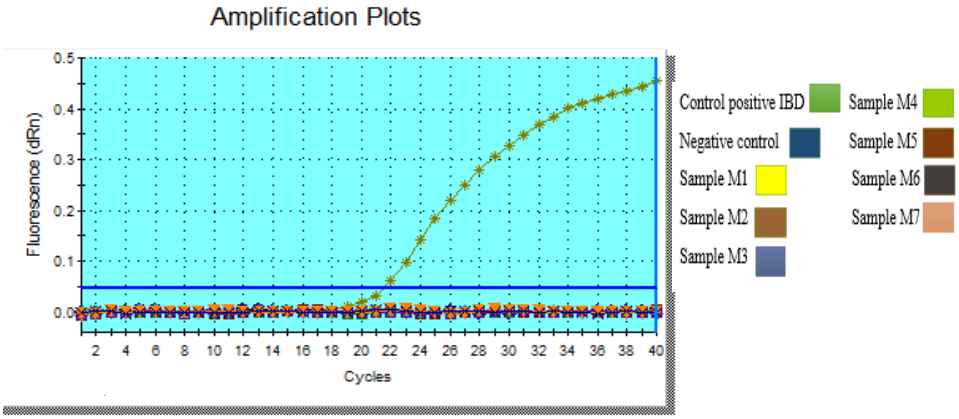

Figure (7): Amplification curve for IBD VP2 gene rRT-PCR of regularly monitored broiler chicken flocks with no cut on ct.

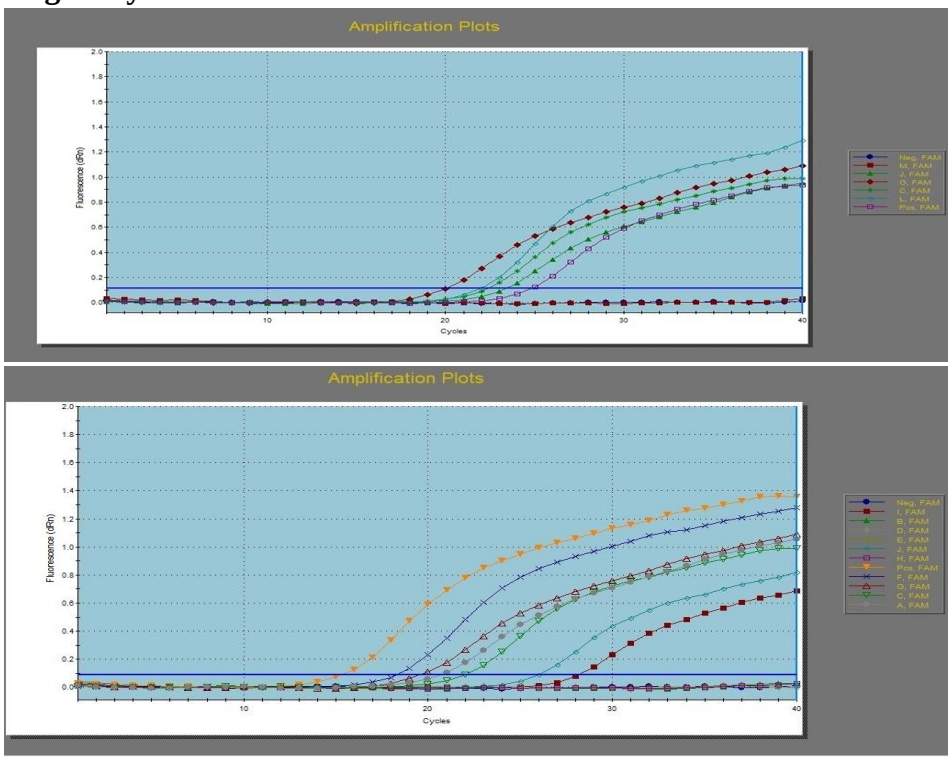

Figure (8): Amplification curve for IBD VP2 gene rRT-PCR for clinically suspected to be infected flocks. The CT (dRn) value ranged from 19.8 to 25

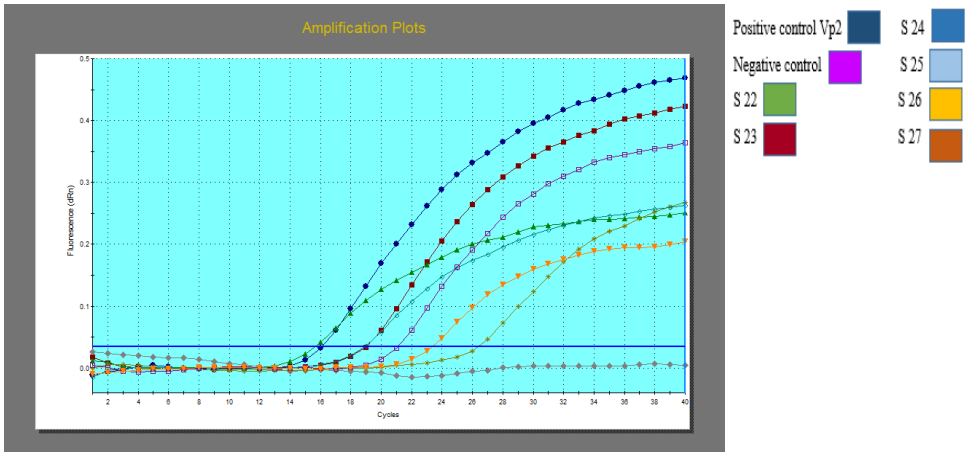

Figure (9): Amplification curve for IBD VP2 gene rRT-PCR in clinical suspected affected with IBDV with ct cut range from 16 to 28.

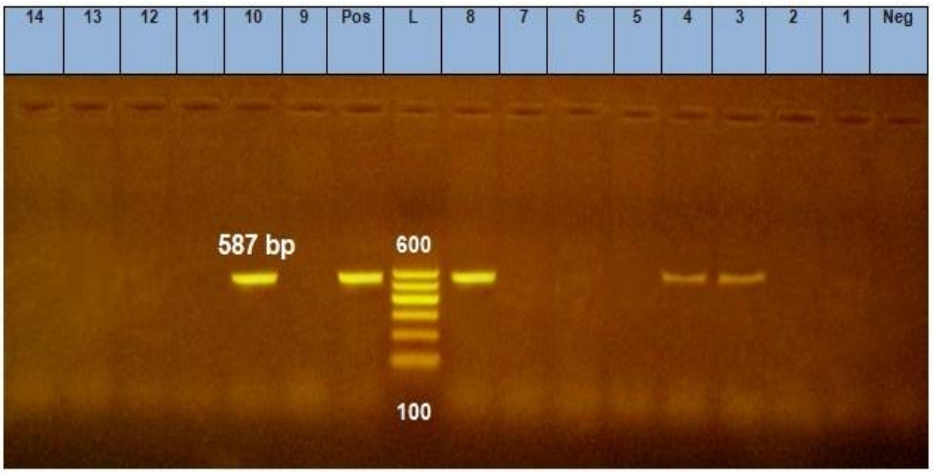

Figure (10): Analysis of IBDV RT-PCR products by gel electrophoresis. Lanes 3-4: faint positive for VP1 lane 8-10: positive for VP1

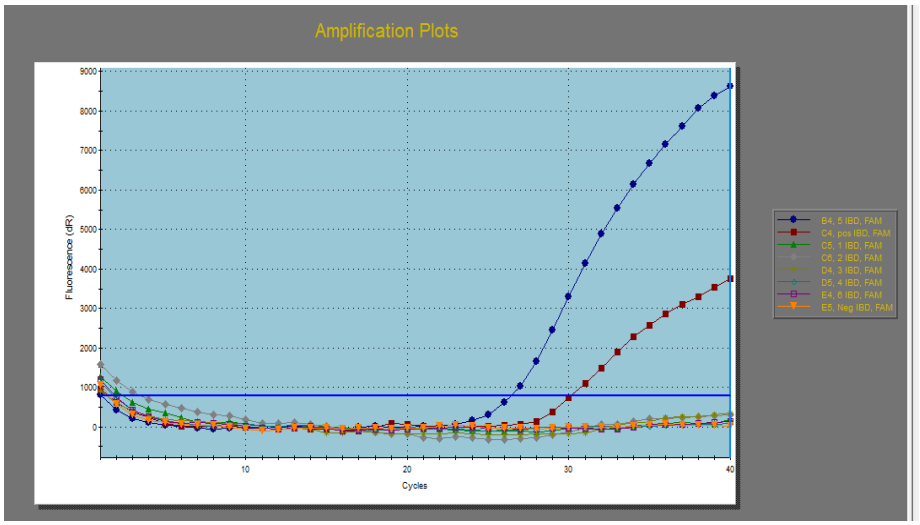

Figure (11): Amplification curve for IBD VP2 gene rRT-PCR in respiratory manifestation farms.

- **The CT (dRn) value at 30.4 Molecular identification of IBDV using rRT-PCR.**

Molecular identification of AI using rRT-PCR

The positive RNA samples were retested by using a specific primer flanking the M gene of AI, then positive samples subjected for H5 and H9 primers.

Regular monitoring

All lung tissue pools collected in both 14 and 21 days of age (100%) from regularly monitored farms have no cut of any i.e. were negative for AI using real time RT-PCR (Figure 12).

Respiratory affected farms

Three of six positive (3/6) for AI virus M gene 50% of respiratory affected farms were positive and 50% were negative using real time RT-PCR. (Figures 13)

When samples were subjected for H5 test in rRT-PCR, all samples were negative. (Figure 14)

When samples were subjected for H9 test in rRT-PCR, 50% of samples were positive and 50% were negative. (Figure 15)

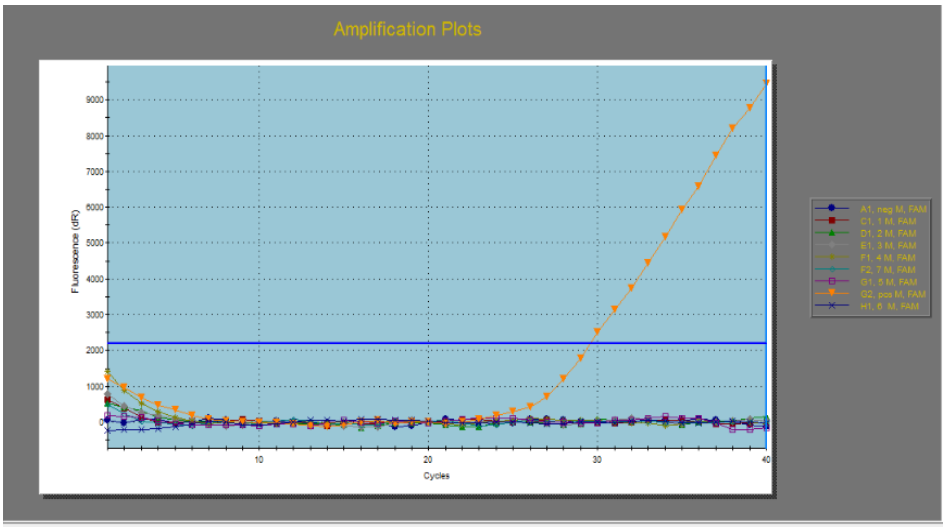

Figure (12): Amplification curve for Matrix AI gene rRT-PCR for Regular monitoring farms

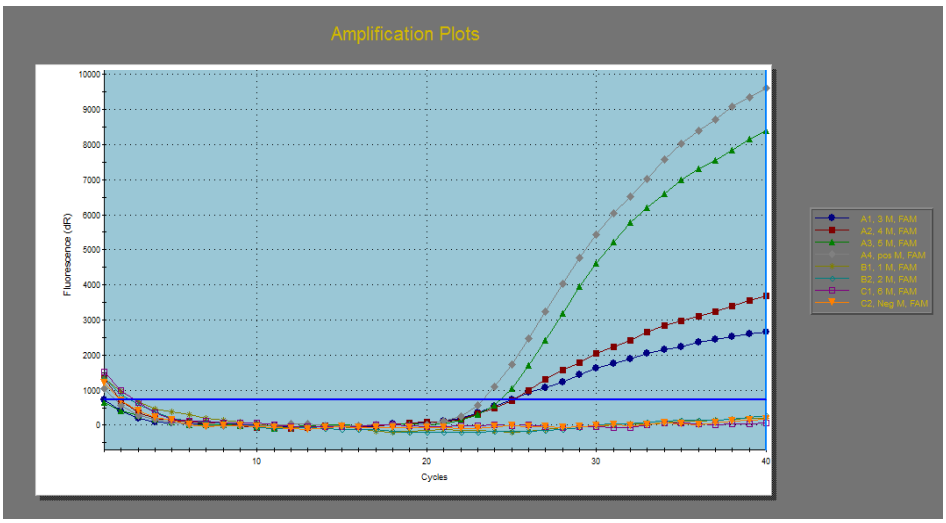

Figure (13): Amplification curve for Matrix AI gene rRT-PCR for respiratory affected farms

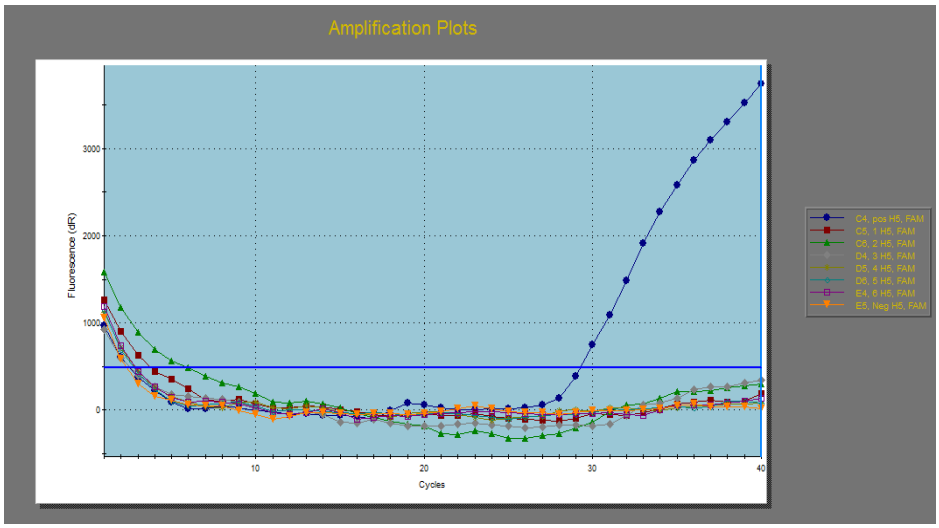

Figure (14): Amplication curve for H5 gene rRT-PCR in respiratory affected farms

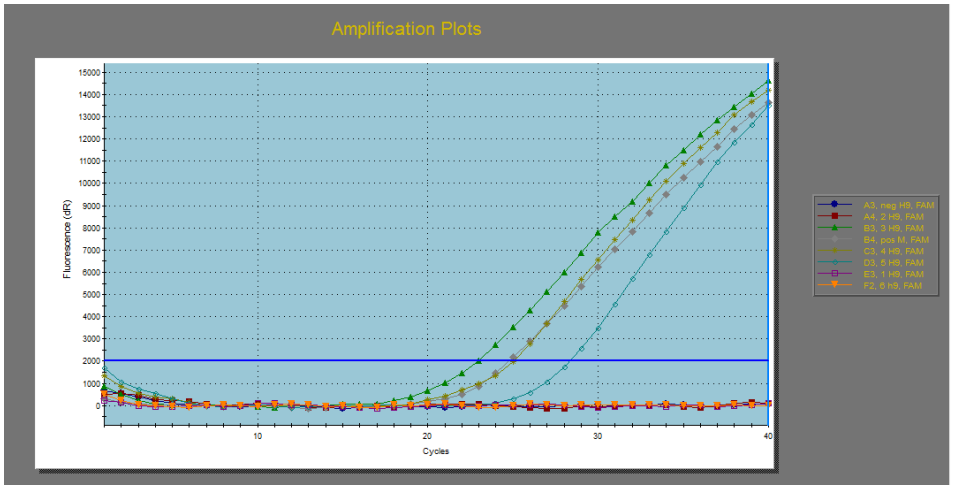

Figure (15): Amplification curve for H9 gene rRT-PCR for respiratory affected farms.

Molecular identification of ND using rRT-PCR

The positive RNA samples were retested by using a specific primer flanking the M gene of ND.

Respiratory affected farms

All lung tissue pools collected in six respiratory affected farms from 18 and 25 days of age were negative for ND using real time RT-PCR (Figure 16).

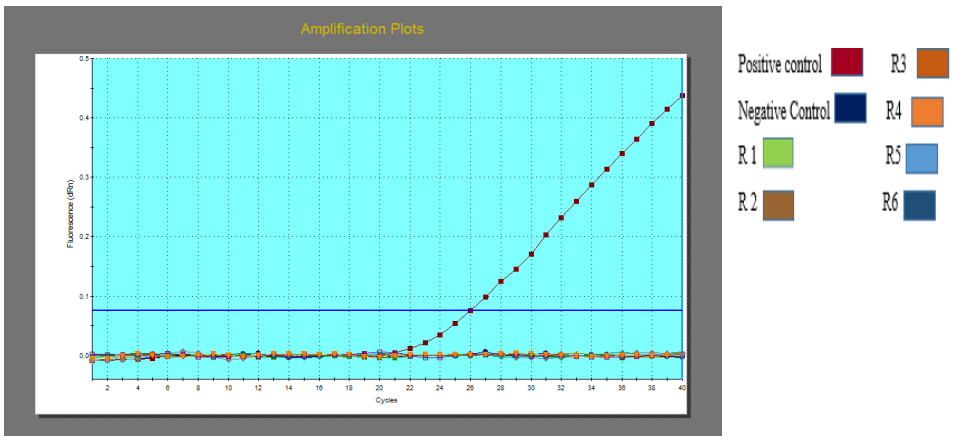

Figure (16): Amplification curve for ND gene rRT-PC for respiratory affected farms with no cut

Sequencing and phylogenetic analysis of VP2 protein of IBD virus:

Fourteen isolates were chosen for genomic sequencing and nucleotid BLASTn analysis. Sequencing of partial length of VP2 protein

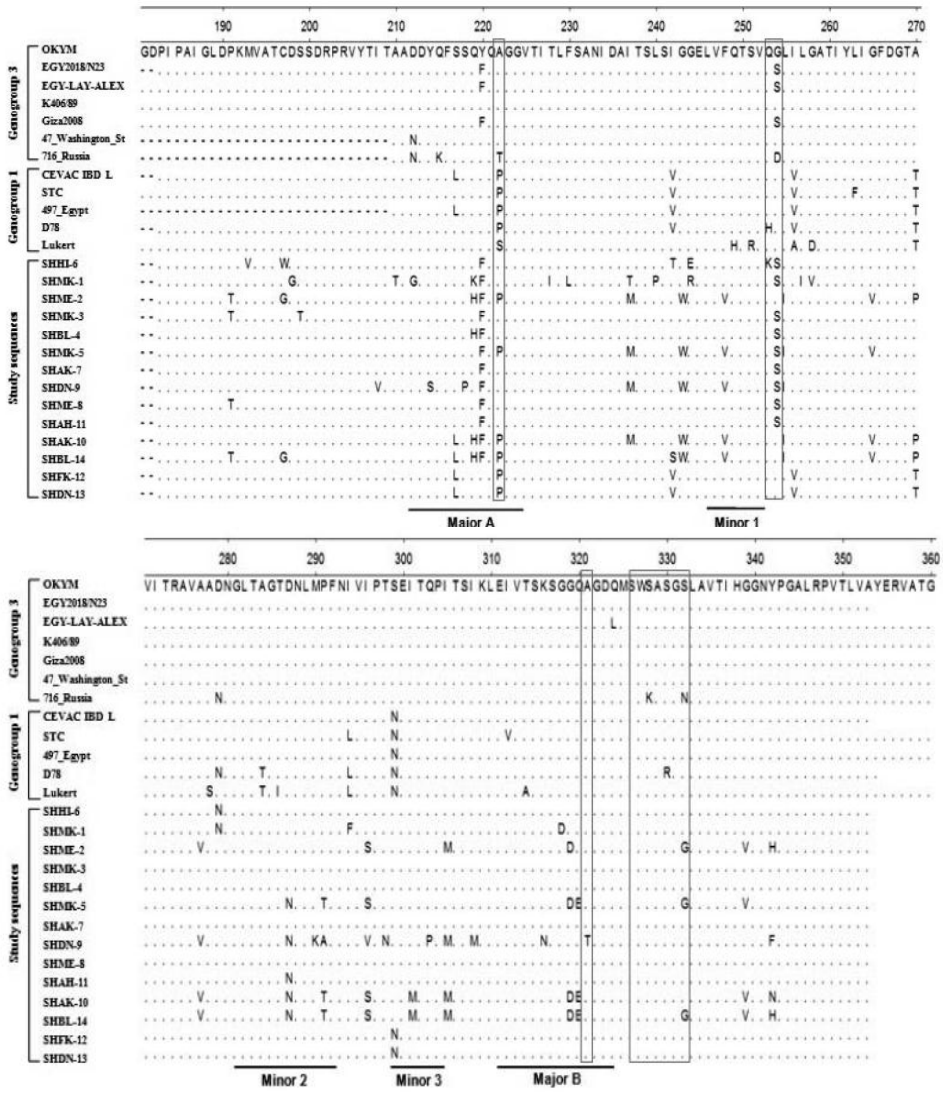

Fig. 4. Alignment of the deduced aa sequences of the VP2 hypervariable region (residues 183–354) of 14 Egyptian IBDV study sequences with other IBDV strains representing genogroup 3, vvIBDV and gengroup 1, and classical IBDV. Dots indicate positions identical to the OKYM sequence. Major hydrophilic peaks A and B and minor hydrophilic peaks 1, 2, and 3 are indicated. Genetic marker positions aa 222, 253–254, 321, and 326–332 are marked with a grey square. The alignment was performed by using ClustalW by MegAlign module of DNAStar software (DNASTAR Lasergene, version 7.2) (31). Numbering of aa sequences is according to Bayliss *et al.* (38).

Figure (17): Alignment of Nucleotide sequences of VP2 gene of 14 Egyptian IBD isolated in comparison to other selected strains.

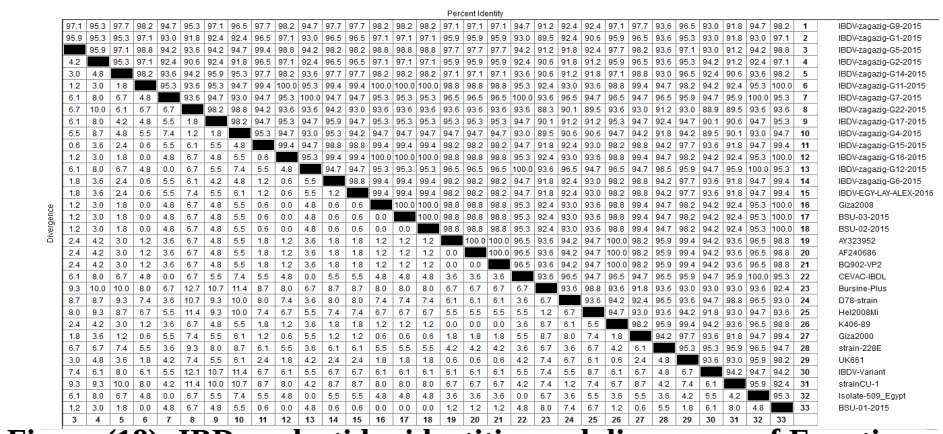

Figure (18): IBD nucleotides identities and divergence of Egyptian IBD isolates in comparison to other selected strains

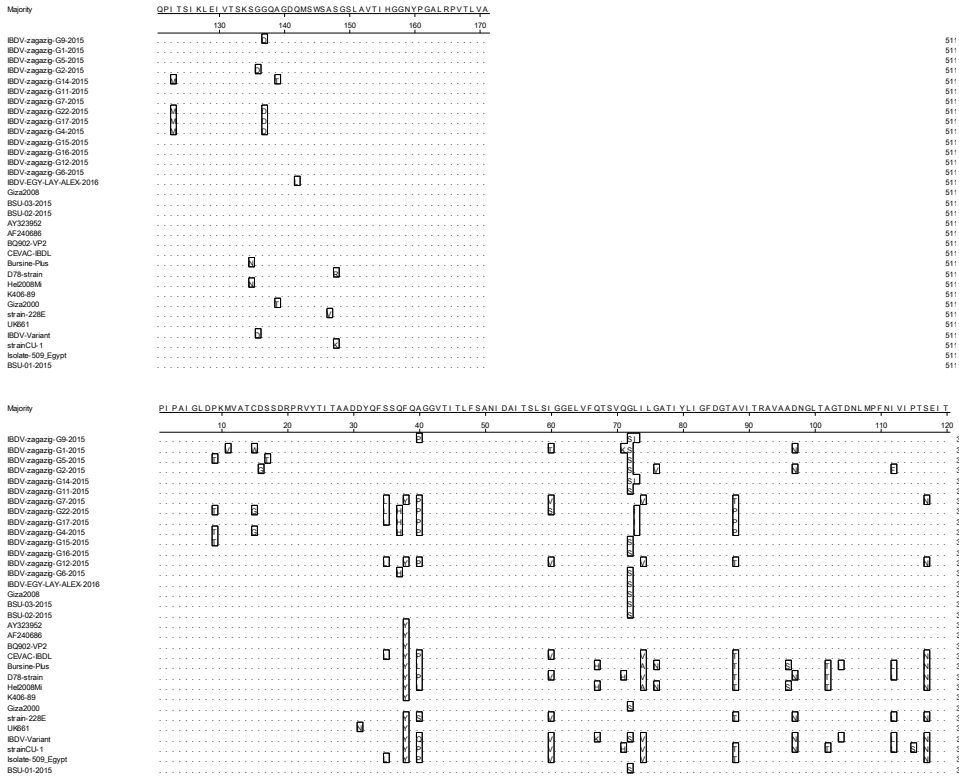

Figure (19): Alignment of Amino acid sequences of IBD protein of 14 Egyptian isolates in comparison to others selected strains.

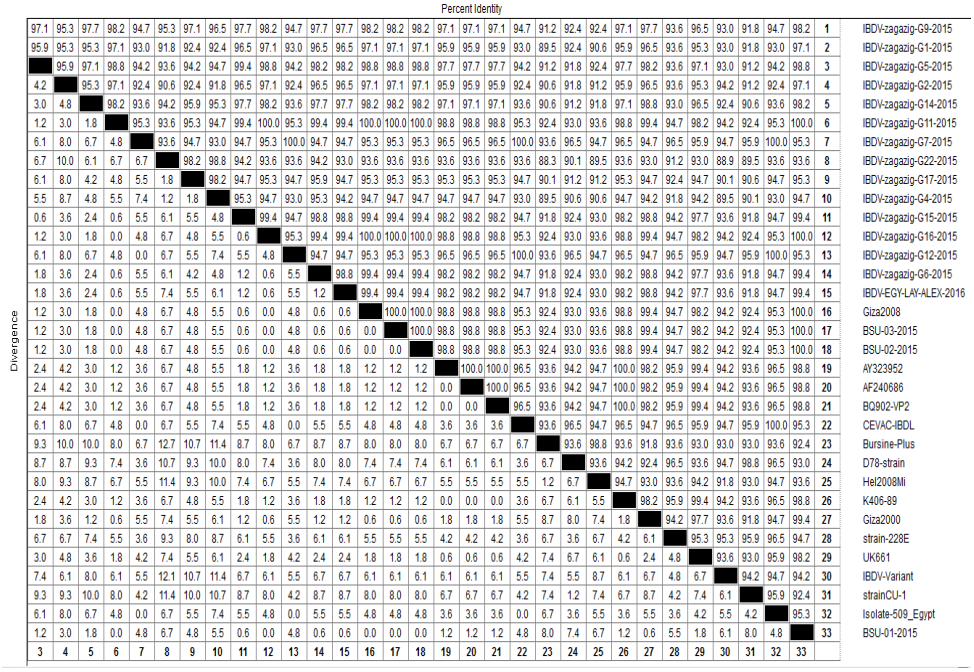

Figure (20): IBD amino acids identities of Egyptian IBD isolates in comparison to other selected strains.

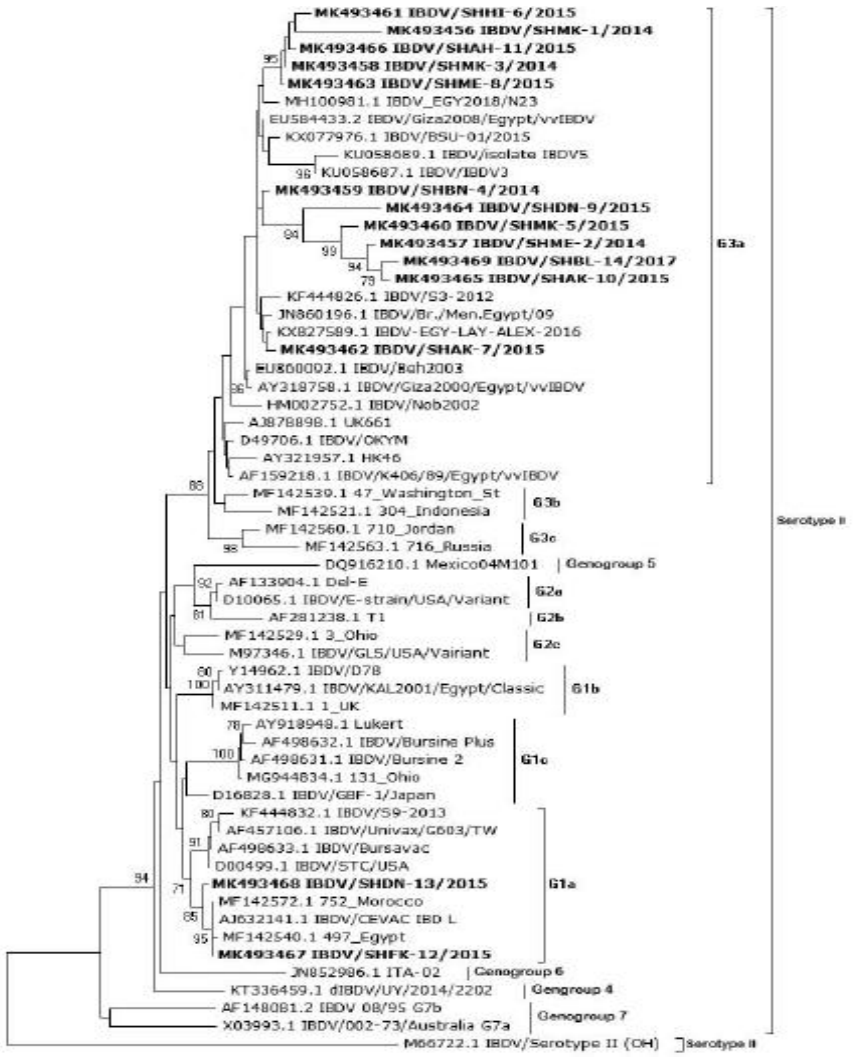

Figure (21): phylogenic analysis of VP2 gene nucleotides sequences of IBD isolates form Sharkia Province, Egypt and other sequences available in Gene Bank. The tree was constructed via multiple alignment of -bp nucleotide sequence of VP2 gene using the neighbor-joining method and the Kimura-2-parameter models in MEGA5. Isolates in the study are marked with solid circle.

On the basis of the nt sequences of the VP2 hyper variable region, the study strains (n¼14) shared identities of 88.3%–99.4% and 81.3%–100% between each other at nt and aa levels, respectively. To determine the origin of the study strains, a phylogenetic tree on the basis of the partial nt sequences of the VP2 gene was constructed, involving representative IBDV strains of different genoroups and sub genogroups of the IBDV serotype I (Fig. 3). Our isolates clustered within genogroup 1(classical strains subgroup G1a; n ¼ 2) and genogroup 3 (vvIBDVstrains subgroup G3a; n¼12). The analysis revealed that two study sequences (SHFK-12 and SHDN-13), which clustered with genogroup 1 were found closely related to IBDV/CEVAC IBD L strain (AJ632141), strain 497_Egypt (MF142540), and 752_Morocco(MF142572), representing a similarity of 94.7%–100%.Those clustered with genogroup 3 vv IBDV strains were closely related to IBDV EGY2018/N23 (MH100981), IBDV/Giza-2008(EU584433), IBDV/BSU-01/2015 (KX077976), IBDV/isolateIBDV5 (KU058689), IBDV/isolate IBDV3 (KU058687), IBDV/S3-2012 (KF444826), IBDV/Br./Men. Egypt/09 (JN860196), andIBDV-EGY-LAY-ALEX-2016 (KX827589; Fig. 24).

Comparative alignment of the VP2 hyper variable region showed that the study sequences share an overall similarity of 87.3%–99.4%with strains of genogroup 3. A comparison of the VP2 gene of the IBDV isolated in this study was carried out with some available vaccines (BursaVac, Bursine 2, Bursine Plus, D78, CEVAC IBD L ,Univax, and GBF-1), and they share a similarity of 87%–99.4%.The highest identity with the IBDV

vaccine was recorded with the IBDV/CEVAC IBD L strain (88.7%–99.4%). However, a lower similarity was observed for Bursine Plus (87%–94.7%). In addition, note that our IBDV strains in G3a were clustered separately.

Deduced aa sequence analysis.

The comparative alignment of the deduced aa sequences of the VP2 hyper variable region of the study IBDVs with reference Gen Bank sequences was performed (Fig. 24).Sequences of IBDV strains of vv IBDV genotype possessed residue sat positions 222A, 242I, 256I, 294I, and 299S, except strainsSHME-2, SHMK-5, SHAK-10, and SHBL-14 had Pro (222P) and242S in strain SHBL-14. In addition, all strains had residues 253Q and 284A, which have been associated with virulence and a pathogenic phenotype of IBDVs, except strain SHHI-6, which had a unique substitution Q253K. Also, the most sequences displaying a vv IBDV genotype analyzed showed G254S. In addition, most of the examined isolates had the Ser-rich heptapeptide (SWSASGS) that was found at positions 326–332, except strains SHME-2, SHMK-5, and SHBL-14 had SWSASGG. Fig. 24 shows aa substitution mutations in the major and minor hydrophilic peaks.

DISCUSSION

V. DISCUSSION

Infectious bursal disease virus (IBDV) belongs to the genus Avibirnavirus within the family Birnaviridae. This non enveloped icosahedral virus has a double-stranded RNA genome consisting of two segments named A and B **(Müller H *et al.*, 1979).**

IBDV is a highly contagious avian pathogen affecting commercial poultry industry worldwide **(Eterradossi and Saif, 2008.).** All pathogenic IBDVs (serotype 1) can be divided into classic, variant, and very virulent strains according to antigenic and pathogenic criteria **(Van den Berg *et al.*, 2004).**

IBDV outbreaks still affect broiler chickens, causing high economic losses.

This study was conducted to investigate the molecular and pathologic features of field IBDVs in vaccinated chicken flocks in the Sharkia Province, Egypt.

To update data and extent of IBD virus in Sharkia Province, total forty commercial broiler farms samples were collected from different age, localities and clinical signs during 2013-2019.

The global situation can be divided into three principal clinical forms, as follows:

a) The classical form, as described since the early 1960s, is caused by the classical virulent strains of IBDV. Specific mortality is relatively low, and the disease is most often

subclinical, occurring after a decline in the level of passive antibodies. **Faragher J.T. (1972)**

b) The immunosuppressive form, principally described in the USA, is caused by low-pathogenicity strains of IBDV, as well as by variant strains, such as the Delaware variant E or GLS strains, which partially resist neutralization by antibodies against the so-called 'classical' viruses**. Jackwood and Saif (1987).**

c) The acute form, first described in Europe, and then in Asia, is caused by 'hypervirulent' strains of IBDV, and is characterized by an acute progressive clinical disease, leading to high mortality rates in affected farms **(Stuart J.C. 1989).**

In regular monitoring farms for sample collection without clear clinical signs for IBDV, while in clinical suspected to be affected IBDV varied mortality rate from (2-20%) with clinical illness of IBDV and in clinically exhibited respiratory manifestation with mortality rate up to 18.7% with swollen head, cyanosis and respiratory signs.

Autopsies performed in regular monitoring farm without any signs of IBDV infection, only clinical signs of protozoal and bacterial diseases, while in clinical suspected to be affected with IBDV showing clinical picture of petticheal hemorrhages on thigh muscle, hemorrhage on proventiculus, hemorrhagic bursae and nephrosis, explaining that these lesions were irrespective of the genotype of the virus. However, the severity, multiplicity, or

reproducibility of pathologic lesions in affected birds are consistent with virulence and antigenic variations of IBDVs. Lesions in the proventriculus and muscles were observed in the studied strains with a vvIBDV genotype, as reported elsewhere (**Mwenda *et al.*, 2018).** Hemorrhages in the pectoral muscles and thighs are frequently observed, probably due to a coagulation disorder **(Skeeles J.K *et al.*, 1980).**

While in respiratory affected farms revealed hemorrhage in trachea, pneumonia, CRD, nephritis and swelling of kidney.

We hypothesize that possible co infections with other viruses, such as AIV and NDV, as well as bacteria that may play a role in exacerbating the clinical picture of IBDV infections in broilers; such results have been reported before (**Hasan *et al.*, 2010)**

The microscopic lesions seen in this study showing Kidney, spleen and bursa of fibricious of suspected to IBDV infected birds showed interstitial hemorrhages with various degrees of degeneration in renal tubules with granular casts in lumina of kidney are similar to those reported by many authors **(Van den Berg, 2000, Oluwayelu *et al.*, 2002, Singh *et al.*, 2015)** who found that the bursa, spleen, and thymus from IBDV-infected birds showed lymphoid depletion, indicating an immunosuppression impact on IBDV. High embryonic death (83.3%) recorded in virus isolation was comparable to 100% mortalities recorded by **(Abdel- Alim and Saif, 2001)**

Samples from regular monitoring farms were collected in two ages (14-21 days) and subjected for viral isolation in embryonated chicken egg without abnormal mortalities in embryo or any pathologic changes in embryo after inspection, on other hand in clinical suspected to be affected farms with high embryo mortality rate range from 40-60% of total embyonated chicken egg.

Results of serology in regular monitoring farms without great variations in geometric mean of H5N1 in two different ages (14-21 day) from zero to 2.75 and 0.75 to 2.25 respectively and in H9N2 from zero to 1, It was recently discovered that highly conserved regions in the HA protein might induce protective antibodies which are able to provide such subtype-independent protection **(Ekiert *et al.*,** 2009**; Wang *et al.*,** 2010**).** While for NDV for two different ages with range 1.6 to 4.25.

Moreover, the very low antibody in Flock S22 of infected farms is due to late vaccination (once at 14 days of age), indicating that birds lack active immunity. Also, the H9 antibodies among this group were less than the respiratory non infected ones with IBDV, although both groups received the same vaccinal regimen against H9N2. Collectively, these findings describe the immunosuppressive effect of IBDV infection.

Sera of suspected to be infected with IBDV flocks results were for H5N1 ranged from 1.83 to 2.50 while for H9N2 ranged from $log_2$1.67 to 2.67 and in NDV ranged from 1.5 to 2.67 in

average age (15-19) days. But Those of exhibiting respiratory manifestation, Although H9N2 AIV is an LPAI virus, it has severe pathogenicity and causes a high mortality rate among farm chickens co-infected with other bacterial and viral diseases **(Bano *et al.*, 2003; Pan *et al.* 2012)**, H5 flock Ab titer ranged from 2.6 to 5.6 on the other hand there is a variation on farms in H9N2 ranged from 0.8 to 4.8 then in NDV ranged from 3.2 to 4.2.

The lower immune response even with ND vaccination in the farms under study could be attributed to the immunosuppressive effect of IBD viruses. The aforesaid rationale was supported by the histopathologic findings in the bursa, kidney and spleen of infected birds. The relatively low mean titer of vaccinated birds clearly contributes to unsatisfactory vaccination results, which may be also related to poor vaccine quality or an unsuitable vaccination regime (**Luc *et al.*, 1992).**

However, vaccination is regarded as an important tool to complement biosecurity efforts. Parent stock vaccination is useful to elicit humoral immunity that will be transmitted to the progeny (passive immunity); it will protect the young chicks for the first few weeks of life **(Maas *et al.*, 2001)**

The ELISA procedure is presently the most commonly used serological test for the evaluation of IBDV antibodies in poultry flocks **(Eterradossi and Saif, 2020)**

AC-ELISA allow the identification of vv IBDV to demonstrate the presence of IBDV specific anti bodies. ELISA

system are commercially available. The virus neutralization assay is the only serological test, which can reliably differentiate IBDV isolate into antigenic serotype and subtype **(Jack wood and Saif, 2004)**

In regular monitoring farms, variable results according to vaccination program and maternal immunity of the flock. At age of 14 days two farms with some variation for IBD immune titer range from 425 to 1452 and after one week all samples were in normal ranged from 1215 to 2039 reflect not present of any changes in farms immunity.

While variation in coefficient variation and mean in clinical suspected to be affected with IBDV, mean titer varied between (117-2543) with different level of coefficient variation which indicate flock infected with IBD, normal titer for IBD mean titer ranged from (2054 to 3065) except last 2 farms ranged above 4000 without high mortality rates and abnormal changes in respiratory signs in exhibited farms.

The probability of perceiving field IBD viruses could be attributed to variation in maternally derived antibody (MDA) levels, as the previous experimental studies indicated that high MDA at the time of IBDV vaccination might interfere with the vaccine response (**Moraes *et al.*, 2005**).

In areas contaminated by IBDV, most broiler flocks have anti-IBDV antibodies when leaving the farm. Current serological tests cannot distinguish between the antibodies induced by pathogenic IBDV and those induced by attenuated vaccine

viruses, so serological diagnosis is of little interest in endemic zones. Nonetheless, the quantification of IBDV-induced antibodies is important for the medical prophylaxis of the disease in young birds, in order to measure the titre of passive antibodies and determine the appropriate date for vaccination **(DeWit, 1999).**

In spite of many reports describing conventional reverse transcriptase (RT)-PCR, sequencing, RT-PCRRE (restriction enzyme), RT-PCR restriction fragment length polymorphism and real-time RT-PCR as being useful tools for management of healthy programmes **(Tham *et al.*, 1995; Kataria *et al.*, 1999; Moody *et al,.* 2000, Jackwood, 2004; Peters *et al.*, 2005; Jackwood& Sommer-Wagner, 2007; Juneja *et al.*, 2008),** the only application of in situ RT-PCR methodology has been described by Liu et al. **(2000), Liu (2000) and Zhang *et al.* (2002),** where incorporated oligonucleotides, named primers, were introduced into the cDNA visualized by in situ hybridization method according to **Chen & Fuggle (1993).**

In regular monitoring farms all samples subjected for RT-PCR for IBDV and AI were negative in both two different age. While, in clinically suspected to be affected farms 19/27 (70.37%) were positive for IBDV. More recently, **(Alkhalefa *et al.*, 2018)** recorded higher percentage of IBDV infection (85%).

Similarly, the studied IBD viruses in Sharkia, Egypt, were genetically diverse. In phylogenetic analysis, the Egyptian IBDVs mostly clustered in the vvIBDV genogroup, explaining

why most IBD outbreaks were found in vaccinated birds. The vvIBDV strains can break through immunity supplied by recent vaccines **(Muller *et al.*, 2012)**

In respiratory diseased on affected farms only one sample 1/6 (16.66%) were positive for IBDV, However, they may produce bursal lesions with the possibility of immunosuppression. Also, they were able to cause virulence and disease conditions **(Jackwood *et al.*, 2008)**

When regular monitoring farms subjected to Rt-PCR for AI, all samples were negative but in respiratory exhibited farms 3/6 (50%) were positive for M gene AI, negative for H5N1 and positive for H9N2 on other hand all samples were negative for NDV.

In this study, two IBD viruses, SHFK-12 and SHDN-13, were genetically related to vaccine strains, suggesting the contribution of vaccinal strains in the epidemiology of IBD in Egypt.

Recently, a phylogenetic analysis on the basis of the hyper variable region of VP2 showed that the isolates were clustered in seven genogroups (**Michel and Jackwood, 2017**). Each genotype contained several subgroups or lineages. Genogroups 1, 2, and 3 were previously categorized as classic, variant, and vvIBDV, respectively. The subgroups within G1 are G1a (STC strain, D00499), G1b (D78 strain, Y14960), and G1c (Lukert strain, AY918948). Genogroup G3 contains three subgroups: G3a, G3b, and G3c defined by UK661 (NC004178),

47_Washington_St, (MF142539.1), and Russia 716 (MF142563.1), respectively **(Jackwood *et al.*, 2018).**

Most of the study strains were located in G3a, while two study strains (SHFK-12 and SHDN-13) were located within G1a. Although the pathogenicity is important for evaluation of the disease severity and degree of immune suppression, we were not able to determine the pathogenicity of viruses under study. Thus, we focused on sequencing of the VP2 hypervariable region, which served as a quick tool to determine phylogenetic relationships **(Le Nouen *et al.*, 2005)**, as well as to determine the mutations in PBC and PHI loops that play vital roles in antigenicity **(Letzel *et al.*, 2007).** Although the majority of our genogroup 3 isolates contained the characteristic aa residues of vvIBDV (222A, 242I, 256I, 294I, and 299S), some of these viruses do not have those typical aa residues, supporting the use of genogroups for accurate classification of IBDVs **(Jackwood *et al.*, 2018).**

Several studies revealed that some residues within the VP2 hypervariable region could be correlated to specific IBDV pathotypes **(Brown *et al.*, 1994).** The predicted aa sequences in this study revealed that most IBDVs had aa residues commonly associated with their characteristic genotypes.

The aa substitutions (G254S and Q324L) were noticed in the PDE and PHI loops. Mutations within the major and minor hydrophilic peaks of the VP2 hypervariable region result in the

emergence of antigenically variant IBDVs, which can escape the immunity bestowed by the parental virus **(Qi *et al.*, 2013).**

At position 254, Ser was observed in most of study strains (n¼ 9), similar to the Italian, American, and Australian variant strains, as well as some of the African ones **(Letzel *et al.*, 2007)**, known to be associated with the reduction of vvIBDV virulence **(Hoque *et al.*, 2001).**

In the PDE loop, G254S was detected previously in the Egyptian isolates, as a contributor to ongoing antigenic drift in IBDV **(Jackwood *et al.*, 2018)** and also from vaccinated chickens with classical IBDV vaccines **(Negash *et al.*, 2012)**, suggesting the role of this mutation in vaccination failure **(Jackwood and Sommer-Wagner, 2011).** Furthermore, the aa mutation S254N was proven to be a contributor to the antigenic drift of Delaware E strain **(Jackwood and Sommer-Wagner, 2011).** In addition, **(Hoque *et al.*, 2001)** recorded that aa substitution G254S and A270E reduced the virulence of vvIBDV strain.

The study sequences (n¼3) have A270P, emphasizing the significance of these alterations in the vvIBDV evolution. Previous studies have revealed that IBDV strains with 253Q and 284A have increased pathogenicity, whereas those with a 253H and 284T are accompanied with reduced pathogenicity **(Jackwood *et al.*, 2008).**

Accordingly, the study strains had 253Q and 284A, which may suggest their virulent nature. Only one strain showed aa substitution Q253K (SHHI-6, MK493461).

At position 321, Ala was detected in 13 IBDV study strains that can bind monoclonal antibody, as those of U.S. variants but not to classical ones **(Letzel *et al.*, 2007).** The aa at positions 318–323 in eight strains is GGQAGD; however, the other six strains have one or two aa substitution at these positions.

Significant aa difference noted in the PBC loop as Y220F in all vvIBDV strains. It was previously reported that mutation at position 212 (D212N) is common in most recent vvIBDV isolates that may influence the structure of VP2 and, consequently, the antigenicity of the virus **(He *et al.*, 2014).**

The unique aa substitution D212G was found in one of the study sequences (SHMK-1). In addition, two strains (SHMK-1 and SHHI-6) were found to contain the mutation D279N similar to the Russian ones. Another substitution (S332G) was found in three sequences. There is a need for additional studies and investigations to explore the potential impact of the observed aa substitutions in this study and their relatedness to pathogenicity, antigenicity, and evolution of IBDVs in Egypt. Due to its immunosuppressive impact, the protection levels obtained in chickens after vaccination are usually affected **(Saif, 1991).**

Thus, the second approach of the present study was to appraise the immunosuppressive role of IBDV in chickens under field conditions. In the current study, the IBD-infected farms

showed the lowest antibody titers against NDV (1.4–2.6 HI log 2 titers) when compared with the apparently healthy ones, as well as those exhibiting respiratory signs. The ND-HI titer of log 3 or above is generally accepted as indicative of specific immunity **(Allan and Gough, 1974).**

Conclusion:

1. Infectious bursal disease is a great threat on poultry production due to high mortality rates and immunosuppression.
2. IBD isolated from vaccinated commercial broiler farms.
3. Monitoring by serological methods is important for all commercial farms.
4. IBD is a cofactor for another viral and bacterial disease in broiler farms.
5. Histopathology reveal changes in lymphoid tissues and suppress immune system.

Recommendations:

1. Regular monitoring of IBD virus by RT-PCR and serological examination.
2. Genomic analysis and sequencing.
3. Serological examination of day old chick to avoid cross immunity and plan for better vaccination program.
4. Biosecurity measures to avoid spread of virus.

SUMMARY

VI. SUMMARY

Infectious bursal disease virus (IBDV) is one of the incriminated etiological agents in both fatal and immunosuppressive disease problems affecting broiler chickens all over the world. In recent years more debate was established about the exact share of this virus in the economic losses in poultry industry in Egypt. This quarrel initiated us to point on the IBD virus infection and its role either as a potential even with regular vaccination or as increasing the susceptibility of other pathogens like avian influenza (AI) subtypes and Newcastle disease (ND) viruses.

For the aforementioned goal, forty broiler flocks at different localities in Sharkia province were investigated for clinical signs and postmortem lesions of IBD virus infection and / or concurrent infections. Alongside, the immune status of the examined birds under different circumstances was explored for the purpose of evaluation of the impact of genetic variation of the isolated IBDV on the increase liability of other viruses.

Clinical inspection among the examined birds revealed no outbreaks in the first category of study farm groups (Regularly monitored broilers), that group was subjected to regular weekly visit for seven broiler chicken farms with total of 56000 birds of various breeds and vaccination programs non-significant mortality rates (0.25%-0.7%) with sporadic sick birds. However, the second group; clinically suspected affected with IBDV (N=27) with a total population of 184000 birds which had an

increase in mortality by (2%-20%) ,General signs of illness, vent picking, huddling together, perfuse watery and whitish diarrhea . the last investigated group included those exhibiting respiratory signs.

Six farms with a total of 46000 birds showed respiratory manifestation and mortality rate up to (18.7%) as well as signs of illness, cyanosis of head, comb and wattles, edema of head and neck with diarrhea.

Necropsy revealed no characteristic lesions of IBD in the first group, but birds of the second group had edematous hemorrhagic bursae (82.1%), hemorrhages (82.1%). in thigh muscles and provetriculus (21.4%) with enlarged inflamed of kidneys with urates (78.6%) of examined birds.

Microscopically, tissue sections of suspected to IBDV infected birds showed interstitial hemorrhages with various degrees of degeneration in renal tubules with granular casts in lumina of kidney and shrunken glomeruli. Spleen exhibited sub-capsular splenic depletion with prominent necrotic changes on most lymphoid follicle and necrotic of lymphocytes with cystic formation beside exudates in the medullary zones of bursal follicles surrounded by inflammatory infiltrations, hemorrhages and edema.

Virus isolation in embryonated chicken eggs (ECE) trials exposed negative detection of IBDV lesions in any tissue pool collected from regularly monitoring farms .All harvested allantoic fluids were HA negative. While, in clinically suspected

affected with infectious bursal disease showed varying degrees of congestion of CAM and embryos. Inflamed swollen kidney, enlarged gall bladder and pale liver were also observed. Embryo deaths ranged from 40% to 60% after 72 to 96 hours post inoculation.

Serological findings using Haemagglutination inhibition test (HI) involve in regularly monitored farms samples were collected in twice, at 14 and 21 days of age . The geometric mean titer (GMT) H5N1 Abs ranged from Zero to 2.75 and 0.75 to 2.25 at 14 and 21 days respectively.The H9N2 Abs were for both 14 days and 21 days (Zero to 1) however NDV testing at 14 days revealed titres from 1.6 to 2.6 and in 21 days from 3 to 4.25 .

Sera of suspected to be infected with IBDV flocks had GMT of Abs against H5N1 (1.83 to 2.50), while for H9N2 (1.67 to 2.67) and in NDV (1.5 to 2.67). But birds suffering from respiratory signs had 2.6 to 5.6 Abs of H5N1; 0.8 to 4.8 for H9N2 and NDV Abs ranged from 3.2 to 4.2.

Using ELISA test in regular monitoring farms, variable Abs against IBDV were recorded bestowing to vaccination program and maternal immunity of the flock. 425 to 1452 in age of 14 days and from 1215 to 2039 at 21 days old.

While, in clinically suspected infected with IBDV, there was a great variation for all farms, mean titer vary between (117-2543) with different levels of coefficient variation which

indicates IBDv flock infection possibility. IBD mean titer ranged from (2054 to 3065) in the respiratory affected birds.

Molecular identification of IBD using rRT-PCR, revealed no VP2 gene of IBDV, (620-bp) in all of the regularly monitored farms. However, 19 (70.37%) IBD isolates were detected in IBD clinically suspected. Selected 6 positive VP2 samples were submitted to RT-PCR for VP1h which presented only in 4 cases farms. One sample from suspected respiratory manifestation farms was positive for IBD and other 5 samples were negative.

Concerning molecular detection of other avian viruses by using real time RT-PCR ,all lung tissue pools collected in both 14 and 21 days of age from regularly monitored farms have no H5or H9 subtypes of AIVs or NDV .

Three(50%) of six samples of respiratory affected farms were positive for AI M gene.When samples were subjected for H5 test in rRT-PCR, all samples were negative. But were H9 positive.

Concerning sequencing and phylogenetic analysis of VP2 protein of IBD virus; Fourteen IBDV isolates were chosen for genomic sequencing and nucleotid BLASTn analysis. Sequencing of partial length of VP2 protein.

On the basis of the nt sequences of the VP2 hypervariable region, the study strains (n¼14) shared identities of 88.3%–99.4% and 81.3%–100% between each other at nt and aa levels, respectively.

Upon phylogenetic analysis of14 Egyptian IBDVs, VP2 hypervariable region of most viruses (n ¼ 12) were clustered within the genogroup 3, although two viruses were closely related to attenuated vaccine isolates in genogroup 1.

The analysis of the amino acid (aa) sequences revealed that most of the strains possessed five consistent aas at the VP2 protein (222A, 242I, 256I, 294I, and 299S), which are characteristic for the very virulent IBDV (vvIBDV).

It could be concluded that IBDV infection is still a threaten for the poultry industry even with regular vaccination. Serology indicated the immunosuppressive effect of IBDV, which is represented by a decrease (1.6–2.6 and 1.4–2.6 mean log 2) in the HI titer of the low pathogenic AIV subtype H9N2 and NDV, respectively. The examined IBDVs showed a high mutation rate within the hypervariable domain of the VP2 peptide. The results highlighted the need for carrying out an inclusive surveillance of IBDV infections in chicken flocks in Egypt.

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نبذة عن تاريخ الباحث

* ولد الطالب في مدينة ههيا - محافظة الشرقية –مصر في ٩ فبراير عام ١٩٨٢
* حصل علي شهادة إتمام الدراسة الإبتدائية من مدرسة مصطفي كامل الإبتدائية بههيا الشرقية عام ١٩٩٢
* حصل علي شهادة إتمام الدراسة الإعدادية من مدرسة ههيا الإعدادية الحديثة بنين بههيا الشرقية عام ١٩٩٥
* حصل علي شهادة الثانوية العامة من مدرسة ههيا الثانوية بنين بههيا الشرقية عام ١٩٩٨
* أتم الطالب دراسته الجامعية بكلية الطب البيطري-جامعة الزقازيق وحصل علي درجة البكالوريوس في العلوم الطبية البيطرية بتقدير جيد دور مايو ٢٠٠٣
* حصل الطالب علي درجة الماجستير في العلوم الطبية البيطرية أمراض الدواجن من قسم طب الطيور والأرانب-كلية الطب البيطري- جامعة الزقازيق في يناير ٢٠١٠
* يعمل الطالب باحث مساعد بالمعمل المرجعي للرقابة البيطرية علي الإنتاج الداجني معهد بحوث الصحة الحيوانية بالزقازيق-محافظة الشرقية حتي تاريخه.

ARABIC SUMMARY

الملخص العربى

يعد فيروس مرض التهاب الكيس الفبراشى المعدي (IBDV) أحد العوامل المسببة للأمراض المميتة و المثبطة للمناعة التي تؤثر على دجاج التسمين في جميع أنحاء العالم. في السنوات الأخيرة تم إجراء المزيد من الدراسات حول الدور الحقيقى لهذا الفيروس في الخسائر الاقتصادية في صناعة الدواجن في مصر. والذى كان الباعث وراء الدراسة الحالية عن ظهور عدوى حقلية لفيروس IBD رغم التحصين المنتظم ضده وكذلك هذا المرض ودوره فى مشاكل الدواجن أو زيادة قابلية مسببات الأمراض الأخرى مثل الأنواع الفرعية لإنفلونزا الطيور (AI) وفيروسات مرض نيوكاسل (ND) وغيرها من الأمراض.

للهدف المذكور أعلاه ، تم دراسة أربعين قطيع من دجاج التسمين في مناطق مختلفة بمحافظة الشرقية بحثًا عن الأعراض الاكلينيكية والصفة التشريحة والتعرف على عدوى فيروس IBD و / أو العدوى لفيروسات المتزامنة. إلى جانب ذلك ، تم تتبع الحالة المناعية للطيور التي تم فحصها في ظل ظروف مختلفة بغرض تقييم تأثير التنوع الجيني للـ IBDV المعزول على زيادة ضراوة المرض الناتج عن الفيروسات الأخرى

كشف الفحص الأكلينيكى بين الطيور بمحموعات قطعان مختلفة عن عدم وجود اعراض فى المجموعة الأولى(٧قطعان) بإجمالي ٥٦٠٠٠ طائر من سلالات مختلفة وبرامج تطعيم متنوعة والتى خضعت للمتابعة الاسبوعية بانتظام وكانت النفوق في الاطار الطبيعى (٠.٢٥٪ -٠.٧٪) مع تسجيل بعض الطيور المريضة (حالات فردية). أما المجموعة الثانية و المشتبه اكلينيكيا بأصابتها بعدوى IBDV بلغ ٢٧ قطيع بإجمالي ١٨٤٠٠٠ طائر والتي عانت من معدل نفوق متزايد فيها بنسبة (٢٪ -٢٠٪) ، وعلامات عامة للمرض ، ومنها نقر في فتحة المجمع مع رعشة وزيادة استهلاك مياه الشرب وقلة استهلاك العلف وتجمع الطيور معًا لارتفاع درجة حرارة الطيور ، واسهال مخاطي أبيض كريمي. كما تم دراسة مجموعة ثالثة تشمل ٦ مزارع بها ٤٦٠٠٠ طائر وتعانى من اعراض تنفسية ومعدل نفوق (١٨.٧٪) بالإضافة إلى إزرقاق وورم الرأس والعرف مع الإسهال.

وبتشريح الطيور لم توجد اى تغيرات مميزة لـ IBD في المجموعة الأولى ، ولكن طيور المجموعة الثانية لديها نزفي وتورم فى الكيس الفبراشى (٨٢.١٪)

ونزيف (٨٢.١٪). في عضلات الفخذ والمعدة الهاضمة(٢١.٤٪) مع تضخم في الكلى وتجمع أملاح اليوريا فى (٧٨.٦٪) من الطيور التي تم فحصها وتشيير باشتباه اصابتها ب IBD.

وبفحص قطاعات الانسجة مجهريا ، أظهرت الطيور المشتبه باصابتها بـ IBDV نزيف خلالي بدرجات مختلفة من الأنابيب الكلوية مع قوالب حبيبية في تجاويفها وانمكاش فى والكبيبات الكلوية. أظهر الطحال اضمحلال تحت المحفظة مع تغيرات نخرية بارزة على معظم الجريبات اللمفاوية ونخر الخلايا الليمفاوية بالكيس الفبراشى بجانب الإفرازات في مناطق النخاع من البصيلات الجدارية لها مع احاطتها بالالتهابات مع انزيفة وتورم.

وبحقن محلول الأنسجة لعزل الفيروس في اجنة بيض الدجاج (ECE) عن عدم وجود علامات لفيروس IBDV في أي من أنسجة المجموعة الاولى بينما المجموعة الثانية و المشتبه بإصابتها ب IBDV أظهرت درجات متفاوتة من احتقان CAM والأجنة. كما لوحظ تورم في الكلى الكبد وشحوب . تراوحت نسب نفوق الأجنة من ٤٠٪ إلى ٦٠٪ بعد ٧٢ إلى ٩٦ ساعة بعد الحقن علي التوالي.

اما الاختبارانت السيرولووجية باستخدام اختبار تثبيط التلازن الدموي (HI) فى المجموعة الاولى في عمر ١٤ و ٢١ يومًا. تراوح عيار المتوسط الهندسي للاجسام المناعية ضد فيروس H5N1 تراوح من صفر إلى ٢.٧٥ ومن ٠.٧٥ الى ٢.٢٥ في اعمار ١٤ و ٢١ يومًا على التوالي ، وكانت الاجسام المضادة ل H9N2 فى عمر كلا من ١٤ ا و ٢١ يومًا (صفر إلى ١) ولكن اختبار NDV في ١٤ يومًا تم الكشف عنه من ١.٦ إلى ٢.٦ وفي ٢١ يومًا من ٣ إلى ٤.٢٥

وكانت اعينات السيرم للمجموعة الثانية المشتبه بإصابتها ب IBDV لديها اجسام مضادة (1.83 2.50) H5N1 ، بينما بالنسبة لـ H9N2 إلى (٢.٦٧-١.٦٧) وفي1.50 NDV إلى ٢.٦٧). لكن المجموعة الثالثة التي تعاني من علامات تنفسية كان لديها ٢.٦ إلى ٥.٦ ضد .H5N1 وتراوحت ٠.٨ إلى ٤.٨ لـ H9N2 وبينما NDVمن ٣.٢ إلى ٤.٢. وباستخدام اختبار ELISA في مزارع المراقبة المنتظمة ، تم تسجيل مستويات متغيرة من الاجسام المناعية ضد IBDV تبعا لبرنامج التحصين ومناعة الأمهات: ٤٢٥ إلى ١٤٥٢ بعمر ١٤ يومًا ومن ١٢١٥ إلى ٢٠٣٩ بعمر ٢١ يومًا. بينما ، في المجموعة المشتبه بها المصابة بـ IBDV ، هناك اختلاف كبير

لجميع المزارع ، متوسط الاجسام المضادة يختلف بين (١١٧-٢٥٤٣) مع مستوى مختلف من معامل الاختلاف مما يشير إلى احتمال إصابة القطيع ب IBDv وتراوح متوسط المناعة ضد IBD من (٢٠٥٤ إلى ٣٠٦٥) فى الطيور المصابة بالجهاز التنفسي.

كشف التعريف الجزيئي لـ IBDV باستخدام rRT-PCR ، عن عدم وجود جين VP2 لـ (620-bp) IBDV في جميع المزارع التي فحصها يشكل دورى (المجموعة الاولى) بينما تم الكشف عن ١٩ عزلة (٧٠.٣٧٪) من فيروس IBD بمجموعة الثانيةوباختبار عدد ٦ عينات VP2 إيجابية إلى RT-PCR لـ وجدت اربعة عينات فقط ايجابية. كانت عينة واحدة من مزارع مظهر الجهاز التنفسي المشتبه بها إيجابية بالنسبة لـ IBD وكانت ٥ عينات أخرى سلبية.

فيما يتعلق بالكشف الجزيئي عن فيروسات الطيور الأخرى باستخدام-RT PCRفإن جميع عينات أنسجة الرئة التي تم جمعها في كل من ١٤ و ٢١ يومًا من المزارع التي تخضع للمراقبة المنتظمة لا تحتوي على أنواع فرعية من H5or H9 من AIVs أو.NDV.

بينما كانت ثلاث (٥٠٪) من ست عينات من المزارع المصابة باعراض تنفسية إيجابية بالنسبة لجين AI- M ، وعندما خضعت العينات لاختبار H5 في rRT-PCR، كانت جميع العينات سلبية. ولكن كانت H9 إيجابية.

فيما يتعلق بالتسلسل والتحليل النشوئي لبروتين VP2 لفيروس IBD ؛ تم اختيار أربعة عشر معزولة IBDV للتسلسل الجينومي وتحليل الحامض النووي. تسلسل الطول الجزئي للبروتينVP2 .

على أساس التسلسل للنيوكليتدات nt الاحماض الامينية aa لمنطقة VP2 شديدة التغير ، شاركت سلالات الدراسة (n¼14) في ٨٨.٣٪ -٩٩.٤٪ و ٨١.٣٪ - ١٠٠٪ بين بعضها البعض عند مستويات للنيوكليتدات الاحماض الامينية على التوالي.

بناء على التحليل الوراثي للسلالات ١٤ IBDVs المصرية ، لوحظ تجميعها منطقة VP2 شديدة التغير من معظم الفيروسات (١٢) ضمن المجموعة الوراثية ٣ ، على الرغم من ارتباط اثنين من الفيروسات ارتباطًا وثيقًا بعترات اللقاح المخفف في المجموعة الوراثية ١.

كشف تحليل تسلسل الأحماض الأمينية أن معظم السلالات تمتلك خمسة احماض امينية متناسقة في بروتين VP2 (222A، 242I، 256I، 294I، و 299S)، والتي هي مميزة لـ IBDVشديد الضراوة.

يمكن استنتاج أن عدوى IBDV لا تزال تهدد صناعة الدواجن حتى مع التحصين المنتظم. أشارت الأمصال إلى التأثير المثبط للمناعة لـ IBDV ، والذي يمثله انخفاض (٢.٦–١.٦ و ٢.٦–١.٤ متوسط لوغ ٢) في مستوى اجسام المناعية HI من النوع الفرعي AIV الممرض المنخفض H9N2 و NDV ، على التوالي. أظهرت الـ IBDVs التي تم فحصها معدل طفرة مرتفع داخل المجال شديد التغير في الببتيد VP2. أبرزت النتائج الحاجة إلى إجراء ترصد شامل للعدوى بالفيروس المسبب لمرض IBDV في قطعان الدجاج في مصر.

(سورة التوبة آيه رقم ١٠٥)

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دور الإختلافات الجينية لفيروس إلتهاب الكيس الفبراشي المعدى فى خطورة إنفلوانزا الطيور

رسالة مقدمة من

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ماجستير العلوم الطبية البيطرية أمراض الطيور والأرانب– جامعة الزقازيق (٢٠١٠)

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للحصول على درجة

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