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Genetic detection of IB and other respiratory viral diseases in chicken flocks in Egypt

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Abstract

Newcastle disease (ND), infectious bronchitis (IB) and avian influenza (AI) are highly contagious and are responsible for huge economic losses in poultry production. Thirteen chicken flocks showed upper respiratory tract manifestation from Ismailia, Sharkia and Dumyat provinces during 2011 and 2012 winter seasons. SPF embryonated chicken eggs were used for virus isolation. Results of HI and PCR indicated that, 13 isolates were positive to IBV and negative for AIV. In addition, 6 isolates were positive to NDV. Six chicken flocks had mixed infection with IBV and NDV, while seven flocks were infected with IBV only. The PCR of IBV targeted ORF1a gene of IBV while targeting the F gene of NDV.

Keywords: NDV, IB, AIV, Chicken, Egypt

Introduction

Among viral poultry diseases, Newcastle disease (ND), infectious bronchitis (IB) and avian influenza (AI) are considered the most contagious and they are responsible for huge economic losses to poultry production (Alexander 2011, Henriques, Fagulha et al. 2011, Xie, Ji et al. 2011). Despite intensive vaccination programs for all of them, outbreaks could re-emerge as a consequence of infections with new variants that differ serologically from the strains used to create the vaccines (Alexander 2011)x(Peyre, Samaha et al. 2009). Infected birds with these diseases present respiratory syndromes and other lesions, such as cough, respiratory distress, poor growth, or production leading to economic losses (Cavanagh and Naqi 1997). Multiple diagnostic methods such as isolation and serology are required for detecting and differentiating viral respiratory infections (Gelb and Jackwood 1998). However, virus isolation methods are time consuming and labor intensive. Furthermore, nonspecific reactions or cross-reactions often hamper serologic tests. Rapid and sensitive methods to detect and differentiate respiratory disease pathogens in poultry are critical for adopting proper preventive and control measures to reduce economic losses. Polymerase chain reaction (PCR) technique, have been used for rapid and sensitive detection of avian pathogens (Cavanagh and Naqi 1997). Controlling respiratory viral disease infection is a problem due to wide variations in the serotypes, a highly contagious nature, the evolution of specific tissue tropism and recombinants due to simultaneous infection of multiple virus types and use of live vaccines **(Bayry, Goudar et al. 2005)** . Outbreaks of infectious bronchitis can occur in vaccinated flocks due to the lack of cross-protection against antigenically unrelated serotypes and variant strains of the virus (Jia, Karaca et al. 1995). In Egypt , in spite of the use of three different types of vaccines (H120,

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MA5, attenuated 4/91) in poultry farms in Egypt for IB , bivalent attenuated vaccine for ND and IB and H5N1 vaccine in first day, outbreaks in vaccinated flocks have been observed that characterized by high mortality in broiler farms **(El-Mahdy, El-Hady et al. 2010, Li, Xue et al. 2010, Mahmood, Sleman et al. 2011, Abdel-Moneim, Afifi et al. 2012)**. Continuous identification of the genotypes and production of new generations of vaccines are crucial. So, the main aim of this study is to identify types of respiratory viral infections that infecting chickens in Egypt.

Material and Methods

Samples: Tissue samples from 13 suspected IBV outbreaks in broiler farms in Ismailia, Sharkia and dumyat were collected. Trachea, lung and kidneys were obtained from 10 birds in each farm and transferred to the laboratory on ice. The birds suffered from respiratory symptoms and lesions as well as kidney damage (enlargement, congestion, and uroletheasis). Ten flocks out of thirteen were vaccinated with attenuated bivalent IB and ND vaccine in drinking water at 1 days age. IB vaccine booster dose were used at 15 days old with IB H120 except M flocks was boostered with 4/91 vaccine. All chicken flocks were vaccinated with inactivated H5N1 subcutaneous injection at 1 day age .

Virus isolation: According to the method described by (Momayez, Pourbakhsh et al. 2002) , pooled tissue samples from each single chicken in one farm were mixed together then homogenized to give approximately 10% (w/v) suspension in PBS pH 7.2 containing 100IU/ml penicillin, 100µg/ml streptomycin, The homogenized samples were centrifuged at 1000g for 15min at 4°C, the supernatant was filtered through a 0.45µm membrane filter . Amount of 0.2 ml of each processed pooled samples were inoculated in the allantoic sac of 10 SPF chicken egg embryos (9–11 days) incubated at 37 ^{o}C/ 3 days examined twice daily. Those that died within 24h after inoculation were discarded. Mortality after 24 hours post inoculation (PI) were considered to be virus specific. After 3 passages , the chorioallantoic fluid was harvested aseptically and used for HA (Mahmood, Siddique et al. 2004) and RNA extraction was performed **(Momayez, Pourbakhsh et al. 2002)**.

RNA extraction: RNA extraction methods for extraction of RNA from allantoic fluid was adopted using commercial RNA extraction kits purchased from (bioteke , CHINA). RNA was extracted from samples according to the instruction of the manufactures.

cDNA synthesis: specific cDNA for each disease was synthesis according to the manufacturer's instruction (bioteke ,CHINA), briefly, 3 µl of total extract was mixed with 2 µl reverse primer (IBV- , H9-808 , H5-2 and NDV(down)) (10 pmole/ul), 1 µl dNTPs (10 mM each) and RNase free distilled water in a total volume of 12.5 µl mixture. The mixture heated to 65 ^{o}C for 5 min then directly chilled on ice, 4 ml from 5`First-strand buffer and 2 ml Dithiotheritol (0.1 mM), 0.5 µl (40 units/ ml) RNase inhibitor were added to the mixture. After incubating the mixture at 42^{o}C for 2 min, 1 µl (200 units/ml) reverse transcriptase (bioteke,CHINA) was added and further incubated at 42 ^{o}C for 50 min, and finally, the enzyme was inactivated at 70 ^{o}C for 15 min. The cDNA was stored at 20 ^{o}C for the following PCR amplifications.

Amplification of ORF1a, F, H5 and H9 genes of IB,ND and AI viruses respectively : separate PCR reactions containing forward and reverse primers targeting specific gene in each virus

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were used to detect viruses in allantoic fluids obtained from the first passage to amplify 143,320,808,456 bp of ORF1a (IBV) ,F (NDV) ,H5,H9 for AI genes respectively. The primers paired used for detection of ORF1a gene was designed specifiy to a conserved region to ensure a wide detection range. The PCR amplification reaction was carried according to (Callison, Hilt et al. 2006) for IB , according to (Pang, Wang et al. 2002) for ND and according to (Ng, Barr et al. 2006) for AI genes. out in 25 µl mixture containing 12.5 µl of Mastermix (Takara , Japan), 2µl of each of 10 pmol forward primer and reverse primer, 3 µl cDNA, the mixture is completed to 25 µl by the addition of 4.5 µl DNase-RNase free distilled water. Amplification was performed with a thermo cycler (Eppindorf, USA) at 94 ºC for 5 min initial denaturation step, 40 cycles (95 ºC for 1 min, 53 ºC for 30 s and 72 ºC for 1 min) and a final extension at 72 ºC for 10 min.

***Table 1*: Oligonucleotides used for Detection of IBV, NDV & AIV**

	Primers	Virus	Sequences 5`-3`	Gene	Position in the gene	Reference
1	f-S1Uni2+ (forward IB)	IB	(CCC) AATTTAAAACTGAACA	S	20,298-20,314	(Adzhar, Shaw et al. 1996)
2	r-XCE2_ (reverse IB)	IB	CTCTATAAACACCCTTACA	S	21,507-21,526	(Adzhar, Gough et al. 1997)
3	S1Unil- (reverse IB)	IB	CCTACTAATTTACCACCAGA	S	21,881-21,901	(Binns, Boursnell et al. 1986)
4	IBP1-(reverse)	IB	CAATTAATTTGGACCTTATCCA	S	22202–22181	(Adzhar, Shaw et al. 1996)
5	IBV+ (forward)	IB	GCT TTT GAG CCT AGC GTT	O	391-408	(Callison, Hilt et al. 2006)
6	IBV- (reverse)	IB	GCC ATG TTG TCA CTG TCT ATT	O	510-530	(Callison, Hilt et al. 2006)
7	H9-1F (forward)	H9N1	AGC AAA AGC AGG GGA AYW WC	H	1-16	(Webster and Kawaoka 1987)
8	H9-808 R (reverse)	H9N1	CCA TAC CAT GGG GCA ATT AG	H	808-827	(Webster and Kawaoka 1987)
9	H5-1 (forward)	H5N1	ACT ATG AAG AAT TGA AAC ACC T	H	335-356	(Ng, Barr et al. 2006)
10	H52(reverse)	H5N1	GCA ATG AAA TTT CCA TTA CTC TC	H	767-791	(Ng, Barr et al. 2006)
11	NDV(up)	ND	GGA GGA TGT TGG CAG CAT T	F	4952-4970	(Pang, Wang et al. 2002)
12	NDV(down)	ND	GTC AAC ATA TAC ACC TCA TC	F	4716-4697	(Pang, Wang et al. 2002)

HA & HI test

To improve the HA propertry of isolated virus , allantoic fluids were treated with trypsin **(Mahmood, Siddique et al. 2004)**. Allantoic fluid from inoculated dead and live embryos was collected 72 hours post-inoculation (PI) and treated directly by mixing 0.25 ml of allantoic fluid from each set of inoculated embryos with 50 ul of trypsin (Sigma Chemical Company St. Louis, MO, USA) . After the addition of trypsin, all samples were held at 37ºC for 30 minutes . Treated antigens were mixed with chicken RBCs 10% and screened for HA property. All three virus passages were examined for HA , HI using reference polyclonal antisera prepared against ND ,IB , AI H5 and H9. HA , HI were carried out according to the methods described by **(Gough, Cox et al. 1996)** . Direct agglutination of the chicken RBCs was read within 5 minutes **(Corbo and**

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Cunningham 1959). Clear and consistent HA was considered as positive.

Results

In this study, 13 broiler flocks in Ismailia, Sharkia and Dumyat were examined. Clinical symptoms and post mortem changes during the outbreaks are likely to be IB virus infection. Morbidity rates in the flocks ranged between 65% and 80% and mortality was ranged from 17-33 % .. Seven flocks out of tested thirteen tested chicken flocks showed IB infection when examined with HA , HI and PCR .The other six flocks showed IBV infection concurrently infected with ND virus infection when examined by HA , HI and PCR. HA , HI and PCR tests for examination of samples collected from 13 chicken flocks showed a negative result for H5 and H9 AI virus.

Virus isolation results

Inoculated pooled chicken samples in SPF ECE of 9 day age are examined In the first passage of 13 samples showed early death of embryos **(Table 2)** in first 48 hours post inoculation. In the second and third passage, the mortalities were declined but stunting, curling and dwarfing of embryos were progressed leading to reduction of embryo size 3 times in comparison with control non inoculated embryos **(**Error! Reference source not found. **,** Error! Reference source not found.**).**

Figure 1. **9 day old chicken embryos showing stunting and curling after 3 passages of IB virus**

Figure 2. **9 day old chicken embryo inoculated with saline**

Table 2 Results of virus isolation in SPF ECE

Isolates no.	Flock	Location	Early death of embryos	Curling of ebmryos	Stunting & dwarfing	Urate deposition in kidney and ureters	HA
1	A	Ismailia	++	-	-	-	+
2	B	Ismailia	++	-	-	-	+
3	C	Ismailia	++	-	-	-	+
4	D	Ismailia	++	-	-	-	+
5	E	Dumyat	-	+	++	+++	+
6	F	Sharkyia	-	+	++	++	+
7	G	Ismailia	-	+	++	+	+
8	H	Dumyat	-	++	++	++	+
9	I	Dumyat	-	++	++	+	+
10	J	Sharkyia	++	-	-	-	+
11	K	Ismailia	++	-	-	-	+
12	L	Shakria	-	+	++	++	+
13	M	Isamailia	-	+	+	+	-

+++ : means curling , dwarfing , stunting with urates deposition in kidneys. ++ : means curling , dwarfing and stunting. + : means dwarfing without curling; no lesions in the embryos

PCR Results

Regarding to amplification of ORF1a,N,H9,H5 of IBV,ND,AI genomes respectively, gel electrophoretic pattern of 13 isolates revealed an amplificationat 143 bp,320 bp ***(****Figure 6,****Figure 3)*** compared to positive and negative samples for IBV and ND. Meaning that all 13 flocks are infected with IB and 6 flocks are infected with ND. Gel electrophoretic pattern of S1 gene trials for amplification didn't show PCR product at 1230 bp .Another one forward and three reverse primers with several combinations between them (about 6 trails) are used but gel electrophoretic pattern didn't show any PCR products to S1 gene of IBV isolates **(** ***Figure*** **4).** Concerning to oligonucleotide primers used for amplification of H9 and H5 gene of AI virus , no PCR amplification reaction were observed and the samples proved to be negative as were failed to

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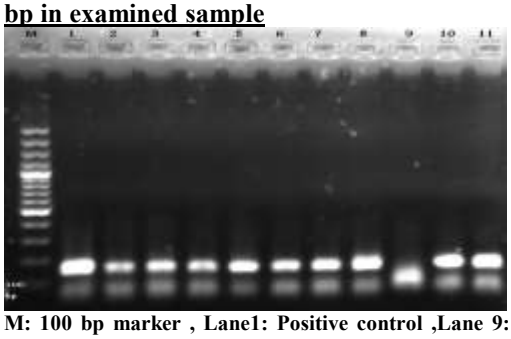
generate an RT-PCR product at 808 , 456 bp for H5 and H9 primers; Respectively (***Figure* 5**)

Figure 3. **Amplifican of ORF1a gene at 143 bp in examined sample**



M: 100 bp marker , Lane1: Positive control ,Lane 9: Negative control , Lane 2, 3, 4, 5, 6, 7, 8, 10 &11: Viral isolates

Figure 4. Gel electrophoresis results of S1 primer combinations.

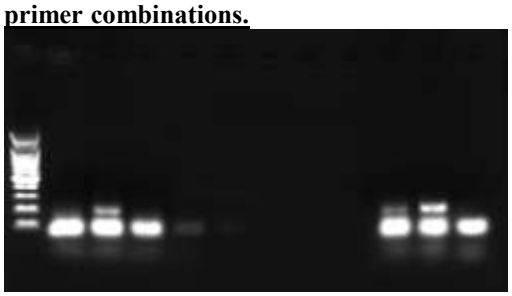
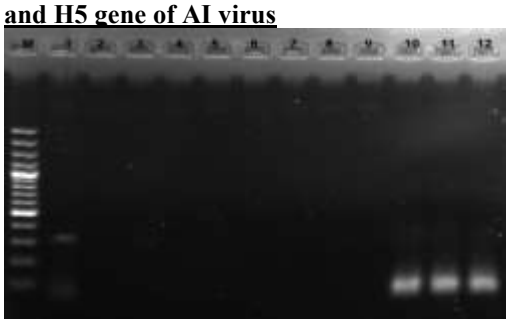
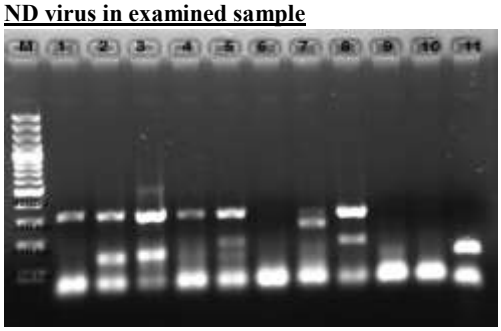


Figure 5. Gel electrophoresis results of H9 and H5 gene of AI virus



M: 100 bp molecular weight marker , Lane1: Positive control ,Lane 9: Negative control , Lane 2, 3, 4, 5, 6, 7, 8, 10 , 11 & 12 : Negative AI samples.

Figure 6. Agarose gel electrophoresis results of ND virus in examined sample



M: 100 bp molecular weight marker , Lane 9: Negative control , Lane 2, 3, 4, 5, 6, 7, 8, 10 &11: Viral isolates

HA & HI Results

The allantoic fluid was exposed to HA test, 12 isolates showed +ve HA (**Table 3**) , while only 1 isolate showed –ve HA when used allantoic fluids treated with trypsin 0.2%. Reference hyperimmune serum to IBV, NDV and AIV are used. Results in (Table **3**) revealed that all HA isolates are positive for IBV, NDV and six flocks are concurrently infected with IBV and NDV.

Table 3. HI comapred to PCR for detection of IBV , NDV and AIV in allantoic fluids

Flocks	ND-HI	AI-HI	IB-HI	PCR result
A	+	-	+	ND +ve IB +ve
B	+	-	+	ND +ve IB +ve
C	+	-	+	ND +ve IB +ve
D	+	-	+	ND +ve IB +ve
E	-	-	+	IB +ve
F	-	-	+	IB +ve
G	-	-	+	IB +ve
H	-	-	+	IB +ve
I	-	-	+	IB +ve
J	+	-	+	ND +ve IB +ve
K	+	-	+	ND +veIB +ve
L	-	-	+	IB +ve
M	-	-	+	IB +ve

Table 4. chicken flocks infected with IBV and NDV

Single IB infection	Mixed IB-ND infection
Flock E (Dumyat)	Flock A (Ismailia)
Flock F (Sharkia)	Flock B (Ismailia)
Flock G (Ismailia)	Flock C (Ismailia)
Flock H (Dumyat)	Flock D (Ismailia)
Flock I (Dumyat)	Flock J (Sharkia)
Flock L (Sharkia)	Flock K (Ismailia)
Flock M (Ismailia)	

Discussion

IBV infect chicken of all ages causing a major economic losses, the IBV has the ability to mutate continusely resulting numerous emerged serotypes and genotypes leading to complicated efforts to control through vaccination (Bayry, Goudar et al. 2005). In Egypt,

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controlling IBV infection is a problem due to several factors. They include a wide variations in the serotypes , virulence of strains, a highly contagious nature , evolution of specific tissue tropisms , recombinants due to simultaneous infection of multiple virus types and use of live vaccines **(Bayry, Goudar et al. 2005)**. Inoculation of samples in SPF eggs resulted in stunted growth and curling of the embryos – a characteristic feature for IBV **(**Error! Reference source not found.**,** Error! Reference source not found.**).** Also, there are severe nephropathiec changes due to change in the tissue tropism of the virus with replacement of the prediction site trachea and respiratory mucosa to another tissue like urinary epithelium , these results are in agreement with **(Susan, M et al. 2010)**. In this study we amplified a 143 bp PCR product from the ORF1a gene of IBV. Gel electrophoretic pattern of 13 IBV isolates showed in **(*Figure 3*)** revealed a clear and specific bands. PCR product size was verified by agarose gel analysis, Compared with positive and negative samples **(*Figure 3*)**, the primer used was targeting a highly conserved region of ORF1a gene and amplified a 143 bp product in all thirteen IBV samples tested. These results appear to be similar to **(Callison, Hilt et al. 2006)** whose developed and validate an RT-PCR based method for rapid detection of IBV in clinical samples that amplifies a 143 bp product in the ORF1a gene of IBV genome and used it to differentiate IBV from other respiratory viral pathogens. In **(*Figure 6*)**, the primer used was targeting a highly conserved region of F gene of ND and amplified a 320 bp product in all six samples tested. These results appear to be similar to (Ng, Barr et al. 2006) whose developed and validate an RT-PCR based method for rapid detection of ND in clinical samples that amplifies a 320 bp product in the F gene of ND genome and used it to differentiate ND from other respiratory viral pathogens.

In this study, allantoic fluid of eggs inoculated with IBV were treated were treated with trypsin and used as viral antigen in HA test. All isolates haeamgglutinate chicken RBCs except the isolate M (**Table 2).** Trypsin induced HA assay was standardized and validated to detect IBV in allantoic fluids , the same results are described by **(Mahmood, Siddique et al. 2004)**. For determination of which type of each virus , further studies should be made amplifying the variable regions enabling us to genotype each of them.

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