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Characterization of real risks of high mortality associated with respiratory disease syndrome (RDS) in commercial broiler chicken in Egypt, 2015

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Abstract

During the last 10 years the broiler sector in Egypt was suffering from high mortality rates accompanied with respiratory manifestations. This problem was identified as a multifactorial respiratory disease syndrome (RDS). In winter 2015, a total of 16 commercial broiler farms of 400,000 chicken's capacity/cycle were suffering from respiratory manifestation and high mortality rates (13% to 62%). The mortalities and respiratory manifestations were started from the 3rd week of age and continued till the end of production cycles, also these farms were suffering on both levels of biosecurity and management practices.

In the current study epidemiological information and field samples (Swabs, blood, feed, and water samples) were collected. Laboratory investigations were conducted via virus detection using PCR, bacterial isolation, feed and water analysis in addition to serological monitoring using ELISA, haemagglutination inhibition (HI) test to identify possible real risks of RDS.

Virus detection using PCR showed (13/16) of tested farms were positive for infectious bronchitis virus (IBV), (7/16) for virulent Newcastle disease virus (vNDV), (7/13) for infectious bursal disease (IBD), (2/16) AI/H9N2, only one case was positive for infectious laryngeo tracheitis (ILT) and all tested farms negative for both highly pathogenic (AI/ H5N1) and avian pneumovirus (APV).

Genetic analysis results confirmed circulation of IBV variant 2,vNDVgenotype VII-d, and very virulent IBD viruses. Bacterial examination confirmed isolation of antibiotics resistant *E.coli and Staph aureus* from the internal organs in both farm 3 and 9, while *Proteus spp.* was isolated from farm 9 only. Feed examination showed positive isolation of *E. coli*. Bad quality water was identified with a high level of chloride, sulfate, total salts, total bacterial count and total coliform count in both water source and farm water.

In conclusion, the main cause of this RDS and high mortalities was a multifactorial risks such as the high challenge of different viruses (IBV, vNDV, IBD, H9N2 and ILT) and antibiotics resistance bacteria (*E. coli and staph aurous*). But the disease situation usually complicated due to the improper biosecurity, bad management, bad quality water and feed, which play key role in birds immunosuppression and mortalities and open the way for other disease causing agent to show their impact. The main corrective and preventive action must be focused on the non-pharmaceutical intervention (such as improvement management practices, selection good quality food and water) and adoption of effective biosecurity and veterinary care programs.

Key words: *"Avian Influenza (AI), Infectious bronchitis virus (IBV), commercial broiler and high mortality".*

Introduction

Egypt was suffering from high mortality rates in different poultry production sectors specially the broiler sector during the last 10 years. These mortalities were correlated to circulation of new different virulent viruses like infectious bronchitis (IBV) variant 2 **(Abdel-Moneim *et al.,* 2012),** virulent NDV genotype VIId **(Abdel-Moneim *et al.,* 2006b and Kilany *et al.,* 2015)**.

This situation became worthiest by winter 2011 after emerging the low pathogenic avian influenza H9N2 **(El-Zoghby *et al.,* 2012)**, as well as the current endemic situation of highly pathogenic avian influenza H5N1 since 2006.
IBV is the most important respiratory disease in chickens of all ages and characterized by severe loss of egg production in mature hens. Some IBV strains cause nephritis in young birds and is occasionally reported to be associated with enteritis **(Gorgyo *et al.,* 1984).** Further studies confirmed isolation of new different IBV strains related to the Massachusetts D3128, D274, D-08880 and 4/91 genotypes at different poultry farms in Egypt **(Abdel-Moneim *et al.* 2006a and Sultan *et al.* 2004).** In addition to the recent introduction and evolution of the Egyptian variant I (Egypt/Beni-Suef/01) and Egyptian variant II that was isolated during 2001 and 2011 **(Abdel-Moneim *et al.* 2002 and Abdel-Moneim *et al.* 2012).**

Newcastle disease virus (NDV) is a highly contagious disease affects poultry industry worldwide. Recently in Egypt sever economic losses were recorded in association with vNDV genotype VIId outbreaks since 2010 **(Abdel-Moneim *et al.* 2006b and Kilany *et al.,* 2015)**. On the other hand infectious Bursal Disease (IBD) is one of the highly contagious and immunosuppressive viral poultry disease that affects birds during the first six week of age. Subclinical IBD infection could cause early immunosupresion before 3rd weeks of age **(Lukert and Saif, 1997)**. In Egypt, evidence of circulating variant IBD strains were isolated from flocks vaccinated using classical IBD vaccines **(Hussein *et al.,* 2003).** The low pathogenic avian influenza (LPAI) H9N2 virus infection recorded in Egypt since 2011 **(El-Zoghby *et al.,* 2012).** The LPAI H9N2 infection didn't cause serious problem among affected birds, unless it's complication with other pathogens (**Naeem *et al.,* 1999).** On the other hand, co-infection with other pathogens such as *Staphylococcus aureus, Haemophilus paragallinarum, E. coli* or infectious bronchitis virus can aggravate H9N2 infections, resulting in high mortality rates **(Bano *et al.,* 2003 and Haghighat-Jahromi *et al.,* 2008).**

Most of recent studies indicated the important immunosuppressive role of secondary bacterial infection that a comparing with different viral infections. g. *Staphylococcus aureus* **(Igbokwe *et al.,* 2012)** which can invade the bird body through the respiratory route and may localized in the lung and spread through blood or lymphatic route to reach the parenchymal organs and leg joints **(Jensen *et al.,* 1987).** *E. coli* is the main cause of colibacillosis in poultry mainly causing of secondary infection when the immunity decreased due to any stress factor **(Barnes *et al.,* 2008)**.

Although *Proteus spp.* is one of normal inhabits at lower intestinal tract, recently some records of septicaemia due to *Proteus* infection had been occurred in quail **(Mohamed, 2004).** Moreover *Proteus* has been associated with respiratory disease in chickens with mortality rate reaching 50% **(Lin *et al.,* 1993).** The role of farm biosecurity, management systems and practices in addition to potential role of feed and water as a source of transmission of many bacterial and viral agents in poultry farms cannot denied **(Whyte *et al.,* 2003).**

Due to this complicated situation, all these risks and possible causes of RDS can produce high mortalities in boiler sectors in Egypt. Accordingly, the present study was dedicated to investigate the main biological and non-biological factors that probably participate in the current problems.

Material and method

Case history

During winter of 2015 a problem of high mortality with sudden death was reported to the reference laboratory for veterinary quality control on poultry production (RLQP). A group of broiler farms (16 farms) with total production capacity 400,000 birds /cycle that present in the same desert district in Giza governorate were suffering from high mortality rate (13-62%) and respiratory signs. These flocks employed two intensive vaccination programs against most common poultry infectious disease as shown in Table 1. A detailed investigation includes flocks history, biosafety practices, and management practices with collecting different random laboratory samples for laboratory diagnosis were adapted. These flocks were using the same water source (underground), and mainly the same feed source supply.

Samples for laboratory investigation

From each flock, 10 tracheal swabs, 10 cloacal swabs and different organs were collected for PCR testing against the following viruses AI/H5N1, AI/H9N2, vNDV, IBV, IBD, ILT and APV. Random 10 serum samples from survived birds were collected for evaluating the immune response. In addition different organs (liver, heart, intestine) from dead birds were collected for bacterial diagnosis and sensitivity testing. In addition, feed samples and water samples were also collected.

Table (1). Main adopted vaccination programs at the studied farms

Program Type	Vaccination age	Name and Type of vaccine		Vaccination Rout
Program 1	1st day of age	Trovac (rFP-AI)	Live	S/C
		Vaxxitek (rHVT-IBD)	Live	S/C
		H9ND	Killed	S/C
		IB primer (H120+D278)	Live	Spray
	Day 8	Re 5 (H5N1)	Killed	I/M
		LaSota	Live	Eye drop
	Day 19	LaSota	Live	Spray
Program 2	1st day of age	Vectormune AI (rHVT-AI)	Live	S/C
		Transmune (IBD)	Live	S/C
		H9ND	Killed	S/C
		IB primer (H120+D278)	Live	Spray
	Day 8	LaSota	Live	Eye drop
	Day 19	LaSota	Live	Spray

Different vaccines and vaccination practices were conducted by the farm owners and under their supervision

Real time RT-PCR for detection of viral agents

RNA was extracted from different field samples using QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's recommendations.

AI/H5, AI/H9, IBV and NDV specific primers were utilized in a 25µl reaction containing 12.5 µl of Quantitect probe rt-PCR master mix (Qiagen, Germany, GmbH), 0.5 µl of each primer of 50pmol concentration, 0.125 µl of the specific probe, 4.5 µl of water, 0.25 µl of the rt-enzyme and 6 µl of RNA extract. The reaction was performed in a Stratagen MX3005P real time PCR machine (Stratagene, USA).

Primers and probes used for Real time RT-PCR identification.

Virus	Primer sequences	Ref.
H5	H5LH1: ACATATGACTAC CCACARTATTCA G H5RH1: AGACCAGCT AYC ATGATTGC H5PRO: [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	**Slomka *et al.*, (2007)**
H9	For: GGA AGA ATT AAT TAT TAT TGG TCG GTA C Rev: GCC ACC TTT TTC AGT CTG ACA TT H9probe:[FAM] AAC CAG GCC AGA CAT TGC GAG TAA GAT CC[TAMRA]	**Ben Shabat *et al.*, (2010)**
IBV	AIBV-fr: ATGCTCAACCTTGTCCCTAGCA AIBV-as: TCAAACTGCGGATCATCACGT AIBV-TM: [FAM]TTGGAAGTAGAGTGACGCCCAAACTTCA [TAMRA]	**Meir *et al.*, (2010)**
NDV	F+4839 TCCGGAGGATACAAGGGTCT F-4939 AGCTGTTGCAACCCCAAG F+4894 [FAM]AAGCGTTTCTGTCTCCTTCCTCCA [TAMRA]	**Wise *et al.*, (2004)**

RT-PCR for detection of IBD and APV

A 25µl reaction containing 12.5µl of Quantitect probe RT-PCR buffer (Qiagen, Germany, GmbH), 0.25µl of Rt-enzyme, 1µl of each primer of 20 pmol concentration, 4.25µl of water and 6µl of template RNA was used. The reactions were performed in a T3 thermal cycler (Biometra). The amplicons were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) along with 100 bp DNA Ladder (Qiagen, Germany, GmbH). The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Virus	Primer sequences	Amplicon size	Ref.
IBD	AUS GU:TCACCGTCCTCATGCTTACCCACATC AUS GL:GGATTTGGGATCAGCTCGAAGTTGC	**620 bp**	**Metwally *et al.*, (2009)**
APV	C1--GATGACTACAGCAAACTAGAG C2—CTTCAGGACATATCTCGTAC	**468 bp**	**Shin *et al.*, (2000)**

The PCR reaction for IBD was performed as follow 20 min at 50°C (RT reaction) 95°C for 15 min (initial PCR reaction) 39 three step cycles of 94°C for 30 sec (denaturation) 59°C for 40 sec (annealing) and 72°C for 10 min (final extension).

The PCR reaction for APV was performed as follow 20 min at 50°C (RT reaction) 95°C for 5 min (initial PCR reaction) 35 three step cycles of 94°C for 30 sec (denaturation) 51°C for 1 min (annealing) extension at 72°C for 2 min, followed by 72°C for 10 min (final extension).

PCR test for ILT virus detection

DNA was extracted using commercially available kit, QIAamp DNA Mini Kit (Catalogue No.51304). PCR amplification for ILT was conducted using 25 µl reaction containing 12.5 µl of Emerald Amp GT PCR master mix (2x premix), 1 µl of 20 pmol conc. specific primers ICP4-1F: ACTGATAGCTTTTCGTACAGCACG and ICP4-1R AGTCATGCGCATCGGGACATTCTCCAGGTAGCA **(Sadeghi *et al.*, 2011)**, 4.5 µl of PCR grade water, and 6 µl of template. The PCR reactions were performed in a Biometra T3 thermal cycler. The conditions for DNA synthesis were: 94°C /3 min for initial denaturation, followed by 35 cycles of 94°C/1min and 62°C /1 min,72°C /1.5 min, and a final extension at 72°C /10 min. finally. The PCR products were separated by electrophoresis on 1.5% agarose gel and photographed by a gel documentation system (Biometra).

Genetic analysis

Genetic analysis was conducted for sample obtained from farm No. 4 which was suffering from high mortality 61%, using the positive Viral RT-PCR product and for the subsequent sequence analysis using forward and reverse PCR primers for amplification. The PCR amplification was performed using Qiagen One Step Enzyme Mix according to the manufacturer's instructions. Gel containing DNA band of the expected size was excised and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer instruction. The purified PCR products were sequenced directly using BigDyeTM Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), The products of the sequencing reactions were cleaned-up using Centrisep purification kit Analyzer (Applied Biosystem, CA - USA). The purified products were sequenced directly using the ABI (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3130 genetic analyzer (Applied Biosystems). The phylogram was drawn using also MEGA 6 software. The alignment of the viruses in the study was done using DNA star MegAlign software. Egyptian viruses and other international reference strains from the Genbank were available from the National Center for Biotechnology Information (NCBI) (http:// www.ncbi). Finally the identity percent and divergence between all viruses was carried out.

Serological monitoring

The randomly collected 10 blood samples from each farm were treated for serum separation by centrifugation at 1800 rpm/5min, separated serum samples were preserved at -20^{o}C till testing at the serology unit.

Enzyme linked immunosorbent assay (ELISA) were used to evaluate humoral antibody response against IBV and IBDV. The ELISA kit was commercially licensed supplied from Biochek Company. ELISA results interpretation was adopted using the Biochek software which permitted to calculate the positivity ratio (S/P) and titer for each sample, as well as geometric mean titers/flock, coefficient variance (CV%) and positivity percentage{(total no of positive individual/total no. of tested samples) X100}.

Haemagglutination inhibition test (HI) was used to evaluate humeral antibody response against AI/H5, AI/H9 and NDV vaccine. The used AI antigens were homologues to different vaccines and also ND antigens were prepared by Reference laboratory for veterinary control on poultry production. HI test was conducted using the two fold serial dilutions technique using 25-µl volumes in 96-well HI V-shape plates. Equal volumes of 4HA units of the antigen were added to diluted serum samples then a 1% suspension of chicken erythrocytes was dispensed into each well, HI titers $\geq$ 3 $\log_2$ were considered positive **(OIE manual 2014 and 2015).**

Bacterial Isolation

All samples (organs, water and feed) were examined bacteriologically for presence of *Salmonella, E. coli* and *Staphylococci*. Isolation and Identification of *Salmonella, E .coli* and *Staphylococcus* were conducted according to standard methods **(ISO 6579:2002; Lee and Arp 1998 and ISO 6888-1:2003)** respectively. Total bacterial count and total coliform count were done for water samples according to **(ISO 4833:2013 and ISO 4832: 2006).**

Bacterial antibiogram test (Antibiotic sensitivity test)

The antibiogram of bacterial isolates were done by disc-diffusion test for *Enterobactraicea isolates* against 10 antibiotics Amoxicillin + Claviolinic acid, Tetracycline, Streptomycin, Ciprofloxacin, Norfloxacin, Trimethoprim-sulfonamethoxazole (SXT), Gentamycin, Nalidixiic acid, Nitroforintin and Chloramphenicol while *Staphylococcus* tested against 11 antibiotics (Oxoid) Penicillin, Amoxicillin+Clavulinic acid, Tetracycline, Ciprofloxacin, Norfloxacin, trimethoprim-sulfonamethoxazole (SXT), Doxycycline, Gentamycin, Chloramphenicol, Amikacin and Erythromy-

cin, according to the Clinical and Laboratory Standards Institute/ Formerly National Committee for Clinical Laboratory Standard **(CLSI/ NCCLS, 2009**).

Results

Farms history

Sixteen broiler frames were suffering from respiratory signs, diarrhea, and mortalities (start from 3rd weeks of age) with intensive use of antibiotics. All affected farms were suffering from improper biosecurity practices (uncontrolled movement of workers and foreigners to these farms, dust was inside the farms, no water treatment or even testing programs, weak veterinary management programs, experience based treatment for the diseases without laboratory support).

Clinical sings and mortality records

All farms showed the same clinical manifestation which start mainly by respiratory symptoms (rales, cough, sneezing), in addition to diarrhea, sometimes facial edema and sinus swelling. The postmortem lesions were showing the same picture of respiratory disease complex with variable degrees in addition to visceral gout and septicemia. Mortalities of all examined farms showed the same pattern **(Figure 1)** as the mortality started day 15 (3rd week) and continued with no stop till end of the rearing cycle. The recorded mortality rates were variable between 13-62%.

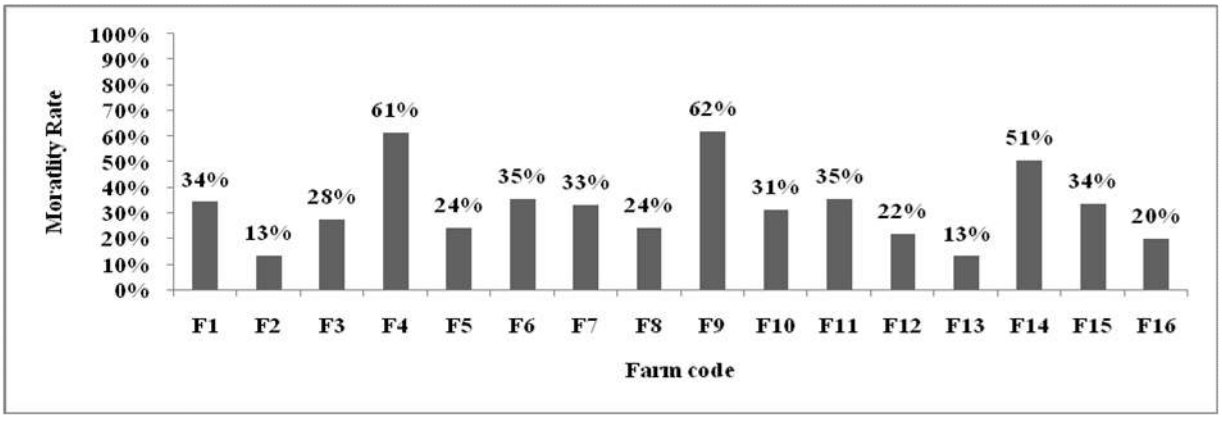

Figure (1). Mortality Rates (%) among different affected farms at 5th weeks of age

Viral detection results

Different PCR results confirmed the possibility of multiple viral infection in the same affected farm, however the highest detection (13/16) and positivity percent (81.2%) was recorded for infectious bronchitis virus (IBV). Virulent Newcastle disease virus (vNDV) was (7/16) with 43.7% positivity. Infectious bursal disease virus (IBD) was (7/13) and a positivity percent (53.8%). Furthermore we have confirmed detection of low pathogenic avian influenza H9N2 (AI/H9)in only two farms 2/16 (12.5%).Only 1 farm was positive for infectious laryngotracheitis virus (ILT) out of 4 tested farms. Furthermore all 16 farms were negative for highly pathogenic avian influenza H5N1 (AI/H5) and avian pneumovirus (APV) (0/2) as shown in **(Table 2).**

The gene analysis results

The genetic analysis results were confirmed the presence of 3 virulent viruses from farm No. 4 as following; IBD virus was indicating that this virus is very virulent IBD virus which showed 99-100% identity with vvIBD Giza 2008 viruses in Egypt. Also the NDV virus was related to the 2015 vNDV genotype VII d viruses isolated from Egypt with an identity of 97-99%. IBV virus showed high identity (99-100%) to IBV variant 2 viruses commonly isolated from Egypt **(Figure 2, 3, 4)**.

Table (2). Results of molecular testing for detection of different viruses using rRT-PCR, RT-PCR and PCR

Farm	Age (day)	Results of viral detection						
		IBV[1]	vNDV[1]	AI/H5[1]	AI/H9[1]	IBD[2]	ILT[3]	APV[2]
F1	29	+	-	-	+	Nd	Nd	Nd
F2	33	+	-	-	+	+	Nd	Nd
F3	28	+	+	-	-	+	Nd	Nd
F4	27	+	+	-	-	+	Nd	Nd
F5	31	-	-	-	-	-	Nd	Nd
F6	32	+	+	-	-	+	-	Nd
F7	28	+	-	-	-	-	-	Nd
F8	30	-	+	-	-	-	Nd	Nd
F9	28	+	+	-	-	+	Nd	Nd
F10	27	-	-	-	-	-	Nd	Nd
F11	25	+	+	-	-	-	Nd	Nd
F12	29	+	-	-	-	Nd	-	-
F13	32	+	-	-	-	Nd	+	-
F14	27	+	+	-	-	-	Nd	Nd
F15	31	+	-	-	-	+	Nd	Nd
F16	32	+	-	-	-	+	Nd	Nd
Total (Post/Total) x100		**13/16 (81.2%)**	**7/16 (43.7%)**	**0/16 (0%)**	**2/16 (12.5%)**	**7/13 (53.8%)**	**1/4 (25%)**	**0/2 (0%)**

(+)=Positive (-) =Negative Nd=not tested

[1]-rRT-PCR were conducted for detection of IBV, vNDV, AI/H5 and AI/H9 viruses

[2]-RT-PCR test were conducted for detection of IBD and APV viruses

[3]-PCR test were conducted for detection of ILT virus

Figure (2). Phylogenetic analysis of IBD virus (Egypt-NLQP-IBD-f158-2015) was showing high correlation with the vvIBD Giza 2008 with identity% (99- 100%) and it was showing clear diversity from the used IBD vaccines (IBD-Sanofi/2512 and CEVA-IBDL) and (Egypt-NLQP-IBD CV33-2015)strain which was isolated 2015.

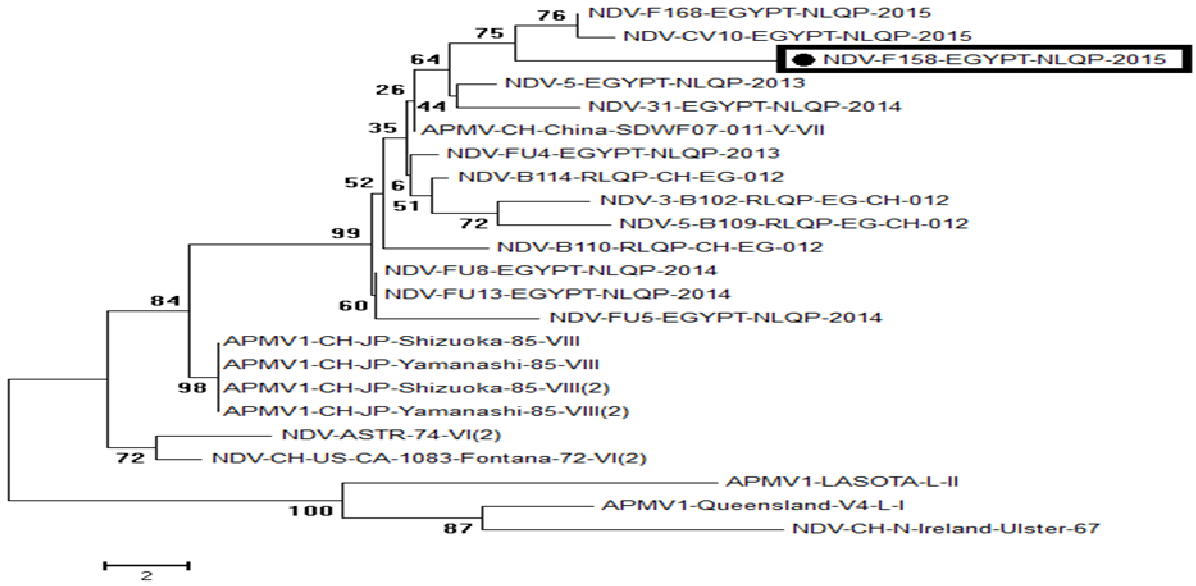

Figure (3). Phylogenetic analysis of NDV revealed that detected virus was closely related to vNDV genotype VII, that is closely related to China SDWF07-011-V-VII and other Egyptian strains which was isolated during 2012-2015 with identity % (97- 99%).

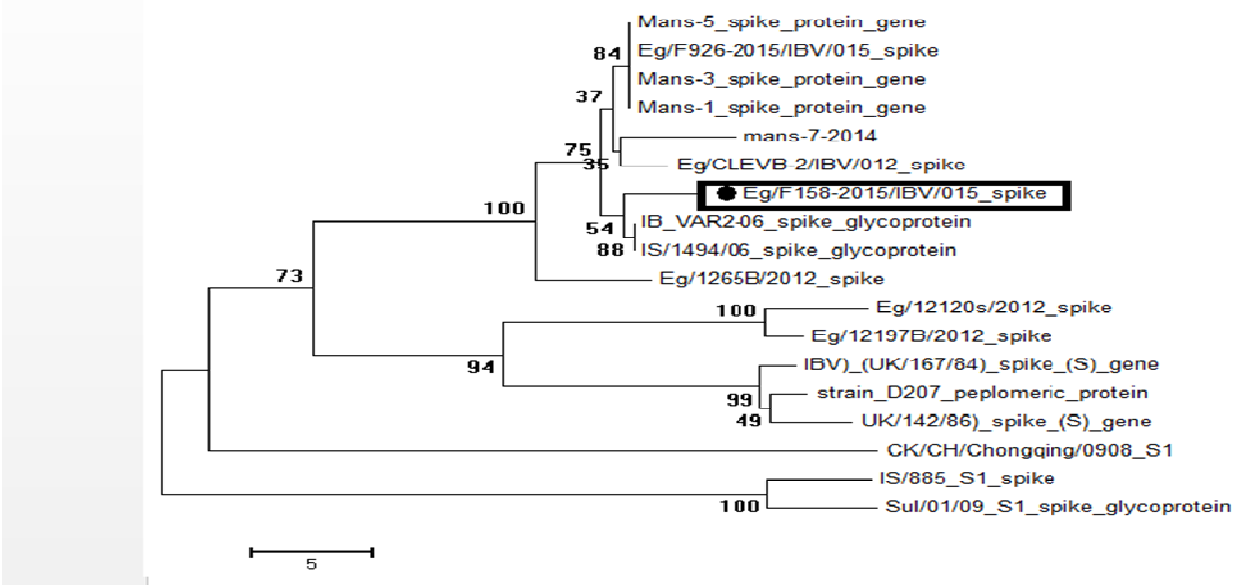

Figure (4). Phylogenetic analysis of IBV revealed that the detected virus was closely related to IBV variant 2 viruses isolated in Egypt (Eg/1265B/2012) with identity % (97- 100%).

Serology monitoring

A total of 60 different serum samples were collected from different 6 farms during the field investigation. The serum samples were examined for detection of humeral antibody response against AI/H5 and AI/H9, NDV, IBV and IBD viruses using either HI or ELISA test. The obtained data were helpful to understand the situation as the results were indicating that the immune system is not working properly for two vaccination program. Immune response against AI/H5 showed variable results ranged from (0-3.8) Log_2 HI titers and positivity range from 0-80%. AI/H9 results were little different as three farms were negative (0-0.3) Log_2 HI titers, However there was one farm showed high sero-conversion (100%) and high mean titer 5.6 log_2. NDV serological monitoring was showing HI mean titers (1.3-3.3) Log_2 and also sero-conversion was ranging from 14-78% as

showed in **Table 3.** In case of both IBV and IBD ELISA results we have recoded seroconversion rates (40-100%) and (85-100%) respectively, in the same time we observed variable GMT (708-6336) and (1462-12542). In addition to high variable coefficient of variance rate (CV%) (50-98%) and (20-98%) for IBV and IBD respectively **as showed in Table 3.**

Table (3). Results of serological monitoring

Farm	Age (day)	HI mean log_2[1]						ELISA GMT titers[2]					
		H5N1/Ag		H9N2/Ag		ND/Ag		IBV			IBD		
		Mean ± SD [a]	Post %[b]	Mean ± SD	Post %	Mean± SD	Post %	GMT	Post %[c]	CV %[d]	GMT	Post %	CV %
3	**29**	0±0	0	0±0	0	1.8±1.9	20	1489	82	91	10342	100	20
4	**27**	1.8±1.2	40	5.6±0.4	100	1.3±0.9	20	1890	80	82	Nd	Nd	Nd
5	**31**	1.2±0.6	10	0±0	0	1.5±1.2	14	708	40	98	1462	85	98
6	**32**	2.2±0.7	40	2.6±0.9	40	2.9±0.9	75	Nd	Nd	Nd	Nd	Nd	Nd
8	**33**	1.4±0.8	10	Nd	Nd	Nd	Nd	Nd	Nd	Nd	12542	100	20
9	**30**	3.8±0.9	80	0.3±2.5	35	3.3±0.7	78	6336	100	50	11152	100	35

[1] HI test were conducted according to OIE 2014-15 using 4HAU standard RBCs and standard HI antigen prepared from locally viruses at RLQP.
[a] Mean ±SD=mean HI log2 titer ±standard deviation among tested samples
[b] Post%= positive seroconversion =(No of tested positive samples ≥3log2/total Number of tested samples)X100
[2] ELISA GMT titer= geometric mean titer using commercial licent ELISA kit for IBD and IBV antibody detection
[c]Post%= (Number of positive samples/total tested samples) X100
[d]CV%= Coefficient variance rate
Nd= not tested as the serum samples wasn't enough for testing.

Bacteriological examination

The bacterial isolation has been conducted on the internal organs collected from two farms (3 and 9). The tested samples showed positive results for isolation and identification of *E. coli* and *Staph aureus* from the internal organs (Liver, Heart and intestine), also we isolated *Proteus spp.* from the livers and hearts of farm 9. *E-coli* isolated from farm 3 was only sensitive to Gentamycin and shows intermediate sensitivity reaction to Levofloxacin and Norfloxacin however *E. coli* isolate from farm 9 was showing intermediate sensitivity reaction for Gentamycin only. Similar sensitivity pattern were observed for the isolated *Staph aureus* bacteria as following; both farm 3 and 9 isolates were sensitive to Gentamycin although they were showed different intermediate sensitivity for Doxycycline and Norfloxacin. While the isolated *Proteus* showed resistance reaction with all tested antibiotic discs as showed in **Table 4.**

Table(4). Results of Bacterial isolation from different tissue samples and its antibiotic sensitivity

Farm	Isolated bacteria	Organ	Sensitivity				
			Gentamycin	Doxycycline	Levofloxacin	Norfloxacin	Other antibiotics
F3	E-coli[1]	Liver and Heart	S	-	IM	IM	R
	Staph aureus [2]	Liver, Heart, Intestine	S	IM	-	R	R
F9	E-coli[1]	Liver and heart	IM	-	R	R	R
	Proteus[1]		R	-	R	R	R
	Staph aureus[2]	liver, Heart, Intestine	S	R	-	IM	R

[1]**Antibiotics sensitivity for Enterobacteriacae was conducted according to (CLSI/NCCLS, 2009**) using following antibiotic discs (Amoxicillin + Clavulinic acid, Chloramphenicol, Ciprofloxacin, Gentamicin, Nalidixic acid, Nitrofurantoin, Norfloxacin, Levofloxacin Streptomycin, Trimethoprim-sulfamethoxazole, Tetracycline)
[2]**Antibiotics sensitivity for *Staph.aureus was conducted according to* (CLSI/NCCLS, 2009**) using following antibiotic discs (Amikacin, Amoxicillin + Clavulinic acid, Chloramphenicol, Ciprofloxacin, Doxycycline, Erythromycin, Gentamicin, Norfloxacin, Penicillin, Trimethoprim-sulfamethoxazole, Tetracycline).
S:sensitive, **R:**resist, **IM:**intermediate.

Feed analysis

The tested feed samples were showed good results and all parameters were in proper permissible limits for protein (21%), Aflatoxin (4.17 ppm), Ochratoxin (0 ppm), negative for *Salmonella* and *Staph*, but it was positive for *E. coli* as showed in **Table 5.**

Table (5). Results of poultry feed analysis

Tested item	Maximum level	Source
Protein	21%	21%
Aflatoxin	5 ppm	4.17 ppm
Ochratoxin	0 ppm	0 ppm
Salmonella	0	Negative
E. coil	0	Positive
Staph	0	Negative

Water analysis results

The obtained results were indicating that the water inside the farm (poultry house) and the original water source is unsuitable for animal use. The two water sources were showing high level for chloride (620 and 650 mg/L), Sulphate (350 and 460 mg/L), Total salts (1.4 and 1.3 mg/L) in farm and source water respectively. Also the microbial testing for both farm and source water were showed high total bacterial count (TBC) ($2.3x10^3$/ml and $25x10^3$/ml) and total coliform count (TCC) ($2x10^2$/ml and $12x10^2$/ml), respectively as showed in **Table 6.** However, the two tested water sources were negative for *salmonella, E. coli* and *staph* isolation.

Table (6). Results of chemical and microbial testing of water source

Tested item		Maximum level	Original Source	Farm water
Chemical testing/ L[1]	Chloride	250 mg/L	650 mg/L	620 mg/L
	Phosphor	-	71.7	75.8
	Nitrite	4 mg/L	Free	Free
	Sulphate	250mg/L	460 mg/L	350 mg/L
	Magnesium	125mg/L	19.76 mg/L	13.21 mg/L
	Total salts	0.18 mg/L	1.3 mg/L	1.4 mg/L
	Ammonia	2 mg/L	0.007 mg/L	0.055 mg/L
	PH	6-8	7.1	7.3
Microbial testing/ ml[2]	T.B.C/ml	200/ml	$25x10^3$/ml	$2.3x10^3$/ml
	T.C.C./ml	50/ml	$12x10^2$/ml	$2x10^2$/ml
	Salmonella	0	Absent	Absent
	E-coil	0	Absent	Absent
	Staph	0	Absent	Absent

[1]Chemical testing/L- the tested parameters were indicated the limits of tested parameters/ liter.
[2]Micorbial testing/ml- the microbial testing parameters were indicates the limits /ml of tested water

Discussion

Infectious diseases in broiler industry lead to high economic losses as a results of the high mortality, massive use of antibiotics and chemotherapeutics in addition to intensive vaccination, moreover, growth retardation and loss of uniformity that increases the slaughter house condemnations rate **(Vandemaele *et al.*, 2002; McKissick, 2006).** During last 5 years the Egyptian poultry industry was facing syndrome of high mortalities in different production sectors**,** this syndrome has been identified as Respiratory disease complex syndrome (RDS) **(RLQP report, 2012)**. Many studies had made highlights for the main causative agents like (IBV, NDV, AI/H9N2, AI/H5N1and APV) and other bacterial causes (*E. coli, Staph, Salmonella, Proteous, mycoplasma Gallisepticum and synoviae*) **(Hassan *et al.*, 2002).** RDS syndromes become one of the important syndromes that affect broiler industry in Egypt due to the high mortality rates that might reach to over 80%. In the current study multiple broiler farms faced the same problem of high mortality (13-62%) during winter season 2015 that associated with respiratory manifestation. Laboratory results were recoded and high detection rate of IBV (81.2%), vNDV (43.7%) and IBD (53.8%) in addition to AI/H9N2 (12.5%). Moreover the detected viruses were mainly in a form of mixed infection models, double to triple viral affection. All these complication increased in case of presence of other immunosuppressive disease like virulent IBD. Immunosuppression caused by IBDV has a significant economic impact especially if it was occurred during early age affecting both humoral and local immune responses of chickens (**Allan *et al.*, 1972 and Saif, 1991**) which will lower flock performance, increase susceptibility for secondary infections, poor feed conversion, and lower expected immune response to vaccines and increase mortality rates and carcass condemnation rates **(Higashihara *et al.*, 1991).** IBD infection alone may cause sporadic severe outbreaks that characterized with 30-60% mortality rates (**Abdel-Alim *et al.*, 2003 and Hussien *et al.*, 2003)**.

Furthermore, the genetic analysis of farm No. 4 detected viruses were as follows; vvIBD (Egypt-NLQP-IBD-f158-2015) detection with 99%- 100% identity to vvIBD Giza 2008 **(Naglaa *et al.*, 2015)**. Also detction of vNDV virus (NDV-F158-Egypt-NLQP-2015) which was closely related to vNDV genotype VII-d China (SDWF07-011-V-VII) and other Egyptian vNDV-VII-d viruses which was isolated during 2012-2015 with (97- 99%) identity % (**Kilany *et al.*, 2015).** In addition to the detec-

tion of IBV variant 2 virus (Eg/F158-2015/ IBV/015) which was showing (97%- 100%) identity % with the Egyptian (Eg/1265B/2012) IBV variant 2 virus **(Abdel-Moneim *et al.*, 2012)** (Figure 2, 3 and 4). These results were indicting the circulation of different virulent, pathogenic and contagious viruses which could be a main cause of either high mortality or immune suppression (vvIBD) and/or respiratory disease manifestations (vNDV-VII and IBV var2 virus).

Serological monitoring results among the tested flocks were indicating immunosuppression in all farms lead to vaccination failure which appear in two forms, the first form refers to bad immune response leads to negative antibodies detection or low antibodies titer levels, low positivity rate and inappropriate CV%, the second form is the picture of early infections due to shooting of antibodies titers for AI/ H9N2 (Farm 4), IBD (Farm 3, 8 and 9).

In this study the isolated bacteria strains (*E. coli, Staphylococcus and Proteus*) were multidrug resistant strains which confirm the current misuse of antibiotics as a medication for bacterial infections in the field. So the treatment of such bacterial infection will be difficult with the current available antibiotics **(Sibi *et al.*, 2011).** Also the Gentamicin sensitivity results cleared the state of the field where there is no misuse of Gentamicin in broiler poultry sector in Egypt.

Both feed and water could play an important role in disease transmission due to contamination by feces, secretions of sick birds, or by pathogenic organisms that originate from other animal species, such as in the case of salmonella and Escherichia coli, respectively **(Carter and Sneed, 1996). Reddy *et al.*, (1995)** confirmed that the maximum number of microorganisms in the drinking water of birds should be 100 CFU/mL for total bacteria and 50 CFU/mL for coliforms.

Inspire of the importance of the causative agents no one can deny the impact of strict biosecurity and working with a one-age system are essential control measures, normally vaccination is an essential tool to increase the resistance of the chickens against challenge with different viral strains.

In conclusion Both field and laboratory collected data have indicated that the current problem in different investigated farms was multifactorial disease agents include many infectious factors like IBV, IBD, vNDV, AI/ H9N2 viral infection and antibiotic resistant infection with *E. coli, staph aureus* and *proteus* in addition to the main and high impact of the non-infectious factors such as bad biosecurity practices, bad management and bad quality water. All these factors were working in synergistic process causing immunosuppresion to the immune system by direct and indirect actions which lead to vaccination failure and increasing mortality rates that reached up to 62% in some farms. So that it is very critical to review and update the current rearing, biosecurity and management programs adopted in different poultry farms. Also we recommend examined water source and feed quality to minimize their effect on poultry heath.

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