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Co-infection between Enterobacteriaceae and Avian Influenza Virus H8N2 in Poultry

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Abstract

Two thousand cloacal swabs were collected from 100 poultry farms (78 broiler farms and 22 layer farms) showing respiratory and diarrheic sings.All samples were examined bacteriologically for isolation of *E. coli* & *Salmonella*. The results revealed detection of *Salmonella* in 31 farms out of 100 examined farms in percentage 31%.*Salmonella* was detected in 23 broiler farms out of 78 examined farms with percentage of 29%. While the organism was detected in 8 layer farms out of 22 examined layer farms with percentage 36%.Isolated serotypes were *S. enteritidis*, 9 isolates which represent 29% of isolated *Salmonellae*, S. Newport was 7 strains with percentage of 23%. *S. Kentucky* that was 6 strains with percentage of 19% from isolated *Salmonellae, S. typhimurium* (n.5) and represent 16% of the detected *Salmonellae, S. Tamale,* it was 4 isolates with percentage of 13%. *S. enteritides* was confirmed with PCR and detection of *sefA* gene was done. Isolation of *E. coli* was carried out and the results revealed isolation of *E. coli* from 67 farms with percentage 67% of examined 100 poultry farms. Incidence of *E. coli* was 68% in broiler farms and 64% layer farms. Serotyping of isolated *E. coli* resulted in O158 (n. 18), O44 (n. 13), O114 (n. 9), O91 (n. 8), O125 (n. 4), O111 (n. 3), O26 (n. 3), O142 (n. 3), O78 and 127 (n. 2 each), O103-O164-O199 (one strain each) and untyped strains were 23 isolates with percentage 26%.Detection of some virulence genes were done and detection of stx_1 gene, *eaeA* gene and *iss* gene was found by using PCR. All collected samples were examined for detection of Avian Influenza virus H9N2 using RRT–PCR. LPAI (H9N2) was detected in 31 farms (24 farms from broiler farms and 7 farms from the layer farms).

Keywords: *Salmonella, E. coli, H9N2, AI virus, Salmonella virulence genes, E.coli virulence genes*

Introduction

Diseases of the respiratory tract are a significant component of the overall disease incidence in poultry. Various pathogens may initiate respiratory disease in poultry and including a variety of viruses, bacteria and environmental factors may augment these pathogens to produce the clinically observed signs and lesions**. (Glisson, 1998).**

E. coli is a member of the family enterobacteriaceae, which may constitute a great hazard to poultry industry causing high mortality, loss of weight and reduction of egg production (**Bandyopadhay and Dhawedkar, 1984**). It is a normal inhabitant of the intestinal tract of birds, under the influence of predisposing factors, like inadequate and faulty ventilation, overcrowding, hunger, and thirst, extremes of temperatures and low vitality, high mortality during rearing, reduced weight gain and condemnation of birds at the time of slaughter (**Kaul *et al.*, 1992**).

On the other hand, Salmonella infection in poultry caused by a variety of Salmonella species and considered as one of the most important bacterial diseases in poultry causing heavy

economic losses through mortality and reduced production. Avian Salmonella infection may occur in poultry either acute or chronic form by one or more member of genus Salmonella, under the family Enterobacteriaceae. **(Hofstad *et al*., 1992).** Although salmonellosis can be a self-limiting disease, the host may become a persistently infected asymptomatic carrier (**Todd *et al.*, 2008**) with intermittent shedding of Salmonella, so can infect other birds and people

Avian influenza is an important poultry disease with the potential to cause major epidemics resulting in significant economic losses. Avian influenza viruses belong to type A have been reported **(Tong *et al*., 2013).** Avian H9N2 influenza viruses have circulated widely in domestic poultry around the world**.** Although avian H9N2 influenza virus is pathotyped as low pathogenic, it can cause serious disease and high mortality in broilers when occur mixed infection with other respiratory pathogens. **(Seifi *et al*., 2010).**

Mixed infections of H9N2 AIV with other respiratory pathogens, particularly IBV, Mycoplasma gallisepticum, Staphylococcus aureus, Avibacterium paragallinarum, Escherichia coli, Ornithobacterium rhinotracheale or immune suppressive agent can exacerbate H9N2 AIV pathogenicity resulting in severe clinical disease and variable mortality **(Nili and Asasi, 2003).**

In this study, we investigate co-infection between Enterobacteriaceae and Avian Influenza Virus in Poultry

Materials and Methods

Samples

A total of 2000 cloacal swabs from 100 commercial chicken farms (20 swabs from each farm). Swabs were collected from broiler and layer farms from diseased chicken showing respiratory and diarrheic manifestation. Chicken farms were from 7 governorates, from 4 Delta governorates: Sharkia, Qalioubia, Dakahlia and Damiatta and from 3 Upper Egypt governorates: Giza, Beni Suif and Fayoum.

Isolation of *E. coli*

The isolation was conducted according to **Swayne *et al*., (1998) and Hitchins *et al*., (1998)**

Serolotyping identification of *E. coli* isolates

It conducted according to **Edwards and Ewing (1972)** using standard polyvalent and monovalent *E. coli* Antisera

Polymerase Chain Reaction (PCR) for detection of *E. coli virulence genes*

Extraction of Nucleic Acid :

QIAamp®DNA Mini Kit (Cat. No. 51304 Qiagen) according to manufacturer instructions.

Primers used in PCR:

Table (1). Showed the used primer sequence and Bp Fragment for *E. coli* virulence genes.

Gene	Primer Sequence (5'- 3')	Bp Fragment	Reference
Increased serum survival gene *ISS*	F:ATGTTATTTTCTGCCGCTCTG R: CTATTGTGAGCAATATACCC	266	**Yaguchi *et al* (2007)**
Attaching and effacing mechanisms gene *eae*A	F: GTGGCGAATACTGGCGAGACT R: CCCCATTCTTTTTCACCGTCG	890	**Fagan *et al* (1999)**
Shiga toxin 1 gene *Stx*1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614	**Fagan *et al* (1999)**

PCR Master Mix

Using of PCR 1.1x Reddy Mix TM Master Mix (Thermo SCIENTIFIC) with Cat. No. (AB0575/LD-A). For conventional PCR (uniplex PCR), DNA samples were amplified in a total of 25 µl that performed in the thermal cycler was adjusted as shown in the following table (2):

Table (2). Thermal cycler of *E. coli* DNA amplifications

1- Initial denaturation	94°C for 5 min.
2- Amplification	(35 cycles).
A- Denaturation	94°C for 1 min.
B-Annealing	54°C for 1 min (*iss* gene), 63°C for 1 min(*iut*A gene), 58°C for 40s (*Stx*1 gene, *Stx*2 gene, *eae* A gene)
C- Extension	72°C for 2 min.

Detection of the amplified product was done using 1.5 % Agarose gel with 0.1 ug/ml ethedium bromide electrophoresis.

Reference cultures were used for quality assurance and PCR specificity as shown in table (3)

Table (3). Reference cultures were used for quality assurance and PCR specificity.

Bacterial species	Source
Escherichia coli	NCIMB-50034, ATCC-43894

Isolation of Salmonella

It was carried out according to the **ISO 6579 (2002)**

Serotyping of *Salmonella* spp.

It was carried out according to **Kauffmann White Scheme (1973).**

Polymerase Chain Reaction (PCR) for detection of Salmonella virulence genes

It was carried out according to **Sambrook *et al.*, 1989**

Nucleic acid extraction: It was done according to **Croci *et al.*, (2004).**

Primers used in PCR: Oligonucleotide Primers sequences used for amplification of DNA for the detection of *Salmonella* species as shown in table (4).

Table (4). Showed the used primer sequence and size of amplified product for Salmonella virulence genes

Primer	Target Gene	Specificity	Primer sequence	Reference	Size of amplified product (bp)
139	*invA*	*Salmonella* species	F-5'GTGAAAT-TATCGCCACGTTCGGGCAA3'	**Oliveira *et al.*, (2002)**	284
141	*invA*		R-5'TCATCGCACCGTCAAAGGAACC3'		284
A058	*sefA*	*S. Enteritidis*	F-5'GATACTGCTGAACGTAGAAGG3'	**Oliveira *et al.*, (2002)**	488
A01	*sefA*		R-5'GCGTAAATCAGCATCTGCAGTAGC3'		488
-	virulence plasmid gene (*SPVC*)	Virulence	F-5'TTGTAGCTGCTTATGGGGCGG-3'	**Joseph *et al.*, 1999 and Bakshi *et al.*, (2003)**	460
-			R-5'-TGGAGAAA CGACGCACTGTACT-GC-3'		460

PCR Master Mix: Brilliant QPCR (Stratagene) Catalog No: 600549. Thermal cycler of *Salmonella* DNA amplifications was illustrated in table (5)

Table (5). Thermal cycler of *Salmonella* DNA amplifications.

1	Initial denaturation	95 °C for 15 min
2	Amplification (35 cycles of)	
	A-Denaturation	94 °C for 1s
	B-Annealing	55°C for 1 s
	C-Extension	72 °C for 21 s
3	Final extension phase	72 °C for 7 min

PCR amplifications were performed with a Thermal cycler. (T3 Thermocycler, Biometra, Germany) and detection of the amplified product was done using 1.5% agarose gel with 0.1 ug/ml ethedium bromide electrophoresis according to **Sambrook *et al*., (1989)**. Reference cultures were used for quality assurance and PCR specificity as shown in table (6).

Table (6). Reference cultures used for quality assurance and PCR specificity.

No.	Bacterial species	Source
1	*Escherichia coli*	NCIMB-50034 ATCC-43894
2	*Salmonella Enteritidis*	ATCC13076

Detection of Avian Influenza virus by RT-PCR:

Extraction of Nucleic Acid:

QIAamp Viral RNA Mini Kit Qiagen was used according to manufacturer instructions. The thermal cycling conditions for gene-specific of influenza virus Type A were recorded in table (7) (**Spackman *et al*., 2002**). Also thermal cycling conditions for gene-specific Probe and Primer sets for influenza virus H9 were recorded in table (8) **(Ben Shabat *et al*., 2010)**

Table (7). Thermal cycling conditions for gene-specific of Influenza virus Type A.

Phase step	Time	Temp	Number of cycles
Reverse transcription	30 min	50°C	1
Heat activation of polymerase	15 min	94°C	1
PCR Denaturation	1sec	94°C	40
Annealing/Extension	20 sec	60°C	

Table (8). Thermal cycling conditions for gene-specific Probe and Primer sets for influenza virus H9.

Stage	Temp	Time	No. of cycles
Reverse transcription	50°C	30 min	1
Primary denaturation	95°C	15 min	1
Amplification			40
a) Secondary denaturation	95°C	15 sec	
b) Annealing and Extention	60°C	45 sec	

The sequences of primers and probes for amplification of part of M gene of AIV type A by Real Time PCR: (Oligo™) were shown in the table (9) and the sequences of primers and probes for amplification of part of H9 gene of AIV-H9 by REAL TIME PCR: (Oligo™) were shown in the table (10).

Table (9). The oligonucleotide for amplification of part of M gene for AIV by REAL TIME PCR **(Spackman *et al*., 2002)**.

Primer/probe	Target gene	Sequence (5`- 3`)
AI M24	M gene	AGA TGA GTC TTC TAA CCG AGG TCG
AI M25	M gene	TGC AAA AAC ATC TTC AAG TCT CTG
AI Probe M	M gene	FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

M24, M25: forward and reverse primers respectively

Table (10). The oligonucleotide for amplification of part of H9 gene for AIV-H9 by REAL TIME PCR **(Ben Shabat *et al*., 2010)**

Primer/probe	Sequence (5'-3')
H9 Forward	GGAAGAATTAATTATTATTGGTCGGTAC
H9 Reverse	GCCACCTTTTTCAGTCTGACATT
H9 Probe	FAM-AACCAGGCCAGACATTGCGAGTAAGATCC–TAMRA

Results and Discussion

Bacteriological and Virological examination demonstrated that the samples collected from poultry farms presenting respiratory manifestations, revealed bacteria and influenza virus presence concomitantly. A number of respiratory viruses like Avian Influenza virus, Infectious Bronchitis, Avian pneumovirus, Newcastle disease virus, in addition to bacteria like *E. coli* may be involved, also environmental factors may act as predisposing factors, the interaction between different viruses and bacteria plays a very important role in respiratory infection in poultry.

The data presented in table (9) for all the tests demonstrated the presence of the influenza virus in samples isolated from poultry farms and co-infection with salmonella and *E. coli.*

Table (9). Illustrated Co-infection of Avian influenza virus with Bacteria.

No.	Governorate	No. of Examined farms	No. of positive farms		
			E. coli	***Salmonella***	**H9**
1	**Sharkia**	10	7	4	4
2	**Dakahlia**	19	12	5	6
3	**Fayoum**	25	17	7	6
4	**Beni Suif**	13	9	4	4
5	**Damiatta**	11	7	3	3
6	**Giza**	9	7	3	3
7	**Qalioubia**	13	8	5	5
Total		**100**	**67**	**31**	**31**

E.coli infections are of significant concern to the poultry industry. It is one of the most important and frequently encountered bacterial avian pathogen causing a wide variety of disease syndrome in birds causing up to 30% of mortality in poultry **(Georgnaras *et al.*, 2001).**

E. coli has been implicated in variety of disease conditions in poultry such as coliseptesemia, coligranuloma, airsaculitis, peritonitis, pericarditis, omphalitis, accounting about 5-50% in poultry flocks **(Edwin and Purushothaman, 2006).**

E. coli infection is most commonly via the respiratory tract, air sacs and is usually secondary to viral infection or mycoplasma causing colisepticaemia which is characterized by the presence of *E. coli* in the blood and colonization of organs including the heart, liver and spleen **(Barnes *et al*., 2003).**

In the present study it was recorded that the prevalence of *E. coli* isolation reached 67 % from the examined 100 broiler and layer farms showing respiratory symptoms. Similar percentage 67% in chickens were reported by (**Syuhada *et al*., 2013),** and relatively low percentage was reported by **(Stella *et al*., 2016),** from the 80 sampled birds, 48 (60%) E. coli was detected. The same results were mentioned by **(Hossain *et al*., 2008)** where it was (60%). Likewise a much lower incidence than the obtained results was reported by **Sripoernomo *et al.* (1992)** where it was 34.3%. Also an 800 chickens suffering from colisepticaemia, collected from different Governorates in Egypt by **(Roshdy *et al.*, 2012),** were examined for pathogenic *E. coli.* The incidence of E. coli isolation was (43.1%) in chickens.

On the other hand, a higher incidence 85.2% was reported by **(Wani *et al.*, 2004),** as well as 88.2% *E. coli* isolates were obtained by (**El-Sukhon *et al.*, 2002),** while a higher percentage of *E. coli* occurrence was detected by (**Albarri *et al.*, 2017)** where it was (93.75%).

In the present study serotyping of 67 *E. coli* isolates was done. 13 different serogroups were identified among *E. coli* isolates and the most predominant one was serotype O158 with 27% followed by O44 and O114 with 19% and 13% respectively. O91 and O125 with 12% and 6% respectively, also O111 and O26 have the same percentage which was 4%. It was found that O142 was similar to O78, O127 where all of them were 3% and O103, O164 and O119 have the same percentage which was 1%.

These result mostly similar to another study was conducted by **(Roshdy *et al.*, 2012)** who investigated the prevalence of different *E. coli* serotypes they found that, the most commonly isolated serogroups were O44, O158, O114, O91, O111, O125, O103, O26, O78 and O127. Likewise **(Bosch *et al*., 1993),** who reported that serogroups O44, O158, O114 and O91 were traditionally associated with colibacillosis in poultry.

In the present study, the amplification of 266 bp fragment of *iss* gene was successful in 11 *E. coli* serogroups in chickens (O158, O44, O114, O91, O125, O111, O142, O127, O103, O164 and O119) except isolates of the O groups 26 and 78, the amplification of *iss* gene was negative. The obtained results were similar to those reported by **Elaine *et al.* (2003),** who examined by PCR the presence of 16 of those genes in 200 colibacillosis isolates from their region and **Zhao *et al*. (2005),** who reported that eighty-four percent of isolated *E. coli* from birds were PCR positive for the increased serum survival (*iss*) gene, **Hassan (2009)** reported that the *iss* gene was detected in all of the 11 serogroups isolated (100%),

In the present study, the amplification of the 890 bp fragment of *eae* A gene (*intimin*) was successful from the extracted DNA of *E. coli*

isolates belonging to the serogroups O44, O158, O125, O114 and O78 whereas isolates representing the O groups O26, O91, O111, O142, O127, O103, O164 and O119 were all negative to this *eae A* gene. These results were similar to those reported by **Janben *et al.* (2001),** who examined 150 *E. coli* strains isolated from visceral organs of poultry had died from colibacillosis for the presence of virulence-associated genes by PCR. **EL-ashker (2006)** examined 10 strains for the presence of *intimin* gene and found 1 out of 10 (10%) *E. coli* isolates contained *intimin* gene.

In the present work, amplification of the 614 bp fragment of *Stx*1 gene (Shiga toxin 1) was negative in all of the tested 13 serogroups except one isolate belonging to serogroup O158 was positive for this gene. **Parreira and Gyles (2002)** determined the presence of *Stx* genes in avian pathogenic *E. coli* (APEC) in 52 out of 97 *E. coli.* One isolate carried *Stx*2 gene, 2 isolates carried both *Stx1* and *Stx2* genes and the remaining 49 isolates+ carried only *stx*1 gene.

Salmonellosis in poultry is an important area of study as it is not only affects the poultry industry but can also occur in humans by consumption of contaminated poultry meat and eggs (**Behravesh *et al*., 2014).** In the present study it was recorded that percentage of positive poultry farms reached 31% out of examined 100 broiler and layer farms showing signs of illness.

On the other hand lower prevalence of Salmonella isolates than the present work was recorded by (**Ozbey and Ertas, 2006**) who isolated Salmonella from chickens by a percentage of 12%, Also a study was conducted by (**Zhao *et al.,* 2016)** to investigate prevalence of Salmonella in free-range chickens in Shandong province, China, where it was found that (38/300, 12.7%) Salmonella isolates were recovered.

Whilst a higher prevalence was recorded by **Bai *et al*., (2015)** who isolated Salmonella from chicken in Henan China by an isolation rate of 45.2%, Furthermore in another study was published by **Hassan *et al*. (2016)** recorded that the presence of Salmonella isolates was in a higher percentage reached to 76%.

Prevalence rates of Salmonella isolates from poultry samples were different in various studies due to multiple factors, such as geographic and seasonal variation, variations in sampling procedures and sample size, animal management practices, hygienic conditions during production or due to differences in isolation methods used (**Naik *et al*., 2015).**

Results of **Marin *et al.* (2011),** using the same procedure we relied in the present investigation (all samples collected were isolated and positive samples were serotyped in accordance with Kauffman White Scheme), showed that all different types of samples collected were contaminated with Salmonella (prevalence ranged between 1.5% and 38.6%). The most prevalent were in decreasing order: S. Enteritidis (52.9%), S. Hadar (17.8%), S. Virchow (8.9%) and S. Ohio (5.4%). These results when compared to the present data that recorded the prevalence of Salmonella in examined poultry farms was 31%. They were serotyped into S. Enteritidis (29%), S. Newport (23%) S. Kentucky (19%) S. Typhimurium (16%) and S. Tamale had the lowest percentage with (13%), indicating predominance of S. Enteritidis.

This roughly similar to several studies conducted by **Mir *et al.* (2015)** who studied the occurrence and serotype diversity of Salmonella isolates in different species of poultry, found that Salmonella Enteritidis was the most predominant serotype, followed by Salmonella Typhimurium, Salmonella Virchow, Salmonella Gallinarum, Salmonella Reading and Salmonella Altona. And (**Zhao *et al.,* 2016**) who found that the most common serotype was S.

Enteritidis (81.6%), followed by S. Indiana (13.2%) and S. Typhimurium (5.3%).
On contrary there were different results recorded by **Santos *et al.* (2007)** who indicated that the most prevalent serotype in turkey isolates were serovar S. Kentucky (26%), followed by S. Senftenberg (19%), S. Muenster (17%), S. Mbandaka (10%), S. Javiana (7%), S. Hadar (5%), S. Heidelberg (5%), S. Agona (2%) and S. Infantis (2%). Also **Alambedji *et al.* (2006)** investigated the incidence of different serotypes of Salmonella in chicken and reported that the most prevalent were S. Kentuchy 30%, S. Muenster (13.3%), S. Brancaster (8.8%), S. Enteritidis and S. Hadar (6.6%).

Virulence of *Salmonella* can be measured by detecting the presence of virulence gene by the polymerase chain reaction (PCR) instead of doing animal inoculation or doing plasmid profiling **(Joseph *et al.,* 1999).** The virulence gene named *sefA* gene found in *Salmonella* Enteritidis, were observed with the extracted DNA of one standard strains of *Salmonella* Enteritidis and the extracted DNA of the positive *Salmonella* Enteritidis isolates while no amplification could be observed with the extracted DNA of other *Salmonella* serotypes.

From the previous results and studies in comparable to the findings of the present work, it was found that there an increasing in the rate of isolation of Salmonella from chicken due to the persistence of S. Enteritidis in the environment. This persistence was studied on a free-range breeding chicken farm which had been depopulated following identification of the organism in breeding birds. The site was sampled periodically for 26 months after depopulation and the organism was found to persist in litter, dried faeces and feed, Salmonella Enteritidis was also found in soil samples after 8 months and in faeces from wild mice and cats (**Davies and Breslin 2003)**

H9N2 virus was introduced for the third time in February 2003 and was isolated sporadically through 2006, primarily from chickens (**Banet-Noach *et al.,* 2007).** H9N2 virus was first reported in Egypt in November 2011; the isolated virus was closely related to viruses of the G1-like lineage isolated from neighboring countries, indicating possible epidemiological links **(El-Zoghby *et al*., 2012).**

In the present work, a virological investigation; at the same time of bacteriological investigation; was conducted on the same cloacal swabs collected from 100 commercial chicken farms from 7 governorates The swabs were collected from 78 broiler farms and 22 layer farms from diseased chicken showing respiratory and diarrheic manifestation.

A total 31 farms out of 100 were positive for LPAI H9N2 (31%), where 31% and 32% of the farms were positive for H9N2 in broilers and layers farms respectively. Nearly similar results were recorded by **Khalifa (2013),** where the LPAI (H9) virus was detected in 39 (%19.5) of examined chicken farms in 2012 - 2013.

Our results agree with the results study conducted by **Bano *et al.,* (2003),** who evaluated the pathogenic potential of low pathogenic AIV(H9N2) serotype in association with other organisms (IBV, Ornithobacterium rhinotracheale and Escherichia coli), which indicated that this low pathogenicity AIV (H9N2) isolate could produce severe infection depending on the type of secondary opportunistic pathogens present under field conditions. Likewise **Mosleh *et al.,* (2017),** who studied the effect of exposure time to Escherichia coli (O2) on the pathogenicity of H9N2 AIV in broiler chickens. Concluding that E. coli infection prior to, after or concurrently with H9N2 virus infection could exacerbate the adverse effects of the virus.

Conclusion:
It was concluded that co-infection can occur between bacteria and influenza virus, thereby causing a potential risk to susceptible hosts,

facilitating either opportunist bacterial invasion into the respiratory tract or exacerbation of the viral infection. Co-infection between viral and bacterial agents was recorded in examined poultry sector. Bacterial infection may be the main cause of the disease or the secondary infection with viral infection which lead to increase morbidity and mortality rates.

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