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MOLECULAR SURVEY OF AVIAN RESPIRATORY VIRUSES IN COMMERCIAL BROILER AND LAYER FLOCKS WITH RESPIRATORY MANIFESTATIONS IN KAFRELSHEIKH GOVERNORATE

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ABSTRACT

Acute respiratory tract infections are generally the main hazard to the poultry industry in Egypt leading to severe economic losses. The present study was aimed to standardize a multiplex polymerase chain (mPCR) reaction that simultaneously detects and differentiates the four major avian respiratory viruses (AIV, NDV, IBV and ILTV) in single reaction, as well as to apply a molecular survey of avian respiratory viruses in commercial broiler and layer flocks suffering from respiratory manifestations with relatively high mortality rate in different localities of Kafrelsheikh governorate using the standardized mPCR assay. The obtained results indicate the high occurrence of mixed infections among these viruses which makes it difficult to differentially diagnose these diseases by clinical manifestations.

INTRODUCTION

Poultry sector is one of the most important segments of the agriculture sector in Egypt, where investment in this industry is about 20 billion Egyptian pounds ***(El Nagar and Ibrahim 2005).*** This sector has faced serious challenges through respiratory viral infections ***(Abdel-Whab and Hafez 2011).*** The etiology of the respiratory disease is complex, often involving more than one pathogen. Several avian viruses have a predilection for the respiratory tract of chickens: Avian Influenza virus (AIV), Newcastle disease virus (NDV), Infectious bronchitis virus (IBV) and Infectious laryngotracheitis virus (ILTV) primarily infect the respiratory tract of chickens ***(Roussan et al., 2008)***. These viruses are of major importance because they can cause disease independently, in association with each other or in association with other bacterial or viral pathogens ***(Ali and Reynolds 2000)***. Unfortunately, due to very close resemblance of lesions produced by several other pathogens in poultry, it is difficult to differentially diagnose clinical manifestations of AI infections from diseases such as ND, IB and ILT ***(Rashid et al., 2009)***. Additionally, mixed infections may occur due to extensive use of multiple live vaccines, high geographic populations and housing densities ***(Pang et al., 2002)***. Therefore, it becomes essential to develop a rapid and sensitive diagnostic technique to detect and differentiate between these diseases which is very important for the control of the disease transmission in poultry and humans ***(Rashid et al., 2009)***.

A multiplex polymerase chain reaction (mPCR) technique that simultaneously detects and differentiates the four major avian respiratory

viruses in a single reaction, will be highly advantageous to the poultry industry and would greatly aid in the diagnosis and control of outbreaks because it offers rapid and specific detection of these viruses without the need for subculture in host system which is laborious and time consuming and cause risk from handling the infectious materials ***(Spackman et. al., 2002).*** Additionally, it eliminates the need to test clinical samples separately for each virus ***(Yashpal et al., 2004)*** and results are generally available within few hours as compared with virus isolation (VI) technique which requires about 4-5 days or sometimes more for definitive diagnosis ***(Tao et al., 2009)***. Furthermore, it provides better detection of the virus from the clinical samples which might appear negative due to inappropriate sampling or loss of infectivity during shipment ***(Siddique et al., 2008)***.

In this study we record a molecular survey of the most important avian respiratory viruses through field samples collected from broiler and layer chicken flocks in Kafrelsheikh governorate during the period from October 2011 till February 2013 using a rapid multiplex PCR technique which detects and differentiates the four major avian respiratory viruses (AIV, IBV, NDV and ILTV) in a single reaction.

MATERIAL AND METHODS

Source and collection of samples:

A total of 100 fresh post mortem (P. M.) tissue samples including lungs, tracheas, and proventriculas were collected from broiler and layer chicken farms suffered from severe respiratory manifestations and

relatively high mortality rate from different localities in Kafrelsheikh governorate during the period from October 2011 till February 2013.Samples were immediately transported on ice to the Central Diagnostic and Research Lab, Faculty of Veterinary Medicine, Kafrelsheikh University and stored at -80°C till used.

Viruses and Vaccines:

These viruses were used as reference control positive during development of multiplex PCR tests:

a) Avian Influenza serotype H5N2 infected allantoic fluid was kindly supplied by Animal Health Research Institute, Dokki, Giza, Egypt.
b) Izovac IB-H120. Freeze-dried live attenuated vaccine against Infectious Bronchitis serotype Massachusetts H120. (Izo S.p.A. Italy).
c) Fowl Laryngeotracheitis. Freeze-dried live attenuated vaccine. LT-IVAX. Shering-Plough Animal Health Corp. (Nebraska USA)
d) NDVvaccine. Freeze-dried live attenuated vaccine against Newcastle disease (Lasota) (HIPRA Laboratories, Girona, Spain).

RNA extraction:

RNA was extracted from AI infected allantoic fluid, IBV and NDVvaccines and field samples using Trizol Easy-Red^{TM} Total RNA extraction Kit (Intron Biotechnology) as per manufacturer's protocol. Briefly, 500 µl of infected allantoic fluid or vaccines were mixed with 750 µl of TRI reagent followed by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to a fresh diethyl pyrocarbonate

(DEPC) treated tube and added with 200 µl of chilled chloroform. The mixture was left at room temperature for 15 min. and centrifuged at 10,000 rpm for 15 min. at 4°C. The upper aqueous phase was then transferred to a fresh tube and 500 µl of chilled isopropanol was added followed by centrifugation at 10,000 rpm for 10 min. at 4°C. The supernatant was discarded and the pellet was first washed with 100% followed by 70% ice-cold ethanol. Finally the pellet was re-suspended in 30 µl RNAse free water and stored at -80°C for further analysis. Moreover, the extracted RNA was analyzed for its quality and quantity using Nano-drop method. Briefly, the equipment was first activated with 2 µl of RNA/DNA free water. Later the same amount of sample was placed on the machine pedestal and the setting for"RNA" selected. The system measures the quality and quantity of RNA in the given sample.

Reverse transcription of extracted RNA to cDNA:

The reverse transcription (RT) of the extracted RNA to cDNA was performed using Maxime^{TM} RT PreMix Kit (Gene On) with oligo (dt)18 primers. Briefly, a mixture of 5 µl of extracted RNA, 1 µl of Oligo dT and 2 µl of Sterile RNAs free water were prepared and incubated at 70 °C for 10 min, then placed on ice for 5 min. Another mixture of 4 µl 5X reaction buffer, 1 µl dNTP mix (10 mM of each =40mM), 1 µl RNAs inhibitor, 1 µl MMLV Reverase (200u/ µl) and 13 µl sterile RNAse free water. Mix I and Mix II were combined, gently vortexed and incubated in heat block at 55°C for 90 min., then at 70°C for 10 min. for inactivation of the enzyme. cDNA was stored at -20°C for further applications.

DNA extraction from field samples and ILTV vaccine:

The DNA was extracted using Gene Jet^{TM} Genomic DNA purification Kit, Fermentas International Inc. as per manufacturer's protocol.Briefly; 20 mg of tissue were grinded in liquid nitrogen using a mortar and pestle. The grinded material was resuspend in 180 µl of Digestion Solution and 20 µl of proteinase K Solution. The sample was incubated at 56°C until the tissue is completely lysed. 20 µl of RNase A Solution were added, incubated for 10 min at room temperature. 200 µl of lyses solution were added and mixed by vortex .Then 400 µl of 50% ethanol were added and mixed .The prepared lysate was transferred to a Gene JET™ Column inserted in a collection tube, centrifuged for 1 min/ 6000xg. 500 µl of Wash Buffer I were added and centrifuged for 1 min/ 8000 xg .Then 500 µl of Wash Buffer II were added and centrifuged for 3 min at (≥12000 xg). The Gene JET™ Column was transferred to a sterile 1.5 ml micro centrifuge tube and 200 µl of Elution Buffer were added to the center of the Gene JET™ Column membrane to elute genomic DNA .The purified DNA was stored at -20 °C for further applications.

Primer designs:

Four sets of oligonucleotide primers that specifically amplify type A influenza virus (Matrix protein gene), S1 gene of IBV, F protein gene of NDV and ILTV were commercially synthesized by (Metabion international AG, Germany) and listed in table 1.The primers were aliquot to a final concentration of 50 pmol and stored at -20°C until use. The functionality and specificity of all primer were tested in single reactions before combining them in a multiplex PCR assay.

Table (1): Oligonucleotide primers used in this study

Primer name	Oligonucleotide sequence	Target gene	Length of amplified fragment	Reference
IBV-F IBV-R	GCTTTT GAG CCT AGC GTT GCC ATGTTGTCACTGTCT ATT	S1 gene	149 bp	***Callison et.al 2006***
NDV-F NDV-R	GGAGGATGT TGGCAGCAT T GTCAACATATACACCTCATC	S1 gene	320 bp	***Pang et.al 2002***
ILTV-F ILTV-R	ACGATGACTCCGACTTTC CGTTGGAGGTAGGTGGTA		647 bp	***Pang et.al 2002***
AIV-F AIV-R	CTTCTAACCGAGGTCGAAACG AGGGCATTTTGGACAAAGCGTCTA	M gene type A	244 bp	***Xie et.al 2006***

Multiplex PCR reaction:

The mPCR technique was optimized using RNA and DNA extracted from live attenuated vaccines for NDV, IBV and ILTV .While RNA of AIV was extracted from infected allantoic fluid. It was performed in 50 µl volumes, in which the reaction mixture consisted of 10 µl of 5X Master Mix (Taq/High Yield-Jena Bioscience, Jena, Germany) (5X Conc.of thermostable DNA Polymerase, dATP, dCTP, dGTP, dTTP, (NH4)SO4, MgCl, Tween 20, Noniodet P-40, stabilizers), 1 µl of forward and reverse primers of each virus, 5 µl of cDNA,5 µl of DNA and 22 µl of PCR grade water. The mPCR was performed in Bio Rad T100 thermal cycler.The cycling protocol consisted of initial denaturation at 96°C for 5 min. followed by 40 cycles of denaturation at 95°C /1 min, annealing at 53 °C /1 min and extension at 70°C /1 min. with a final extension at 72 °C /10 min. Throughout the development of the mPCR, many modifications were applied to the annealing temperature, extension time and the number of cycles. A negative control containing only PCR master mix, primers and PCR grade water was also included.

Detection of PCR product by gel electrophoresis:

PCR products were analyzed by agarose gel electrophoresis using 2% agarose. Amplified products were visualized by ultraviolet light transillumination after staining with 0.1µg/ml ethidium bromide. A 100 bp ladder (GeneON, GmbH, Germany) was used as a molecular weight marker.

RESULTS

The mPCR technique was succssesfully able to amplify specific genes of AIV, NDV, IBV and ILTV with expected product size of 244 bp, 320 bp, 149 bp and 647 bp respectively in one single reaction which were visualized by agarose gel electrophoresis. Only the target sequences were amplified and no non specific PCR products were noticed. Negative control sample (containing only buffer system and primers) was negative.

To validate the use of these mPCR assays in field samples, it was applied on 100 field samples collected from broiler and layer chicken flocks suffering from severe respiratory signs and relatively high mortality in Kafrelsheikh governorate**.**

The total number of positive samples was 61 out of 100 examined samples (61%). The number of samples with single viral infection was 45 sample with a percentage of (45% from total samples) and (73.7% from positive samples) while number of samples with mixed viral infection was 16 sample (16% from total samples) and (26.3% from positive samples).15 samples were mixed infections with two different viruses while only one sample contained mixed infection with 3 viruses.

The total number of samples positive for AIV infection was 30 samples (30% from total samples) and (49.1% from positive samples) ,16

of them were single infection while in 14 samples it was combined with other viruses (8 samples co infected with IBV, 5 samples co infected with NDV and 1 sample mixed with both IBV and NDV.)

The total number of positive samples for IBV infection was 25 samples out of 100 examined samples (25% from total samples) and (40 % from positive samples).14 of the were single IBV infection while in 11 samples it was mixed with other viruses (8 samples with AIV, 2 samples with NDV and one samples was mixed with both AIV and NDV).

NDV was detected in 23 samples out of 100 collected samples (23% from total samples) and (37.7% from positive samples). 15 of them were single NDV infection while in 8 samples it was mixed with other viruses (5 samples with AIV, 2 samples with IBV and in one samples it was combined with both AIV and IBV). While ILTV could not be detected in this study.

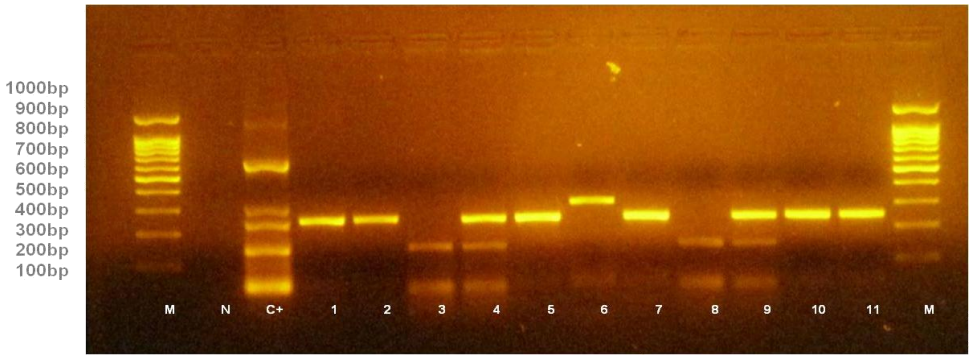

Fig. (1): Agarose gel electrophoresis of multiplex PCR amplified products for purified RNA and DNA of field samples. Lane M; 100 bp DNA size marker. Lane N; Negative control. Lane C;positive control .Lane 1, AIV, Lane 2; AIV.Lane 3;IBV. Lane 4; IBV+AIV. Lane 5;AIV.Lane 6;NDV.Lane 7;AIV.Lane 8;IBV.Lane 9;IBV+AIV.Lane 10;AIV. Lane 11; AIV.

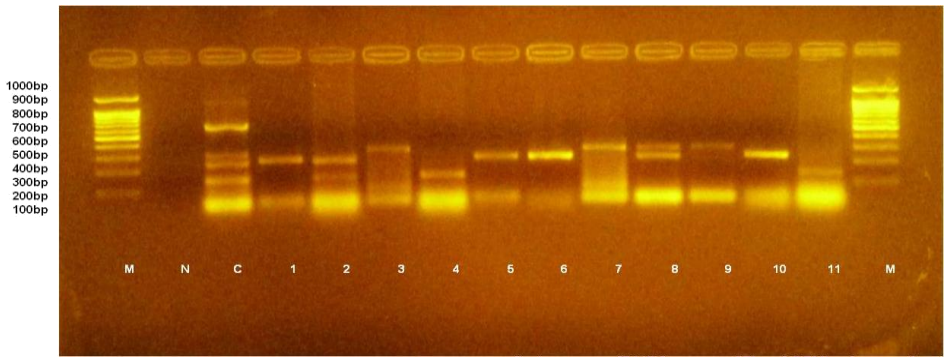

Fig. (2): Agarose gel electrophoresis of multiplex PCR amplified products from purified RNA and DNA of field samples. Lane M ;100 bp DNA size marker. Lane N; Negative control .Lane 1,AIV, Lane 2; AIV+IBV.Lane 3;NDV. Lane 4; IBV. Lane 5; AIV. Lane 6; AIV.Lane 7; NDV.Lane 8; AIV+NDV.Lane 9; NDV .Lane 10; AIV. Lane 11; IBV.

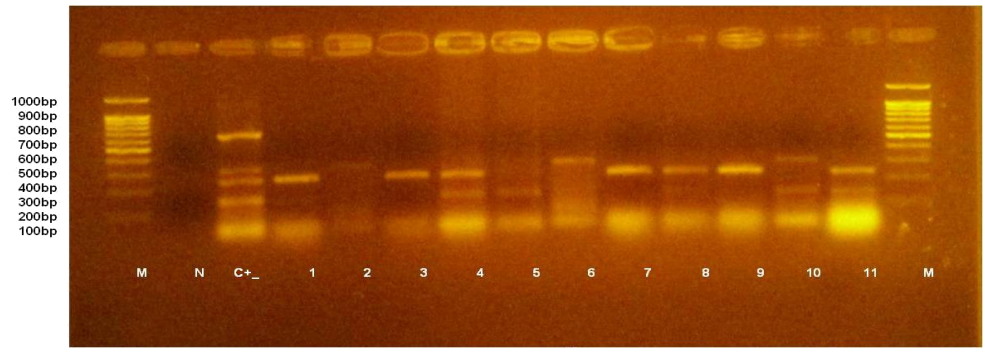

Fig. (3): Agarose gel electrophoresis of multiplex PCR amplified products from purified RNA and DNA of field samples. Lane M; 100 bp DNA size marker. Lane N; Negative control .Lane 1,AIV, Lane 2; NDV.Lane 3;AIV. Lane 4; IBV+AIV. Lane 5; IBV. Lane 6; NDV.Lane 7; AIV.Lane 8;AIV.Lane 9;AIV .Lane 10;NDV+IBv. Lane 11; IBV+AIV.

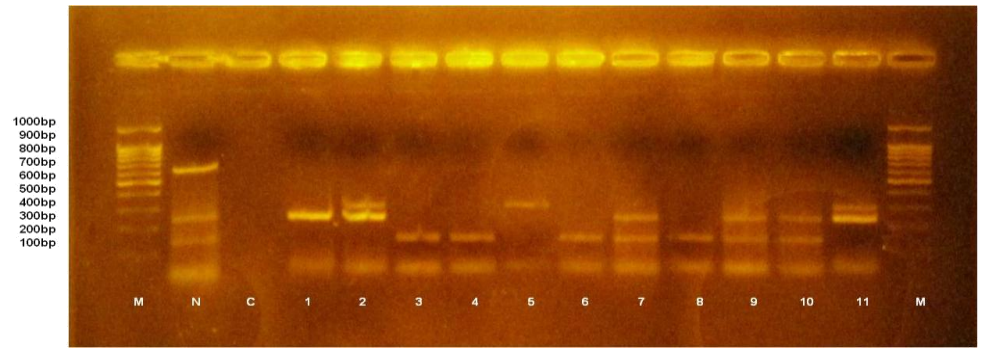

Fig. (4): Agarose gel electrophoresis of multiplex PCR amplified products from purified RNA and DNA of field samples. Lane M; 100 bp DNA size marker. Lane N; Negative control .Lane 1, AIV, Lane 2; AIV+ NDV. Lane 3; IBV. Lane 4; IBV. Lane 5; NDV. Lane 6; IBV.Lane 7; AIV +IBV.Lane 8; IBV.Lane 9; AIV+IBV .Lane 10; AIV+IBV. Lane 11; IBV+NDV.

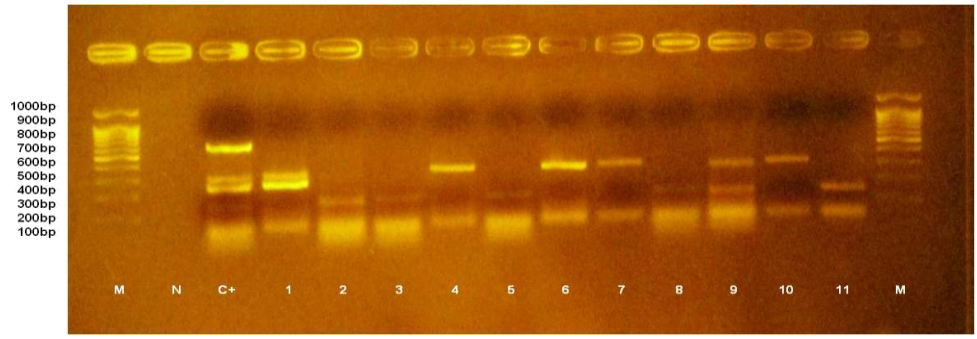

Fig. (5): Agarose gel electrophoresis of multiplex PCR amplified products from purified RNA and DNA of field samples. Lane M; 100 bp DNA size marker. Lane N; Negative control .Lane 1, AIV+NDV, Lane 2; IBV.Lane 3; IBV. Lane 4; NDV. Lane 5; IBV. Lane 6; NDV.Lane 7; NDV.Lane 8; IBV.Lane 9; NDV+IBV .Lane 10;NDV. Lane 11; IBV.

Table (2): Multiplex PCR results of field samples collected from Kafrelsheikh governorate.

Type of respiratory viral infection	Total No. of examined samples	No. of positive samples	%	Type frespiratory viral infection	Total No. of examined samples	No. of positive samples	%
AIV (total number of AIV)	100	30	30%	IBV+NDV (mixed infection)	100	2	2%
IBV (total number of IBV)	100	25	25%	AIV+NDV (mixed infection)	100	5	6%
NDV (total number of NDV)	100	23	23%	AIV+NDV+IBV (mixed infection)	100	1	1%
AIV (single infection)	100	16	16%	ILTV infection	100	0	0%
IBV (single infection)	100	14	14%	Total number of positive samples	100	61	61%
NDV (single infection)	100	15	15%	Total number of mixed infections	100	16	16%

Table (3): The incidence of mixed infections in the examined field samples detected by mPCR assay in relation to the total number of positive samples:

Type of mixed infection	Number of positive samples	Total number of positive samples	Percentage in relation to total number of positive samples (61)
AIV +IBV	8	61	13.1%
AIV +NDV	5	61	8.1%
IBV+ NDV	2	61	3.2%
AIV+IBV+NDV	1	61	1.6%

Table (4): The incidence of mixed infections in the examined samples detected by mPCR assay in relation to the total number samples with mixed infections:

Type of mixed infection	Number of positive samples	Total number of samples with mixed infections	Percentage in relation to number of samples with mixed infections
AIV +IBV	8	16	50%
AIV +NDV	5	16	31.25%
IBV+ NDV	2	16	12.5%
AIV+IBV+NDV	1	16	6.25%

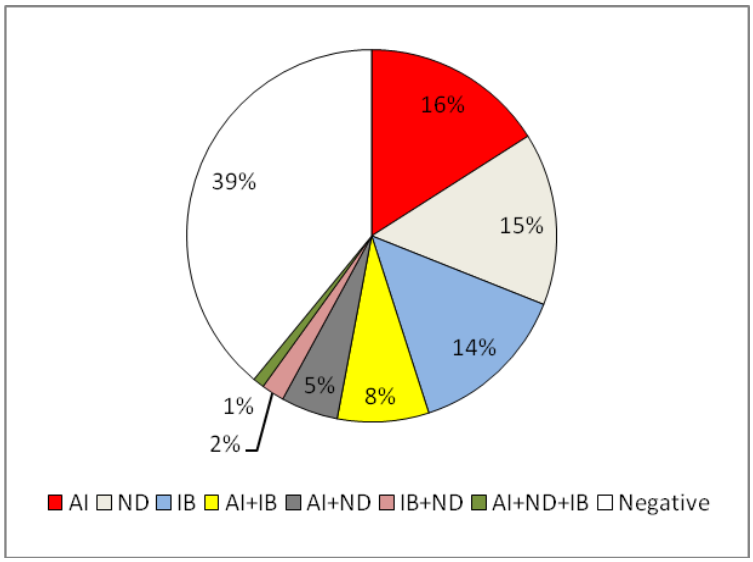

Fig. (7): Percentage of detected respiratory viral infections in relation to the total number of examined samples.

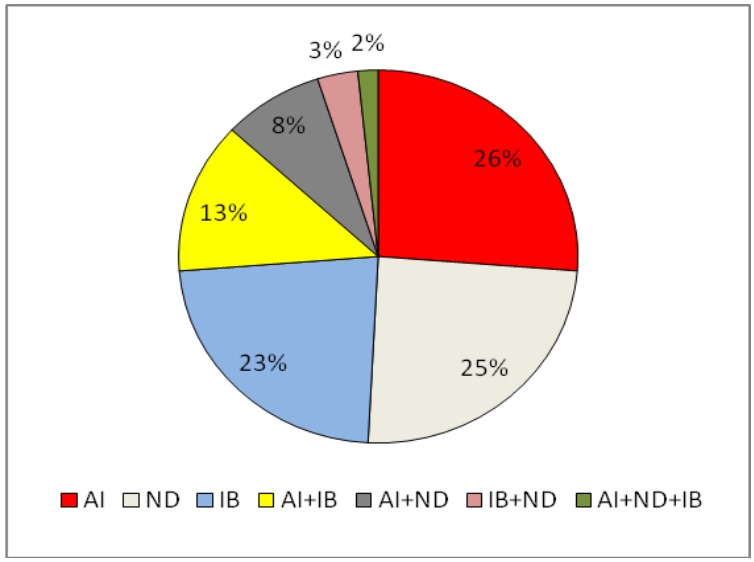

Fig. (8): Percentage of detected respiratory viral infections in relation to the number of positive samples.

DISCUSSION

Respiratory tract infections are of paramount importance in poultry industry and causing high morbidity and mortality in poultry farms throughout the world ***(Ahmed at al., 2009).*** A wide variety of pathogens have been associated with respiratory infections in poultry, including AIV**,** IBV, NDV and ILTV. Mixed viral infections are usually observed in poultry farms; as one or more viruses are involved in the respiratory complex that makes it difficult to determine the main viral causative agent which produces sever disease ***(Rafiei et al., 2009).*** Therefore, a multiplex polymerase chain reaction (mPCR) technique that simultaneously detects and differentiates the four major avian respiratory viruses will be highly advantageous to the poultry industry and would greatly aid in the diagnosis and control of outbreaks.

This study was undertaken to develop and evaluate a mPCR for the detection of AIV, NDV, IBV and ILTV in field samples taken from chickens of various outbreaks in Kafrelsheikh governorate during the period from October 2011 till February 2013. The mPCR in the present study was able to amplify nucleic acid of AI, ND, IB and ILT viruses in one single reaction. Similar technique was developed and standardized by ***Pang et al., (2002)*** who used mRT-PCR for detection of 6 avian respiratory pathogens, with specific primers for IBV, AIV, ILTV, NDV, Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS). Also ***Yashpal et al., (2004)*** reported the development and evaluation of a single-tube multiplex RT-PCR (mRT-PCR) assay for simultaneous and specific detection of Avian Pneumo virus (APV), AIV, and NDV. The mRT-PCR assay was found to be as sensitive and specific as sRT-PCR which also agreed with our study.

To validate the use of these mPCR assays in field samples, it was applied on 100 field samples collected from broiler and layer chicken flocks suffering from severe respiratory signs and relatively high mortality in Kafrelsheikh governorate. The mPCR was successfully able to detect and differentiate single and mixed infections with two or more viruses in a single reaction. The total number of positive samples was 61 out of 100 examined samples (61%). The number of samples with single viral infection was 45 with a percentage of 45% from total samplesand 73.7% from positive samples while number of samples with mixed viral infection was 16 (16% from total samples) and (26.3% from positive samples). The result of high percentage of mixed infections was similar to that obtained by ***Pang et al., (2002); Ahmed et al.,(2009); Tao et al., (2009); Yashpal et al., (2004); Rashid et al.,(2009); Roussan et al., (2008) and Rafiei et al., (2009)*** who reported that the etiology of the respiratory disease is complex, often involving more than one pathogen, which makes it difficult to determine the main viral causative agent which produces severe disease. These mixed infections may occur due to extensive use of multiple live vaccines, high geographic populations and housing densities ***(Pang et al., 2002),*** despite the use of IBV, NDV and AIV vaccines, it is common to find NDV, IBV and AIV infections in vaccinated broiler flocks ***(Mehrabanpour et al., 2011)***. AIV was evidenced in 30% of the examined samples with percentage of 49.1% from positive samples which was in accordance with ***Hegazy et al., (2011) and Omar et al., (2011)*** who noted that AIV remains one of the greatest health concerns for both human and poultry and they suggested an endemic situation of AIV in Egypt. In 14 samples AIV were

combined with other viruses (8 samples co infected with IBV, 5 with NDV).Similar results were obtained by ***Tao et al., (2009); Farkas et al., (2007); Mehrabanpour et al., (2011) and El Zowalaty and Goyal (2011)*** who reported that co-infection of AIV and NDV occurs frequently in field cases .Our results of frequent cases of AIV and IBV co-infection agreed with that of ***Haghighat-Jahromi et al., (2008)*** and ***Seifi et al., (2009)***. IBV infection was evidenced in 25% of examined samples, which reflects the endemic nature of the disease as described by ***(Mahgoub et al., (2010)*** and ***Lebdah et al., (2004).*** Despite the regular vaccination programs in chicken farms mostly with Mass H120 strain ***(Hoda et al., 2013)***; IBV still has a severe adverse effect on the poultry industry. Concerning NDV, the total number of samples positive for NDV was 23 out of 100 collected samples with a percentage of 23% from total samples and 37.7% from positive samples which was similar to that obtained by ***Mohamed et al., 2011*** who reported that ND outbreaks occur frequently in Egypt.

The present study is conclusive with the fact that the etiology of respiratory organisms is very complex usually involves more than one pathogen. Based upon the results obtained in the present study, this mPCR assay for simultaneous detection of AIV, IBV, NDV and ILTV, could be a useful instrument for rapid screening and surveillance in wild and domestic birds. Furthermore, it is also concluded that AI, IB and ND viruses are wide spread among broiler and layer chicken farms in Kafrelsheikh governorate, despite the vaccination effort and the strict biosecurity measures being employed which lead to severe respiratory disease or they predispose to secondary bacterial infection after suppression of the chicken's immune system.

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مسح جزيئي على الأمراض التنفسية الفيروسية في الدواجن في محافظة كفر الشيخ

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تعتبر الأمراض التنفسية من أكثر الأمراض انتشارا في مزارع الدجاج فى مصر مسببه خسائر اقتصاديه كبيره نتيجة لنقص الإنتاج الداجني من اللحم الأبيض والبيض. ومن أهم الفيروسات المسببة للأمراض التنفسية فى الدجاج فيروس إنفلونزا الطيور، فيروس النويكاسل، فيروس التهاب الشعب الهوائية المعدي وفيروس التهاب الحنجرة والقصبة الهوائية المعدي فى الدجاج. وغالبا ما يكون سبب المرض هو خليط ما بين هذه الفيروسات وبعضها أو بينها و بين ميكروبات أخرى. وقد صممت هذه الدراسة لتطوير اختبار تفاعل البلمرة المتسلسل المتعدد للكشف عن وجود هذه الفيروسات الأربعة في اختبار واحد وذلك باستخدام بوادئ عامه للكشف عن الحامض النووي الخاص بهذه الفيروسات. أثبتت الدراسة أن هذا الاختبار قادر وبنجاح على تحديد والتفريق بين هذه الفيروسات سواء كانت العدوى فرديه أو مختلطة وذلك باستخدام بوادئ عامه للفيروسات الأربعة. وللكشف عن مدى نجاح وفاعليه هذه الاختبارات عند تطبيقها على العينات الحقلية، فقد تم تجميع 100 عينه (رئة وقصبه هوائيه) من مزارع دجاج مصابه بأعراض تنفسيه ونسب نفوق عاليه من مناطق مختلفة فى محافظه كفر الشيخ. وأظهرت النتائج أن نسبه الإصابة الكلية بهذه الفيروسات الأربعة 61% وقد وجد أن الإصابة الفردية بنوع و احد من هذه الفيروسات كان 45% بينما نسبه العدوى المختلطة بنوعين أو ثلاثة كان 16%. نسبه الإصابة الكلية بفيروس إنفلونزا الطيور كانت 30%و بفيروس النيوكاسل 23% وبفيروس التهاب الشعب الهوائية المعدي 25% بينما لم يتم تشخيص فيروس التهاب الحنجرة والقصبة الهوائية المعدي في هذه الدراسة.