

Concurrent Infectious Bronchitis and Newcastle Disease Infection in Egypt

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Abstract: Infectious bronchitis (IB) and Newcastle disease (ND) are two major causes of economic losses in the poultry industry. 150 samples were collected from chicken showing respiratory manifestations and mortalities to study the prevalence of NDV and IBV in four Egyptian Governorates Al Daqhaliya, Al Qalyoubiya, Al Sharqiya and Alfayoum. From five broiler farms and six layer farms. Swabs (tracheal and cloacal swabs) and organs (trachea, kidney and lung) were collected from dead birds. Tissue samples (trachea, kidney and lung) were inoculated in specific pathogen free (SPF) eggs via allantoic cavity with candling daily. ELISA test was performed to screen each virus alone. Then RT-PCR was performed. Clinical examination of infected chicken showed that 59.33% (n=89) of examined chicken showing dyspnea, diarrhea, generalized paralysis, decreased egg production, ataxia and a significant ($P < 0.01$) 33.3% of chicken (n=50) showed more than one sign. The causative agents caused curling, dwarfing and cherry embryos in SPF eggs. ELISA test was repeated twice for each sample where once for IB and another one for NDV. The results showed that 56.67% were infected with NDV this indicates either infection with NDV alone or in association with IBV, while 50% were infected with IBV, also this indicates either infection with IB alone or in association with NDV. By assessment of samples, data revealed that, 46.67% of them were infected by both viruses. RT-PCR results showed that out of 71 samples taken from layers chicken, three were infected with NDV, one was infected with IBV and 32 samples were positive to both viruses. On the other hand, out of 79 samples taken from broilers, 11 were infected by NDV, 4 by IB and 38 by both viruses and the rest were negative for both viruses.

Key words: Newcastle Disease • NDV • Infectious Bronchitis • IBV

INTRODUCTION

Avian infectious bronchitis virus (IBV) is a member in the genus *Coronavirus* and its genome consists of a 27.6 kb single stranded positive-sense RNA molecule that encodes four structural proteins. The nucleoprotein (N) is responsible for the nucleo-capsid formation, whereas the remaining structural proteins, the spike (SP), small membrane (M) and envelope proteins (E), are inserted in the envelope surrounding the nucleocapsid [1].

Newcastle disease virus (NDV) is designated avian *paramyxovirus*, which belongs to the genus *Avulavirus* within the family *Paramyxoviridae* [2, 3]. The genome comprises a single stranded negative sense RNA that encodes the RNA-dependent RNA polymerase (L gene), the haemagglutinin-neuraminidase (HN gene),

the fusion (F gene) and matrix (M gene) proteins, the phosphoprotein (P gene) and the nucleoprotein (NP gene) [4].

Infectious bronchitis (IB) and Newcastle disease (ND) are two major causes of economic losses in the poultry industry [5- 7]. Clinical signs occurring in these avian respiratory diseases are often non-specific. IB and ND are characterized by respiratory signs including gasping, coughing, sneezing, tracheal rales and nasal discharge and they are believed to be involved in poor egg production in layers and acute highly contagious respiratory diseases in infected chickens [8, 9, 10].

This study aimed to investigate mixed infections of infectious bronchitis virus and Newcastle disease virus in Egyptian chicken field and seeks suitable fast and accurate method for diagnosis of these field strains viruses.

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MATERIALS AND METHODS

Samples Collection: In the current study 150 samples were collected from chicken farms showing respiratory manifestations and mortalities during the period from January to June 2013, to study the prevalence of NDV and IBV in four Egyptian Governorates Al Daqhaliya, Al Qalyoubiya, Al Sharqiya and AlFayoum, from five broiler farms and six layer farms that complain about the presence of infected chicken. Swabs (tracheal and cloacal swabs) and organs (trachea, kidney and lung) were collected from dead birds. A questionnaire regarding the vaccination status of chickens was administered to domestic poultry owners during sampling. All swabs were soaked in virus transport medium (VTM: RPMI + bovine serum albumin + penicillin + streptomycin + amphotericin B) as stated in Choi *et al.* [11] The samples were stored at -70°C until further processing.

Virus Isolation and Propagation: Samples were inoculated in specific pathogen free (SPF) eggs (Koum Oshiem SPF chicken farm, Fayoum, Egypt). 200µl of each sample was inoculated into allantoic cavity or chorioallantoic sac then incubated at 37°C with candling daily. Allantoic fluids were harvested at 96 h post inoculation (PI). The allantoic fluids were harvested and stored at -70°C with examination of embryo for curling and dwarfism [12].

Viruses and Sera: Reference virus and sera were kindly supplied by Animal Vaccines and Sera Institute, Abasia, Egypt.

Indirect ELISA: This method was done according to Madbouly *et al.* [13] and Afonso *et al.* [14]. Virus-infected allantoic fluid was collected and clarified by centrifugation at 4°C at 3000×g for 20 min. Virus was concentrated by pelleting at 48,000×g (Sorvall™ MX Plus Floor Model Micro-Ultracentrifuge) for 2 h and the pellet was resuspended to 1/100 of the original volume with PBS. ELISA test was performed for screening each virus individually. The conjugate used was peroxidase (Sigma, Germany). The optical density (OD) at 450nm was read using an automated plate reader (Bio-Tek EL312E reader, Bio-Tek Instruments).

Virus Diagnosis by RT-PCR: Viral RNA was extracted from the supernatants of 10% w/v sample suspensions and allantoic fluid with the RNA extraction kit QiAamp viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

Two steps RT-PCR (Qiagen, Germany) were performed where the first step was to amplify N gene of IBV using the primer reported by Akin *et al.* [15] in a highly sensitive multiplex PCR format (5' GTC TAC CAG GCA TTC GCT TCC AGG AAC AGC AGA AGA AGG 3') and (5' TGG GTG ACT CAA TTC TGC TGT TGG ACG TGT ACC TACA CCA 3'). While at the second step using primers encoding F gene of NDV described by Kho *et al.* [16] (5' GTC TAC CAG GCA TTC GCT TCT TCT ACC AGG ATC CCA GCA 3') and (5' TGG GTG ACT CAA TTC TGC TGA TGC CTC TAA TGG GGC TTT 3'). The multiplex RT-PCR optimization strategy in this study follows the step-by-step protocol described by Henegariu *et al.* [17].

RESULT AND DISCUSSION

Virus Isolation and Propagation on SPF-ECE: The viruses cause curling, dwarfing and cherry embryos 96 hours (hrs) post inoculation (PI) (Fig. 1). The same findings of curling and dwarfing and cherry embryos after 96hrs PI were reported by Selim *et al.* [12] and Maminaiina *et al.* [4].

Clinical Signs: Clinical examination of infected chicken showed 5.33% dyspnea, 6.67% diarrhea, 5.33% generalized paralysis, 2% decreased egg production, 6.67% ataxia and a significant ($P<0.01$) 33.3% of chicken showed more than one sign (Table1). The results match with the results reported by Chen *et al.* [18] and Hughes and Gorton [10] as they reported the same symptoms reported in the current study for both viruses.

Indirect ELISA for Virus Diagnosis: ELISA was conducted for each virus individually. The results of ELISA plates tested for NDV showed that 56.67% were infected with the tested virus (Table 2). Meanwhile, ELISA plates tested for IBV showed 50% were infected with IBV (Table 2). While Table 3 showed that 46.67% of the tested samples were infected by both viruses. Chen *et al.* [19] reported the high sensitivity of ELISA test for detection of IBV even in high dilution. De Oliveira *et al.* [20] argued the sensitivity of ELISA for detection of NDV in serum.

RT-PCR Results: Electrophoretic results showed illuminating bands at 236 and 386 bp, respectively (Fig. 3), which comes in agreement with the results obtained by Akin *et al.* [15] who reported a band at 386bp encoding N gene of IBV and Kho *et al.* [16] who reported a same band

Table 1: Clinical signs of infected chicken, some chicken showed more than one symptoms

Symptoms	Numbers of infected chicken	Percentage
Dyspnea	8	5.33%**
Diarrhea	10	6.67%
Decrease in egg production	3	2%
Ataxia	10	6.67%
Generalized paralysis	8	5.33%
Chicken show more than one sign	50	33.33%**
χ^2 value		32.2**

**P<0.01

Table 2: ELISA results for IBV and NDV

Virus tested	Number of birds tested	Positive results		Negative results	
		Number	Percentage	Number	Percentage
IBV	150	75	50%**	75	50%
NDV	150	85	56.67%**	65	43.33%
χ^2 value			27.7**		

**P<0.01

Table 3: Results of samples tested by ELISA

Number of samples	Samples were IBV and NDV positive		Samples were IBV only positive		Samples were NDV only positive		Samples which are both viruses negative	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
150	70	46.6%	5	3.33%	20	13.33%	55	36.%

Table 4: Results of RT-PCR

Governorates	Total number	Broiler					Layers				
		Total number	Age	Positive cases			Total number	Age	Positive cases		
				For NDV	For IBV	For Both			For NDV	For IBV	For Both
Al Daqhaliya	32	20	29-33D	3	1	10**	12	12-14M	2	-	4
Al Qalyoubiya	29	11	26-29D	1	1	5	18	15-16M	-	-	7
Al Sharqiya	35	30	30D	4	1	15**	5	13M	-	-	3
Al Fayoum.	54	18	26-27D	3	1	8	36	16M	2	1	18**
χ^2 value						37.2**					

**P<0.01

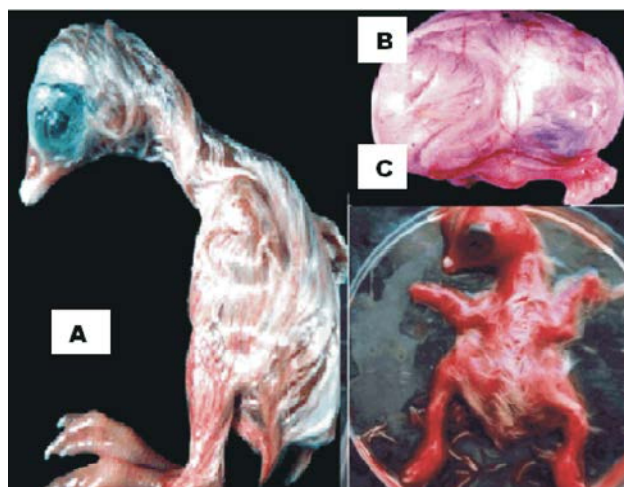


Fig. 1: Photo shows normal ECE. Photo B shows dwarfing 96 hrs PI. Photo C shows cherry embryo96 hrs PI

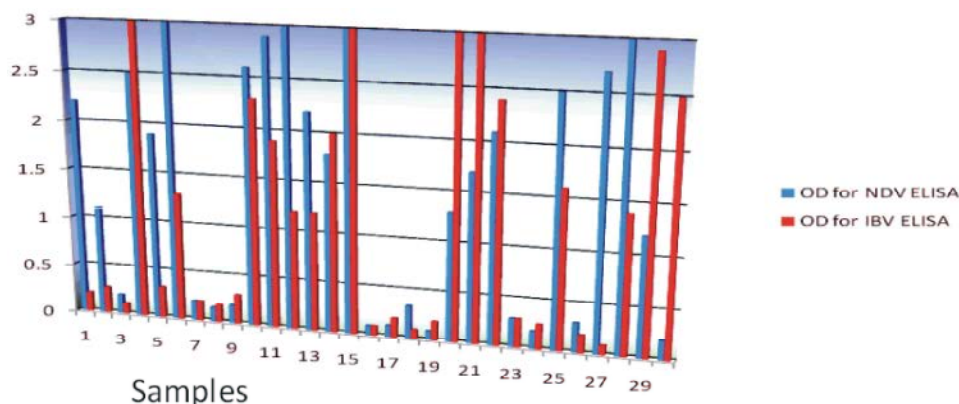


Fig. 2: Shows different OD of ELISA results of each sample for both viruses

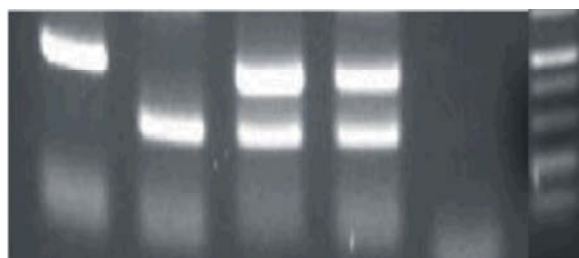


Fig. 3: Agrose gel show bands at 236bp encoding F gene of NDV (lane 2, 3 and 4) and bands at 386bp encoding N gene of IBV (lane 1, 3 and 4) lane 5 negative sample and lane 6 contain marker ladder.

at 236 bp encoding F gene of NDV. Moreover, RT-PCR results showed that out of 71 samples taken from layers farms, 3 were infected with NDV, one was infected with IBV and 32 samples were positive to both viruses. On the other hand, out of 79 samples taken from broilers farms, 11 were infected with NDV, 4 with IBV and 38 with both viruses (Table 4). The prevalence of both viruses in the current study is relatively high and it is of concern because these viruses are two of the most dangerous viruses in poultry. While there was significant geographical variation in the prevalence of NDV, no geographic variation concerning IBV, all observed cases in the four Governorates occurred in vaccinated domestic poultry. This could be a result of the low number of IBV-positive cases in the current study. Tarnagda *et al.* [21] also reported the multi-infection IBV and NDV and H5N1 in Burkina Faso. The main problem of these two viruses is that they have many variants that evolves and independent evolution in Egypt and persistence of divergent strains currently circulating in the country [12]. Moreover, other variants gain access through transportation from nearby countries. For example in Libya, Awad *et al.* [22] reported that the variant isolated were with 100% relatedness to Eg/CLEVB-2/IBV/012 and

IS/1494/06 (Egyptian variant). The sum of all above made vaccination very difficult. There is an urgent need for the implementation of routine laboratory surveillance for poultry disease and for the development of strategies to improve the quality of the vaccines and to develop vaccination of new variant to safeguard the important poultry industry in Egypt. Also a new study should be adopted to predict the new variant of each virus to eliminate them.

The current study recommends ELISA technique for the identification and screening of both viruses and RT-PCR as a confirmatory test. Further studies should be conducted on quasispecies model for both viruses to predict new variant and performing accurate vaccines for proper control of the two diseases in Egypt.

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