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Effect of *E. coli* Proteolytic enzymes on Virulence and Pathogencity of Avian Influenza Virus

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Abstract

In this study the effect of the *E. coli* proteolytic enzymes on the avian influenza virulence and pathogenicity was estimated. Results showed that *E. coli* prevalence was 67% from 100 tested poultry farms. Serotyping of the isolates revealed 13 different serogroups of *E. coli*. The virulence gene named *iss* was detected in 11 out of the 13 serotype using PCR. On the other hand the virulence gene named *eae* A gene (*intimin*) was detected in 5 serogroups of *E. coli* out of 13 serogroups. Another virulent gene named *Stx1* was tested by PCR and the result was positive in O158 and negative for 12 serogroups. 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 using real time PCR. AI virus H9N2 is low pathogenic and has no effect on tissue culture cells. In this study AI H9N2 was inoculated on MDCK with and without proteolytic enzyme extracted from *E. coli*. MDCK cells inoculated with low pathogenic H9N2 and the proteolytic enzyme showed CPE of sloughing and cell death compared to cells inoculated with low pathogenic H9N2 alone. In vivo study in SPF chickens, mortality of chickens inoculated with low pathogenic H9N2 and *E. coli* reached 80% while in chickens inoculated with *E. coli* only reached 30% in contrast chickens inoculated with low pathogenic H9N2 had no mortality. In conclusion the proteolytic enzymes of *E. coli* increases the virulence and pathogenicity of AI.

Keywords: *E. coli, proteolytic enzymes, H9N2, AI virus, pathogencity, E. coli virulence genes.*

Introduction

Virus–Bacteria associated infections have been reported to be one of the major causes of field problem in commercial farms.

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**).

E. coli is a normal inhabitant of the intestinal tract of birds, under the influence of predisposing factors, like inadequate and faulty ventilation, overcrowding, hunger, 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**).

Bacteria present in the respiratory tract are potential sources of proteases that could contribute to cleavage influenza virus in vivo (**Dalva *et al*., 2005**).

From 2003 to 2012, Highly Pathogenic Avian Influenza (HPAI), H5N1 subtype outbreak was reported in 61 countries in both domestic poultry and wild birds (**OIE Manual, 2012**). In mid-February 2006, H5N1 infection was reported in Egypt among domesticated poultry

in the most governorates resulting in sever losses for poultry industry (**Aly *et al*. 2006; Kilany, 2007).**

Widespread prevalence of Low Pathogenic Avian Influenza (LPAI) H9N2 subtype in the Middle East region and its detection in Egypt in quail in early summer 2011 added another risk factor to the Egyptian poultry industry in addition to highly pathogenic H5N1 subtype so this situation increases the need for further surveillance and investigation of H9N2 viruses in commercial and household chickens **(Arafa *et al*., 2012).**

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

In this study, we investigate the role of proteolytic enzymes produced by *E. coli* species on enhancement of low pathogenic Avian Influenza H9N2 pathogencity and virulence.

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 and 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

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 (4) (**Spackman *et al*., 2002**). Also thermal cycling conditions for gene-specific Probe and Primer sets for influenza virus H9 were recorded in table (5) **(Ben Shabat *et al*., 2010).**

Table (4). 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 (5). 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	

Cytopathic effect of H9N2 on cell culture in the presence of bacterial proteolytic enzymes

Method:

Confluent monolayer of Madin-Darby Canine Kidney cells (MDCK) were grown in 6-well tissue culture plate and the cells were checked under microscope. Growth medium was removed by pipette into a discard container. The cells were washed three times with 1 ml PBS then the washing solution was decanted into a discard container. Tryptic Soy Broth (TSB) was prepared using the following ingredients tryptone, soy, NaCl, dipotassium phosphate, glucose and 1 liter of distilled water, these ingredients were dissolved under gentle heat and then autoclaved for 15 minutes at 121°C. Virus/bacterial proteolytic enzymes mixture was prepared by cultured the bacterial isolates in TSB and centrifuged at 12.000g for 15 min at 4°C **(Dalva *et al.*, 2008)**. Then it was filtered through a Millipore 0.45 µm pore diameter syringe filter, the clarified supernatant was tested for proteolytic activity, mixing of 0.1 ml (100 µl) from clarified supernatant with 0.1 ml (100 µl) 10^6 EID50 H9N2 AIV and incubated at 25ºC for 60 minutes (1 hour), the inoculation of the cells as follow:

- **1st well:** negative control (Non-inoculated cells).
- **2nd well**: 200 µl of H9N2 AIV of 10^6 EID50.
- **3rd well**: 200 µl *E. coli* proteolytic enzymes.
- **4th well**: 200 µl H9N2 mixed with E-coli proteolytic enzymes.

The plates was gently rocked to coat the cell monolayer with the inoculum dilutions to avoid dry spots on the cell monolayer and incubated for 30 minutes for absorbance. 2 ml of maintenance media was added to each well. The plates was incubated in a 37ºC, 5% CO2 incubator for 48 hours and the inoculated cells were daily observed & cytopathic effect was recorded.

In vivo study

A total of 40 SPF chickens divided into 4 groups, each group contained 10 SPF chickens 3 weeks old. Bacteria inoculation in the bird was per OS and the rout of AI viruses inoculation in the bird was intra nasal**.** The challenge day of AI viruses was 7 days after inoculation of *E. coli* spp. The birds were observed for 14 days post challenge and clinical signs, mortality rates, postmortem lesions were recorded. Liver, spleen, ovary, cecum and cloacal swabs were examined for re-isolation and identification of *E. coli* isolates used in the experiment by PCR at the end of the experiment. The experiment groups and challenge doses were illustrated in table (6).

Table (6). The experiment groups and challenge doses.

Group	Bacteria challenge doses			AI viruses challenge doses		
	Type	Dose	Age	Type	Dose	Age
1	*E. coli* positive control	10^6 CFU/bird	28 days	-	-	35 days
2	*E. coli* co-infection with H9N2 infection			AI/H9N2	10^6 EID50/bird	
3	-	-		AI/H9N2 control	10^6 EID50/bird	
4	Control group	-		-	-	

Results and Discussion

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. Poor ventilation, dust, ammonia, other gases and other factors associated with, may act as predisposing factors, the interaction between different viruses and bacteria plays a very important role in respiratory infection in poultry, so the aim of this study is to throw spots up on virus–bacteria associated infections and excogitate the role of both of them.

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.

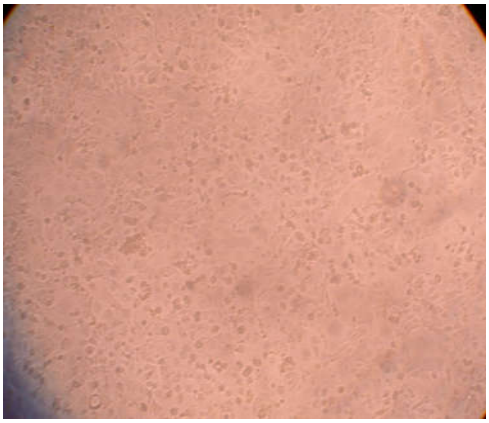
H9N2 virus was introduced for the third time in February 2003 and was isolated sporadically through 2006, primarily from chickens (**Banet-Noach *et al.,* 2007).** H9N2 virus was first reported in Egypt in November 2011; the isolated virus was closely related to viruses of the G1-like lineage isolated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

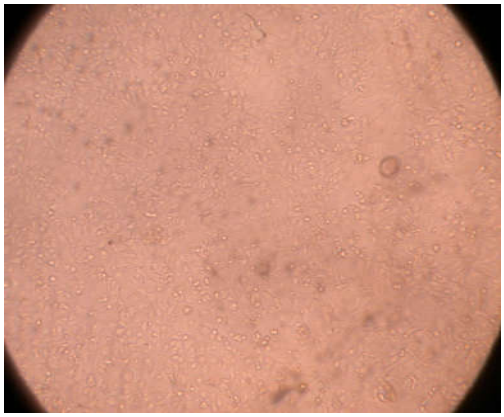
A total 31 farms out of 100 were positive for LPAI H9N2 (31%), where 31% and 32% of the farms were positive for H9N2 in broilers and layers farms respectively. Nearly similar results were recorded by **Khalifa (2013),** where the LPAI (H9) virus was detected in 39 (%19.5) of examined chicken farms in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

The cytopathic effect of LPA1 H9N2 on cell culture in the presence of bacterial proteolytic enzymes was studied on MDCK cells. Daily microscopic examination of infected cell showed cell round and vacuoles after 24 hours post infection as showed in fig. (1). Proteolytic enzymes of *E. coli* induced rounding and slight sloughing of the cell sheet (30-40%). While LPAI (H9N2) with the enzymes showed rounding and sloughing of 70% - 80% of cell sheet. At the same time AIV (H9N2) and negative control (non) inoculated cells had no cytopathic effect on the MDCK cell (confluent sheet). The obtained results were completely agreed with **Dalva *et al.,* (2008)** who studied the effect of bacterial protease from bacteria isolated during co-infection with influenza viruses in human on MDCK cells with the virus and they found corroborated that virus versus bacteria synergism could be able to potentiate respiratory infection which increasing damage to infected hosts. Also this results were supported by the study carried out by **Marcus *et al.,* (2011)** who treated the un-cleaved influenza virus with 44 bacterial supernatants on MDCK cell.

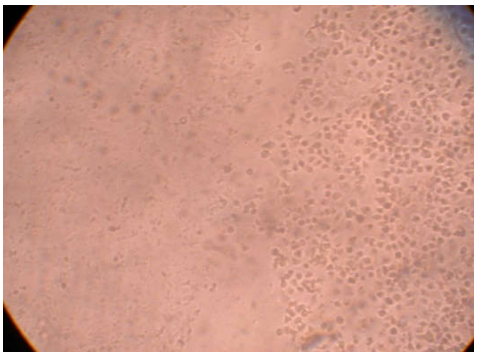
Fig. (1): Cytopathic effect of the Avian Influenza virus (H9N2) with and without proteolytic enzymes on MDCK cells (20X).



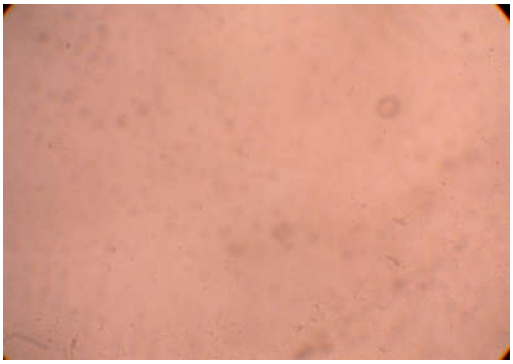
No. 1



No. 2



No. 3



No. 4

The pervious photos illustrated the following results:

Photo No. 1: Negative control (Non-inoculated cells) (**Note: confluent sheet**).
Photo No. 2: AIV H9N2 (**Note: confluent sheet**).
Photo No. 3: *E. coli* proteolytic enzymes **(Note: rounding and slight sloughing of the cell sheet 30-40%).**
Photo No. 4: AIV H9N2 mixed with E-coli proteolytic enzymes **(Note: rounding and slight sloughing of the cell sheet 70-80%).**

The analysis of obtained results revealed that bacterial isolate was toxic to MDCK cells due to bacterial proteolytic enzymes.

To study the co-infection of AI virus1 in vivo with proteolytic bacteria an experiment was done in SPF chicks after **Gast *et al.*, (2004)**.

In our vivo study, the results were as follows; in group inoculated with *E. coli* only, the death started in 5thdpc (3 birds died, 2 birds were sick) and in the following day till the end of experiment, only 3 birds died and 2 birds were sick showing clear signs of *E. coli* infection. The cumulative specific mortality (3/10) reaching 30%. In group inoculated by *E. coli* and H9N2, the challenged birds were showing increasing in the clinical signs, while the mortality started from 4thdpc (3 birds died, 3 birds were sick) and in the following day 5 birds died, 3 birds were sick. The cumulative specific mortality (8/10) reaching 80% and in group inoculated with H9N2 only or non-inoculated group, there is no death birds showing no clinical signs.

At the end of the vivo study, liver, spleen, ovary, cecum and cloacal swabs were exam-

ined for re-isolation and identification of *E. coli* isolates used in the experiment by PCR. Overall percentage of re-isolation of *E. coli* from different organs of SPF chicken, necropsied on day 6, 8 and 10th day post-viral infection was shown in table (7) .

Table (7). Percentage of re-isolation of *E. coli* from organs of SPF chicken.

Organs	*E. coli* isolation			
	G1	G2	G3	G4
Liver	60	75	0	0
Lung	50	77	0	0
Spleen	37.5	75	0	0
Cecum	37.5	65	0	0

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.

Results of viral shedding in experimental infection revealed that mixed infection associated with a higher incidence of LPAI H9N2 shedding comparing to single LPAI H9N2 infection. However within mixed infection there was marked differences in viral shedding, where group infected with LPAI H9N2 then *E. coli* revealed a higher early and prolonged shedding comparable to other infected group table (8).

Table (8). Mean of virus shedding at 6th, 8th and 10th post viral inoculation.

Group	6 day	8 day	10 day
LPAI H9N2	6.986	0.419	0.085
LPAI H9N2 and *E. coli*	14.928	10.730	7.480

Conclusion:

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. Presence of proteolytic bacteria with influenza virus infection had bad effect on tissue culture and in vivo the bacteria accelerate the viral infection. The bacterial infection increased morbidity and mortality rates. The proteolytic enzymes of *E. coli* increases the virulence ad pathogenicity of LPAI H9N2

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