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RESEARCH ARTICLE

Molecular Identification of Some Respiratory Pathogens from Broiler Chickens in Sharkia Governorate, Egypt

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Abstract

In this study, we investigated the prevelance of avian influenza virus (AIV) (subtypes H5 and H9), infectious bronchitis virus (IBV), Newcastle disease virus (ND) and Mycoplasma gallisepticum (MG) incriminated in respiratory troubles among chicken broilers at different localities in Sharkia Governorate during 2013-2017 outbreaks. Out of 53 broiler flocks, 225 chickens showing respiratory manifestations were examined. Viral isolation from respiratory tissues, serological and molecular identification were done. Based on hemagglutination inhibition (HI) test, for 42 positive haemagglutinating allantoic fluids, the prevalence of NDV and AIV (H9 and H5 subtypes) was 64.3%, 45.3%and 2,4% respectively. Two diagnostic polymerase chain reaction (PCR) assays were used. Based on TaqMan probe base real-time PCR, 17/19, 10/42 and 1/42 flocks were confirmed to be infected with IBV, AIV subtype H9 and H5 respectively. Moreover, conventional PCR amplification for detection of Mycoplasma gallisepticum in 25 flocks was performed and revealed 56% (14/25) positive flocks. Subsequently, amplified products of three *M. gallisepticum* representative isolates were sequenced and phylogenitically analyzed with other strains on GenBank. The results revealed that the three examined isolates were wild type (field isolates).We could conclude that there were continuous circulation of different respiratory agents (viral (NDV, AIVH9 & H5, and IBV) and bacterial (MG) in broiler flocks in Sharkia Governorate either as single or mixed infection although wide using of different vaccination regimes and antibiotics so early diagnosis of these pathogens is considered the most effective tool in prevention and control of diseases.

Keywords: Broilers, AIV subtype H5, H9, HI, Real-time PCR, *M. gallisepticum*; Moderate exercise

Introduction

Respiratory pathognes cause tremendous losses to poultry industry in Egypt which may be due to inadequate biosecurity measures that increase the susceptibility to infection or co infection with one or more respiratory pathogens. Highly and low pathogenic avian influenza (HPAI & LPAI) viruses, infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) as well as other bacterial infections such as Mycoplasma species and Avian pathogenic *Escherichia coli* (*E. coli*) were frequently recorded as causes of mortalities and decreased productivity in chicken flocks [1].

Avian influenza (AI) is a highly contagious disease that affects a wide varities of birds and mammals and has a worldwide distributon. It is caused by Influenza A virus that belongs to family orthomyxoviridae that causes high economic losses [2]. HPAI subtype H5N1 virus is endemic in Egypt, where, several genetically and antigenically distinct H5N1 lineages co-circulate [3]. Low pathogenic AI subtype H9N2 was first reported in Egypt in November 2011. The isolated virus was closely

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related to viruses of the G1-like lineage isolated from neighboring countries, indicating possible epidemiological links [4]. The HPAIV- H5N8 originated in Far East China and spread across Central Asia into Europe and Africa reaching Egypt in 2016 [5].

There are persistent evolution of the AIVs subtypes (H5 and H9) that complicates the condition with other respiratory problems in Egyptian commercial chicken flocks [6]. During 2013-2015, Awad *et al.*, [7] recorded avian influenza infection with HPAIV-H5N1 in; 12% of broilers, 28.8% of layers and 50% of duck flocks in Alexandria and EL Behera Governorates. Howerver, they documented LPAIV- H9N2 infection only in 9.8% of broiler flocks. The incidence of H5N1 subtype was fairly stable in 2017 (12.8%) and 2018 (10.2%), while, H5N8 subtype increased from 23% in 2017 to 66.6% during 2018. The proportion of H9N2-positive samples was constantly high (100% in 2017 and 63% in 2018), and H9N2 co-circulated with HPAIV- H5N8 (56.8%) [8].

Infectious bronchitis virus belongs to the order Nidovirales, family Coronaviridae, genus Gamma coronavirus [9], positive sense RNA genome, approximately 27.6 kb in size [10]. There are a wide genetic diversity and generation of new variants of the virus due to continuous evolution resulted from mutations mainly at the spike protein (S) which is considered the main genomic region indicating the evolution processes of IBV [11]. In Egypt, IBVs circulating during 2012 were classified into two groups. First group was clearly variant from Israeli variant IS/885 and the second group was more closer to variant vaccine viruses like CR/88121 and 4/91 indicating independent evolution of IBVs and continuous evolution of divergent strains [12].

Mycoplasma gallisepticum (MG) is the prevalent pathogen that has increased the presence of *E. coli* and NDV along with IBVand AIV in association with respiratory disease in broilers. Maintenance of Mycoplasma free flocks is very important tool in eradication of these problems and this can be accomplished by early detection of MG and other respiratory pathogens [13].

Screening of broiler flocks for *Mycoplasma gallisepticum* was done at different districts in Egypt. In 2006, the incidence was 63.49% in Sharkia and Dakahlia Governorates [14], while an incidence of 62.89% was recorded in Dakahlia Governorate from November 2016 to April 2017 [15]. Sequencing the Mgc2 gene is a powerful tool in differentiation between field and vaccine strains of *M. gallisepticum* in vaccinated and non vaccinated flocks [16, 17].

The present study aimed to investigate the prevalence of infectious bronchitis, AIV subtypes (H5 and H9) as well as *Mycoplasma gallisepticum* in different broiler flocks with different breeds and ages in Sharkia Governorate using various diagnostic techniques as well as sequencing and and phylogenetic analysis of representative *Mycoplasma gallisepticum* isolates from different diseased flocks.

Materials and Methods

Clinical examination, necropsy and sampling

Fifty three broiler flocks of different breeds (29 Cobb, 10 Hubbard, 8 Ross, 3 Avian 48, 2 Sasso and 1 Arbo broiler flocks) from different localities in Sharkia governorate were investigated for the presence of respiratory manifestations during 2013- 2017. From these flocks, 225 broiler chickens (10-37 days of age) suffering from respiratory troubles and mortalities of 0-25% were subjected to clinical and postmortem examination. Fifty three tissue sample pools were harvested from sick birds, each tissues pool representing lungs and trachea from 4-5 sick birds per flock.

Isolation and serological identification of AIVs and IBV

Pooled tissue sample from each flock were prepared in tissue suspention 1:10 W/V and centrifuged. Antibioticts [Penicillin (2000 units/ml)/Streptomycin (2 mg/ml)] were added to the superntent and 0.2 mL/each was inoculated via allantoic cavity route in 9-11 day embryonated chicken egg (ECE) which were then incubated at 37 ˚C for 4 days at the Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University and according to OIE terrestrial manual [18, 19]. Harvested amnioallantoic fluids (AAF) were screened for direct haemagglutination activity followed by microtitre plate hemagglutination test and haemagglutination inhibition (HI) using three different antisera against ND, H5 and H9

according to OIE World Organization for Animal Health [20]. Reference antigens and antisera were kindly supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. The AAF samples that were negative for HA were further inoculated for 2-3 passages for detection of haemagglutination activity or embryos curling and dwarfing.

Molecular identification of IBV, AIV subtypes H5 and H9

The RNA extraction was performed from 42 AAF samples positive for HA and HI in case of AIV and directly from 19 respiratory tissue pool samples for IBV using BIOLINE isolate II RNA mini kit, Catalouge NO BIO-52072. For detection of IBV, AIV subtype H5 and H9, a uniplex one-step RT-PCR using MyTaq™ RT-PCR Kit BIOLINE (Ameridian Life Science Company) was done as following; 2x master mix (25 µL), forward primer (2 µL), reverse primer (2 µL), reverse transcriptase (0.5 µL), ribosafe RNase inhibitor (1 µL), template (5 µL) and H2O up to 50 µL. The cycling conditions were Reverse transcription 45 ˚C for 20 min then Polymerase activation (1 min at 95 °C) then 40 cycles of; denaturation (10 s at 95 ˚C), annealing (10 s at 60 ˚C) and extension (30 s at 72^{o}c). The primer sequences were summarized in Table 1.

Table (1): Oligonucleotide primer sequences and thermal cycling conditions for detection of *Mycoplasma gallisepticum*, IBV, Avian influenza subtype H5 and H9

Type	Target gene	Primers	Size of amplified products	References
*H9	HA Haemagglutinin protein	H9F:GGAAGAATTAATTATTATTGGTCGGTAC H9R: GCCACCTTTTTCAGTCTGACATT H9PRO FAM-5 – AACCAGGCCAGACATTGCGAGTAAGATCC-3-TAMRA		[21]
*H5	HA	F: ACATATGACTACCCACARTATTCAG R: AGACCAGCTAYCATGATTGC H5PRO FAM-5 - TCWACA GTGGCGAGT TCCCTAGCA -3-[TAMRA]	-	[22]
*IBV	NP Nucleocapsid protein	F: GCTTTTGAGCCTAGCGTT R: GCC ATGTTGTCACTGTCTATT IBV5-G Probe-5 FAM-CACCACCAGAACCTGTCACCTC-BHQ1		[23]
MG	mgc2 a second cytadhesin protein	F: CGCAATTTGGTCCTAATCCCCAACA R: TAAACCCACCTCCAGCTTTATTTCC	300bp	[25]

.* Uniplex real time PCR for detection of avian influenza subtype H5 and H9 as well as infectious bronchitis virus.

Molecular identification of Mycoplasma gallisepticum

Samples from 25 flocks were selected from different loaclities, breeds, ages and during different seasons were grown in pleuro-pneumonia-like organism PPLO broth. After 24-48 h of incubation, the chromosomal DNA samples of all cultures were extracted according to Liu *et al*., [24]. Master mix consisted of a total volume of 50 µL final concentration as the following: 0.1 mM dNTPs, 2.0 mM MgCl2, 100 nM primers (2 µL) of each primer 2.5 units of Taq polymerase (5 U/l) (Promega, Madison, WI) and 5 µL of templet RNase Free Water up to 50 µL. The cycling conditions were an initial denaturation (180 s at 95 ˚C), followed by 35 cycles of denaturation (20 s at 94 ˚C), annealing (40 s at 58 ˚C), extension at (1 min at 72 ˚C) and final extension (90 s at 72 ˚C).The primer sequences was summarized in Table 1.

Sequencing and phylogenetic analysis of M. gallisepticum Cytoadhesion 2 gene (MGC2)

Purification of three representative identified MG isolates was done using QIAquick PCR purification Kits (Qiagen Inc. Valencia CA), following the manufacturer's instructions.

Purified products were sequenced in the forward and/ or reverse directions using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Cat. No. 4336817. The sequencing was done on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Firstly, a BLAST® analysis was done to establish sequence identity with previously published sequences on gene bank (http://www.ncbi.nlm.nih.gov/ BLAST). The CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign model of Lasergene DNA Star software Pairwise which was designed by Thompson *et al.*, [26] for comparative analysis of sequences. MEGA6 was used for phylogenetic analyses using maximum likelihood, neighbour joining and maximum parsimony [27]. The nucleotide sequences of three isolates strain sharkia/2017, strain sharkia /2014/1 and strain sharkia/ 2014/2 were submitted to GenBank with accession numbers of MK310101, Mk310102 and MK 310103, respectively compared with other local and international *MG* published sequences on GenBank which summarized in Table (2).

Table 2. The *Mycoplasma gallisepticum* reference strains published in Gene Bank and used in this study

No	Strain name	Country	Year	Accession no
1	Eis10-17	Egypt	2017	KY421065
2	ZA_MG B293_15	SouthAfrica	2015	KY362286
3	F/2015 MGC2	Thailand	2015	KX268633
4	S6/2015 MGC2	Thailand	2015	KX268634
5	ZA_MG B726_06	South Africa	2006	KY362207
6	AHRU2003CU5113.2/2015	Thailand	2015	KX268625
7	Algeria/26/mg2	Algeria	2013	KP892755
8	Eid1.mg-TK-EG014	Egypt	2014	KP691072
9	USA/K6001B/CK07	U.S.A	2007	KC247882
10	6-85 Mgc2	Australia	2013	JQ770178
11	MG DSD-4	Israel	2011	JN113346
12	MG HZ-19	Israel	2011	JN113342
13	Eis4-C-10 (field isolate)	Egypt	2010	HQ591356
14	RabE1-08	Egypt	2008	FJ234839
15	ISR/K3868/CK95	Israel	2010	HQ143377
16	Eis6-T-10 (field isolate)	Egypt	2010	HQ591357
17	JOR/4/CKA	Jordan	2010	HQ143378
18	MG031008 Mgc2	Russia	2009	FJ965796
19	EgPk1UAF08 Mgc2	Pakistan	2008	FJ395202
20	USA/65099/PF08	U.S.A	2008	KC2478839
21	Eis-8-CK-14	Egypt	2014	KP135564
22	MG-70 cytoadhesin (vaccine	Brazil	2014	KJ019171
23	CK.MG.UDL.PK.2013.4	Pakistan	2013	KF874279
24	MGS1167 cytadhesin	India	2014	KP300758
25	MGS 927 cytadhesin	India	2014	KP261894
26	Nouh-C-15-mgC2	Egypt	2015	KT992784
27	F/2015 MGC2	Thailand	2016	KX268633
28	AHRU2003CU5713.2/2015 MGC2	Thailand	2016	KX268630
29	IR/RH1376.49/16 Mgc2	Iran	2016	KY651218
30	Man-Reh.2/Mg/CK/EG016	Egypt	2016	KY404987
31	ZA_MG B1102_03	South Africa	2003	KY362212
32	IR/RH1376.61/16	Iran	2016	KY651222
33	K4649ATK98 Mgc2	U.S.A	2005	AY556265
34	K2101CK84 Mgc2	U.S.A	2004	AY556238
35	HF51 Mgc2	U.S.A	2004	AY556233
36	ts11 Mgc2	U.S.A	2004	AY556232
37	strain F Mgc2	U.S.A	2004	AY556230
38	strain S6 Mgc2	U.S.A	2004	AY556229
39	strain A5969Mgc2	U.S.A	2004	AY556227
40	Eis 7-c-10 (vaccine strain F)	Egypt	2011	HQ591358
41	Strain 6/85	U.S.A	2004	AY556231

Results

Clinical and post mortem findings

All the examined broiler chicken flocks suffered from general signs of illness ruffled feather, reduced feed and water intake, and depression. In addition, whitish and/ or greenish diarrhea or both with respiratory signs (sneezing, coughing, rales, nasal and ocular discharge) were recorded. In addition to the respiratory signs, one flock showed nervous signs. The post mortem examination revealed many lesions. Most of these birds showed picture of septicemia including congestion in subcutaneous tissues, trachea, larynx, lungs, liver, spleen and kidneys with chronic respiratory disease picture (CRD) fibrenous perihepatitis, pericarditis and airsaccultitius Caseated plugs in trachea and bronchioles of lung were observed in in 35 flocks. Nephrosis of kidney was recorded in 31 flocks. Petechial haemorrhages on papillae of proventriculus and coronary fat of heart were recorded in 3 flocks (Figure 1).

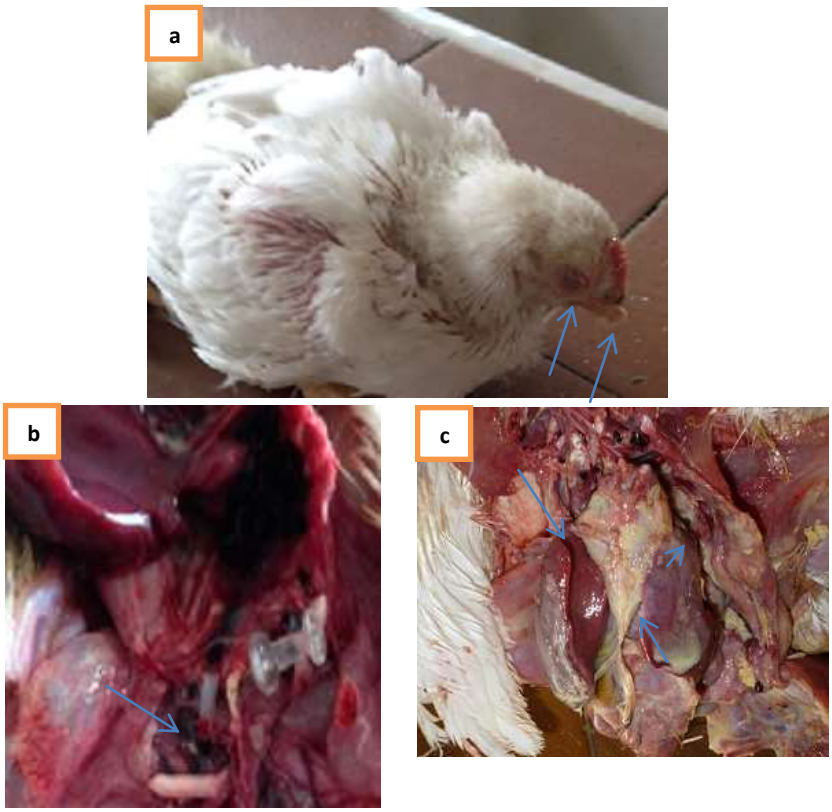

Figure 1. Clinical signs and postmortem lesions of examined chickens: a) 25 days old Cobb broiler chick, flock No 42 . shows closed eyes and general signs of illness b) caseated plug inside trachea of 26 day old chick, flock No.14 c) fibrinous pericarditis, fibrinous perihepatitis and fibrinous airsacculitis in 30 days old Hubbard chick, flock No. 15.

Isolation and serological identification of AIVs and IBV

The virus isolation trials revealed variable embryo lesions including dead congested embryos (42), small size embryos (1) and normal embryos (10). Fourty two (79.2%) of the harvested amnioallantoic fluids were positive for HA. The hemagglutination inhibition test identified 27 NDV isolates, 20 AIV with percentage of 64.4% and 47.6% respectively. 27 NDV were 22 alone (52.4%), 20 AIV were 14 H9 alone (33.3%), (5/42, 11.9 %) showed mixed infection of AIV and ND (Samples number 12, 50, 51, 52 and 53) and one flock showed mixed infection by both AIV subtype H9 and AIV subtype H5 (sample number 6) 2.4%.

Molecular identification of IBV, AIV subtypes H5 and H9

Based on the results of clinical examination, embryo lesions, HA activity and HI test, 19 respiratory tissues samples were directly tested for IBV infection using RT-PCR and 17 of them were found positive, however, 42 hemagglutination positive AAF samples were subjected to RT-PCR for identification of AIV subtype H9 and H5, of which, 10 samples were identified as H9 and only one was H5.

Molecular identification of Mycoplasma gallisepticum

Twenty five samples representing 25 broiler flocks (from different localities and different seasons suffered from respiratory signs (out of a total of 53 investigated flocks were screened for presence of *Mycoplasma gallisepticum* by specific primers against MGC2 gene using conventional PCR. Fourteen flocks' samples were found positive for *Mycoplasma gallisepticum* (56 %).

Sequencing and phylogenetic analysis of M. gallisepticum Cytoadhesion 2 gene (MGC2)

The sequencing data showed that there was high similarity between isolates of 2014 (1, 2) with accession no (Mk310102 and MK 310103) and isolates of 2017 accession no (MK310101) as the percent of nucleotide identity between them was 99%, while the percent of nucleotide identity between two isolates of 2014 was 100%.

Comparison of three isolates of the current study with other Egyptian published sequences of *MG* mgc2 field isolates on GenBank exposed 96.3- 99.5% , 90.8-91.7 % similarity with E7-C-10 strain which resembles F vaccinal strain.

The results indicated that variable range of similarity between the three tested isolates and four commercial vaccinal strains such as 6/85(65.9-76.1%), F(88.9- 89.8%), TS-11(95.6-95.7 %) and MG-70 (96.7- 98.5 %).

The partial sequence of the MGC*2* gene of the three isolates revealed 90-99.5% nucleotide identity with previously published sequences of MG strains isolated from middle east such as strains Iran (IR/RH1376.49/16 and IR/RH1376.61/16), Israel (MG DSD-4, MG HZ-19 and ISR/K3868/CK95) and Jordan (JOR/4/CKA).

A wide range of identity percent were detected between our isolates and other published sequences of other countries such as (Pakistan, Brazil, India, Thailand, Russia and Algeria) was 92.9% – 99% similarity ,South Africa (81.7 %- 85.7%) similarity, USA (80.7-98.9%) similarity and Australlia (65.9%-76.1%)% similarity which resemble 6/85 vaccinal strain. Phylogentic tree pattern illustrated in (Figure 2).

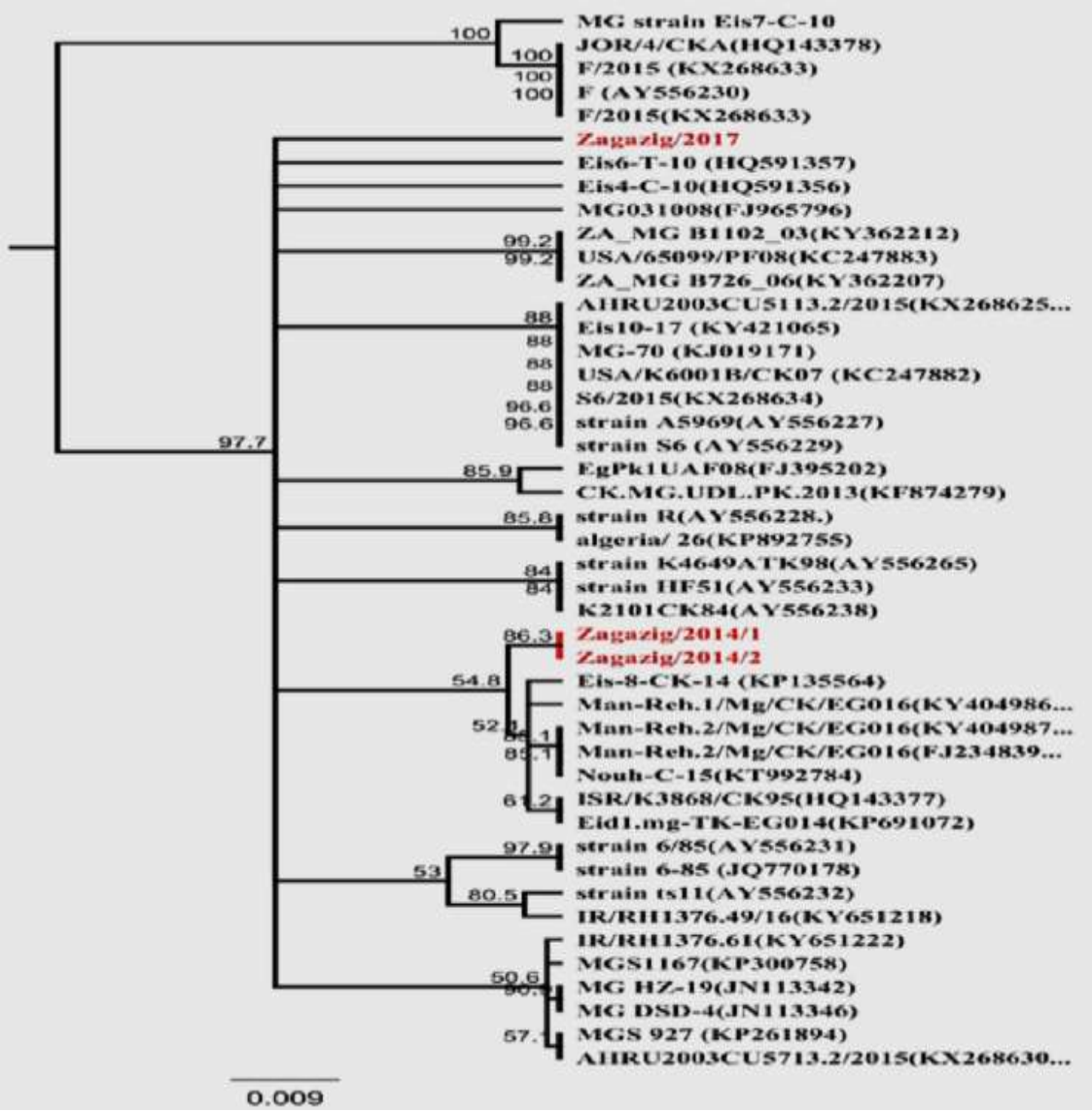

Figure 2. Phylogenetic tree based on *M. gallisepticum* mgc2 gene sequence for three representative isolates obtained in this study (red color) with other Egyptian and international published sequences on GenBank.

Discussion

Egyptian poultry farms are frequently affected by a wide range of avian respiratory pathogens either single or mixed infection. The routine identification and monitoring of field avian pathogens is among the most important tools for disease prevention and control. The recorded clinical signs were in the form of general signs of illness (ruffled feather, loss of feed and water intake and loss of weight gain), respiratory signs (dyspnea, gasping, coughing, sneezing, nasal discharge, eye lacrimation and rales) and swollen head with closed eyes. The forementioned clinical findings were previously shown to be associated with *M. gallisepticum* infection, NDV, AIV (H9, H5), IBV and reported by many authors in chickens [28-39]. Greenish diarrhea as well as cyanosis of comb and wattle were recorded in the examined flocks, similar clinical picture was reported in birds infected with NDV and AIV [37, 38]. Nervous signs were recorded in one flock which is associated with NDV infection. The neurotropic strains of NDV causes paralysis and torticollis [39].The above mentioned clinical signs were varied in their severity and mortality percents according to the presence of

one or more than one of these causative agents. Flock no 6 (AIV H9, H5, IBV and MG) showed respiratory signs, 100% morbidity and 7.5% mortality, flock no 13 (NDV, IBV and MG) showed nervous signs, respiratory signs and 25% mortality, flock no15 (NDV, IBV, MG) showed respiratory signs, 50% morbidity and and 4.3% mortality, flock no 49 (NDV,IBV, MG) showed respiratory signs and mortality 20% while flock no 34 (AI H9 and IB) showed respiratory signs and 11.1% mortality, flock no 40 (NDV and MG) showed respiratory signs, greenish diarrhea and 25% mortality, other flocks showed low mortality percents either infected by one or two causative agents. This may be attributed to using of vaccines agaist these agents and the mortality percents (which were calculated in 1st day of observation or during 1st 3 days of course of disease based on data collected from owner).

On necropsy, most of the examined broiler flocks had septicemic lesions in the form of congested subcutaneous tissues (S/C), tracheitis, congested lung with frothy exudate in some cases, pancreatitis with necrotic foci, congested spleen with mottling appearance in some cases, thymus congestion, fibrinous pericarditis, fibrinous air sacculitis, fibrinous perihepatitis, haemorrhage on coronary fat, caecal tonsils and proventriculus.

Particularly Flock no 6 (AIV H9, H5, IBV and MG) showed septicemia, CRD, caseated plug in trachea and in bronchiolesas well as nephrosis of kidney, flock no 49 (NDV, IBVand MG) showed septicemia, fibrinous airsacculitis, caseated plug in trachea and in bronchioles and haemorrhage at caecal tonsils and on coronary fat.

Similar findings had been reported in chicken flocks associated with NDV, AIV (H9, H5), IBV and *M. gallisepticum* mixed infection [28, 30]. In addition, 35 flocks showed caseated plug at tracheal bifurcation and in bronchioles of lung, caseated abdominal air sacs, swollen kidney with nephritis and ureters distended with urate deposits. Many studies investigated the occurrence of IBV in broiler flocks in Egypt and recorded the gross lesions as mucous or caseated material in trachea and bronchi, fibrinous pericarditis, perihepatitis and airsaculitis. Pale or congested and enlarged kidneys with urates deposition and the ureters with urates were seen [32-34].

Table 3: Cumulative results of ECE inoculation, haemagglutination, HI, RT-PCR and conventional PCR used for identification of investigated pathogens

Sample code	Breed/ age (Day)	Date of collection	Flock capacity	Vaccination	Mortality N and %	Locality	ECE*	HA	HI	RT- PCR			PCR-MG	Total no of pathogens
										IBV	AIVH9	AIVH5		
1	Cobb/22	22/5/2013	5000	ND+AI +IB	40 (0.8%)	AboHamad	N	-ve	-ve	+ve	-	-ve	+ve	IB, MG
2	Avian/15	5/6/2013	170	IB+ND	0.0%	Abo Kabeer	DC	+ve	H9	Nd	+ve	-ve	Nd	H9
3	Hubbard/18	5/11/2013	130	IB+ND	1(0.8%)	Abo Kabeer	DC	+ve	H9	Nd	nd	Nd	Nd	H9
4	Hubbard/35	20/11/2013	3500	ND+AI	25 (0.7%)	AboHamad	DC	+ve	H9	+ve	nd	Nd	Nd	H9, IB
5	Cobb/32	22/11/2013	12,000	ND+AI	60 (0.5%)	Minia alkamah	DC	+ve	ND	Nd	-	-ve	+ve	ND, MG
6	Ross/29	28/11/2013	16,000	ND+AI	1200 (7.5%)	Zagazig	DC	+ve	H9, H5	+ve	+ve	+ve	+ve	H9,H5,IB,MG
7	Hubbard/26	28/11/2013	6500	ND+IB	181 (2.8%)	Abo Hamad	DC	+ve	ND	Nd	-	-ve	Nd	ND
8	Hubbard/30	1/12/2013	6000	ND+AI	160 (2.6%)	Abo Hamad	DC	+ve	ND	Nd	-	-ve	Nd	ND
9	Cobb/27	2/12/2013	5000	ND+AI	270 (5.4%)	Abo Hamad	DC	+ve	ND	nd	-	-ve	Nd	ND
10	Cobb/29	3/12/2013	4800	ND	55 (1.1%)	Abo Hamad	DC	+ve	H9	nd	+ve	-ve	Nd	H9
11	Cobb/31	4/12/2013	11,500	ND+IB	30(0.2%)	Abo Hamad	DC	+ve	H9	nd	+ve	-ve	+ve	H9, MG
12	Hubbard/29	5/12/2013	4000	ND	100 (2.5%)	Abo Hamad	DC	+ve	ND+H9	nd	nd	Nd	Nd	ND, H9
13	Ross/37	17/12/2013	4000	ND+IB	1000(25%)	Belbis	DC	+ve	ND	+ve	-	-ve	+ve	ND,IB,MG
14	Cobb/26	22/12/2013	5000	ND+IB	12(0.2%)	Hehia	DC	+ve	H9	+ve	nd	Nd	Nd	H9,IB
15	Hubbard/30	23/12/2013	6000	ND	260(4.3%)	Hehia	DC	+ve	ND	+ve	-	-ve	+ve	ND,IB,MG
16	Cobb/34	29/12/2013	5000	ND+IB	140 (2.8%)	Belbis	DC	+ve	ND	nd.	-	-ve	+ve	ND, MG
17	Arbo/27	31/12/2013	8000	ND+AI+IB	195(2.4%)	Abo Hamad	DC	+ve	H9	nd	nd	Nd	Nd	H9
18	Cobb/30	8/1/2014	6000	ND+IB	115(1,7%)	Diarb Negem	DC	+ve	ND	nd	-	-ve	Nd	ND
19	Cobb/34	8/1/2014	5000	ND+IB	60(1,2%)	Diarb Negem	DC	+ve	H9	+ve	+ve	-ve	+ve	H9, IB, MG
20	Cobb/24	8/1/2014	4750	ND+IB	37(0.7%)	Faqous	DC	+ve	H9	nd	nd	Nd	-ve	H9
21	Cobb/26	9/1/2014	5000	ND	70 (1.4%)	Hehia	DC	+ve	H9	+ve	+ve	-ve	-ve	IB, H9
22	Avian/21	14/1/ 2014	3500	ND+IB	60 (1.7%)	Al Hessinia	DC	+ve	ND	nd	-	-ve	-ve	ND
23	Cobb/27	20 /1/ 2014	1500	ND+IB	13(0.8%)	Al Salheia	DC	+ve	H9	nd	nd	Nd	Nd	H9
24	Cobb/29	20 /1/2014	2000	ND+IB	18 (0.9%)	Al Hessinia	DC	+ve	ND	nd	-	-ve	-ve	ND
25	Hubbard/22	21/1/2014	4500	ND+IB	20(0.4%)	San El Hager	DC	+ve	ND	nd	-	-ve	-ve	ND
26	Cobb/29	24/1/2014	3000	ND+IB	100 (3.3%)	Al Salheia	DC	+ve	ND	nd	-	-ve	-ve	ND
27	Cobb/31	24/1/2014	4000	ND	50 (1.25%)	San El Hager	DC	+ve	ND	nd	-	-ve	-ve	ND
28	Cobb/30	29/1/2014	5000	ND+IB	3(0.06%)	Al Salheia	DC	+ve	ND	nd	-	-ve	Nd	ND
29	Cobb/30	29/1/2014	2500	ND+IB	20(0.8%)	Al Salheia	N	-ve	-ve	+ve	-	-ve	Nd	IB
30	Cobb/35	29/1/2014	7000	ND+IB	60(0.8%)	Al Salheia	DC	+ve	ND	nd	-	-ve	Nd	ND
31	Cobb/35	29/1/2014'	12000	ND+IB	15(0.1%)	Al Salheia	N	-ve	-	nd	-	-ve	Nd	-
32	Avian	31/3/2014	5300	ND	54(1%)	Diarb Negem	N	-ve	-ve	+ve	-	-ve	Nd	IB

	48/34													
33	Cobb/22	9/4/2014	2000	ND+AI	33(1.6%)	Hehia	N	-ve	-ve	-ve	-	-ve	-ve	-
34	Hubbard/36	14/4/2014	4500	ND+IB	500(11.1%)	Minia ElKamah	DC	+ve	H9	+ve	+ve	-ve	Nd	H9, IB
35	Hubbard/30	15/4/2014	6000	ND+AI	160 (2.6%)	Abo Hamad	N	-ve	-ve	+ve	-	-ve	-ve	IB
36	Cobb/24	8/5/2014	4000	ND+AI+IB	18(0.4%)	Abo Hamad	DC	+ve	H9	nd	+ve	-ve	-ve	H9
37	Cobb/30	8/5/2014	4000	ND	70(1.7%)	Hehia	DC	+ve	H9	-ve	nd	Nd	-ve	H9
38	Hubbard/10	12/5 /2014	1500	ND+IB	30(2%)	Faqous	Ss	-ve	-VE	+ve	-	-ve	Nd	IB
39	Ross/21	15/5/2014	6000	ND+IB	10(0.1%)	Faqous	N	-ve	-VE	+ve	-	-ve	Nd	IB
40	Ross/26	20/5/2014	2000	ND+AI	500(25%)	Faqous	DC	+ve	ND	nd	-	-ve	+ve	ND, MG
41	Ross/21	2/3/2015	2800	ND	83(3%)	Zagazig)	DC	+ve	ND	nd	-	-ve	+ve	ND, MG
42	Cobb/25	2/3/2015	4500	ND	18(0.4%)	Zagazig	DC	+ve	ND	nd	-	-ve	+ve	ND, MG
43	Ross/22	3/3/2015	500	ND	15(3%)	Zagazig	DC	+ve	ND	nd	-	-ve	+ve	ND, MG
44	Ross/18	26/4/2015	250	Non V*	5(2%)	Zagazig	DC	+ve	ND	nd	-	-ve	Nd	ND
45	Saso/35	1/5/2015	1000	ND	20(2%)	Zagazig	DC	+ve	ND	nd	-	-ve	Nd	ND
46	Cobb/32	8/6/2015	12000	ND+IB	50 (0.4%)	MashtuElsaugh	N	-ve	-ve	nd	-	-ve	Nd	-
47	Cobb/25	20/1/2016	19.5000	ND+AI	60(0.3%)	Abo Hamad	N	-ve	-ve	+ve	-	-ve	Nd	IB
48	Cobb/26	22/1/2016	300	NONV	5(1.6%)	Zagazig	N	-ve	-ve	nd	-	-ve	Nd	-
49	Cobb/27	14/2/2017	500	ND	100(20%)	Zagazig	DC	+ve	ND	+ve	-	-ve	+ve	ND, IB, MG
50	Ross/34	16/2/2017	3000	ND	30 (1%)	Zagazig	DC	+ve	ND+H9	nd	+ve	-ve	+ve	ND, H9, MG
51	Cobb/25	29/2/2017	5000	ND	100(2%)	Minia El Kamh	DC	+ve	ND+H9	nd	nd	Nd	Nd	ND, H9
52	Cobb/33	4/3/2017	14,000	ND+IB	240(1,71%)	Abo hamad	DC	+ve	ND+H9	+ve	+ve	-ve	Nd	ND, H9,IBV
53	Saso/29	16/3/2017	7,500	ND+AI	50(0.6%)	Zagazig	DC	+ve	ND+H9	nd	nd	Nd	Nd	ND, H9
Total	53							42		17/19	10/42	1/42	14/25	

*N= Normal embryo without any obvious changes; DC= Dead and congested embryos after 1-3 passages while mortalities less than 24 hours were excluded and regarded as non-specific deaths; ss = small size ; -ve = negative; +ve = positive; nd= not done NonV= non vaccinated ND= Newcastle disease AI= avian influenza IB = infectious bronchitis

In the present study, the HI results revealed 27/42 flocks were infected by NDV 22 were alone, 20/42 flocks were infected by H9 14 were alone, 5 flocks were infected by both NDV and AIV subtype H9 and only one flock was infected by both AIV subtypes H9 and H5. There are continuous circulation of NDV and AIVs either single or in a mixed manner in Egyptian poultry flocks as reported by previous studies [28, 29, 35]. The mixed infection with NDV and LPAI (H9N2) was recorded by other studies [30, 45, 46]. Co-infection with AIV Subtypes H9 and H5 was reported by Hassan *et al*., [8] who found that co-circulation of HPAIV H5N8 and H9N2 was the most commonly documented mixed infection (11/39 farms).

Real-time RT-PCR is the most sensitive and specific diagnostic method for identifying the presence of antigen [47]. Regarding to this study, 19 flocks with clear caseated plug in trachea were subjected to real time PCR for detection of IBV infection. Seventeen samples were found to be positive for IBV. Therefore the OIE confirmed that the prob based RT-PCR targeting nucleoprotein is considered the most accurate method for IBV identification [41]. Detection of IBV directly from suspected tissues was done following EL-Baz [48] who identified IBVdirectly in 9 tissues suspected to be infected with IBV by RT-PCR out of 44 with a history of presence of caseated plug at tracheal bifurcation in 28 flock and 22 flocks showed kidney nephrosis and nephritis. RT-PCR screening of allantoic fluids of the same 9 positive samples previously examined revealed the presence of IBV infection only in 2 samples, although these samples made reduction in embryo size upto 2 cm on ECE incomparsion to its negative control after 3-6 blind passages. So identification of IBV directly from tissues is better for diagnosis.

The results of HI test were in agreement with the results of Real time –PCR. Helmi and Hyrnato [49] recorded that the positivity of 7 samples for AIV type A either by serological analysis using HA and HI tests or molecular analysis by RT-PCR amplification for matrix gene and haemagglutinin.

Utilizing both HI, Real time PCR and conventional PCR data the results revealed that 13 flock harbored NDV, 8 were AI subtype H9 positive, 6 carrying both NDV+MG, 3 were positive for NDV+IBV+MG, 1 has mixed infection with AI subtype H9 and MG, 4 were AI subtype H9 and IBV positive samples, 1 harbored mixed infection with AI subtype H9, IBV and MG,1 was positive for AI subtype H9, NDV and IBV, 3 were carrying NDV and AI subtype H9,1 was IBV and MG positive, 6 were infected only with IBV, 1 harbored AI subtype H9, H5, IBV and MG, 1 had NDV, AI subtype H9 and MG mixed infection and 4 with no identified agents, may be infect by another pathogen. These results are similar to those recorded by Roussan *et al*., [30]. They investigated a total of 115 commercial broiler flocks with a history of respiratory disease, the results showed that 13 and 14.8% of these flocks were infected with NDVand IBV, respectively, whereas 5.2, 6, 9.6, 10.4, 11.3, and15.7% of these flocks were infected with both NDV+MG, MG+(avian pneumovirus) APV, IBV+NDV, IBV+MG, NDV+AIV and IBV+AIV, respectively. Furthermore, 2.6% of these flocks were infected with IBV, NDV, and APV at the same time. On the other hand, 11.3% of these flocks were negative for the above respiratory diseases. Infectious bronchitis virus was recorded by several studies as being associated either with AIV (HPAIV or LPAIV) or NDV in natural infection in Egypt and other countries [28, 30].

The results of sequencing and phylogentic analysis of three isolated *M. gallisepticum* strains Zagazig /2014/1(Mk310102), Zagazig /2014/2 (MK 310103)and Zagazig /2017 (MK310101) revealed high similarity (96.3-99.5%) with other Egyptian published sequences of MG mgc2 (field isolates).. At the same concern, Younis *et al.,* [15] found that the similarity of two isolated sequences (RAG-2 – MG-CK-EG017 & RAG-1 – MG-CK-EG017) with other published strains was (93.1- 96.6 %).While the similarity with other commercial vaccines were varied. High similarity were recorded with ts11 vaccine and MG70 (96.7- 98.5 %) than 6/85(65.9-

76.1%), F(88.9- 89.8%). So the sequence analysis of this study revealed sequences of field isolates. These results resembled those stated by Eissa *et al*., [50] who recorded that the partial sequence of the *mgc2* gene of NLQP-MG-1 in comparison with universal vaccine strains showed a 97% identity with the vaccine strains 6/85 and TS-11 and an 87% identity with the MG F-strain (AY556230.1) from which we concluded that this strain is a wild-type infection and not a vaccinal strain. Also with Eissa *et al.*, [51] who found that Egyptian MG (11 isolates) showed 88-92%, 90-95% and 91-94% homology when compared to vaccine strains (F, TS-11 and 6/85, respectively).

Conclusion

There is continuous circulation of different respiratory agents in broiler flocks in Sharkia Governorate either as single or mixed infection confirming the importance of utilizing faster diagnostic tools as well as new preventive strategies.

Conflict of interest

None of the authors have any conflict of interest.

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الملخص العربي

التعرف الجزيئي علي بعض مسببات الأمراض التنفسية من دجاج التسمين في محافظة الشرقية – مصر

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في هذه الدراسة ، تم الكشف عن انتشار فيروس أنفلونزا الطيور (AIV) (النوعان الفرعيان H5 و H9) ، وفيروس التهاب الشعب الهوائية المعدى (IBV)، وفيروس مرض نيوكاسل و المايكوبلازما جاليسبتكم (*Mycoplasmagallisepticum (MG*) االمسئولة عن المشاكل التنفسية في قطعان دجاج التسمين في مناطق مختلفة من محافظة الشرقية خلال ٢٠١٣-٢٠١٧. من بين ٥٣قطيعا، تم فحص ٢٢٥ طائر تعاني من اعراض تنفسية. تم عزل الفيروس من أنسجة الجهاز التنفسي وعمل الفحص السيرولوجي والجزيئي . بناءً على اختبار مانع تلازن الدم ,كان معدل انتشار NDV و AIV عترة H9 و H5 ٦و٥٠% و ٣٧.٧٪ و ١.٩٪ على التوالي ؛ وكان هناك عدوي ثنائية ل AIV مع NDV وكذلك AIV النوع الفرعي H5 مع H9 بنسبة ٤و٩ ٪ و ١.٨ ٪ علي التوالي . واستخدمت نوعان من الاختبارات التشخيصية سلسلة البوليميريز (PCR). استنادًا إلى Real time PCR، تم تأكيد إصابة ١٧ من ١٩عينة ، ١٠ من ٤٢ و ٤٢/١ بـ IBV، AIV ، النوع الفرعي ٩ و ٥ على التوالي. علاوة على ذلك ، تم إجراء اختبار PCR التقليدي للكشف عن الميكوبلازما جاليسبتكم في ٢٥ قطيع وكشف ٥٦ ٪ (٢٥/١٤) قطعان إيجابية. في وقت لاحق ، تم عمل تتابع جيني لثلاث عزلات تمثيلية الميكوبلازما جاليسبتكم ومقارنتها مع سلالات أخرى على بنك الجينات. أظهرت النتائج أن العزلات الثلاثة التي تم فحصها كانت من النوع البري (عزلات حقلية).من هذه الدراسة نستنتج ان هناك تواجد مستمر لمسببات الامراض التنفسية الفيروسية (فيروس مرض نيوكاسل و أنفلونزا الطيور(النوعان الفرعيان H5 و H9) و الالتهاب الشعبي المعدي) وكذلك البكتيرية مثال (المايكوبلازما جاليسبتكم) في محافظة الشرقية في صورة عدوي مفردة او عدوي متعددة علي الرغم من استخدام برامج التحصينات والمضادات الحيوية المختلفة لذلك التشخيص المبكر لمثل هذه العدوي تعتبر اهم اداة في السيطرة ومنع هذه الامراض .