Advanced Studies on Diagnosis of Single *M. gallisepticum* Infection and Combined with *E. coli* in Chickens

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ABSTRACT

Mycoplasma gallisepticum (*MG*) is a persistent, highly transmissible chicken pathogen. It predisposes the birds to other infections such as *Escherichia coli* yielding significant losses in performance and associated economics to all sectors of the poultry industry. This study aims to evaluate the role of *MG* and *E. coli* virulence genes in occurrence of respiratory diseases in chickens using advanced techniques. It was noted that *MG* and/or *E. coli* were accused of CRD in naturally infected chickens. PPLO medium, Digitonin test and biochemical reactions were used for characterization of Mycoplasma while *E. coli* was identified through isolation on MacConkey`s and EMB agar media, biochemical tests and rapid API 20E. Serotyping of E. *coli* isolates showed that O78 was the most predominant serotype (33.33%) followed by O27 (22.22%), other serotypes as O86a, O115, O29, O125 and O146 were also included. Conventional PCR on *MG* isolates showed the presence of 16S rRNA and *mgc*2 genes with characteristic bands at 330 and 824 bp, respectively. Uniplex PCR on APEC showed that *iuc*D virulence gene was detected in O27, O78, and O86a but not in O115, while *fim*C was detected in the four mentioned serotypes with amplicon sizes of 714 and 497 bp, respectively. Multiplex PCR on APEC showed that O27 had the four virulence genes (*iuc*D, *irp*2, *iss* and *tsh*) with characteristic bands at 714, 413, 309 and 824 bp, respectively, O78 and O86a had two virulence genes (*iuc*D and *iss*) while O115 had *iss* virulence gene only. The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed has made real-time PCR technology an appealing alternative to conventional PCR and it could detect and quantify *iuc*D virulence gene of *E. coli* O115 which was negative when detected by conventional PCR.

INTRODUCTION

M. gallisepticum infection is commonly designated as chronic respiratory disease (CRD) of chickens. It affects the entire respiratory tract, particularly the air sacs, where it is localized. It is mainly characterized by respiratory rales, coughing and nasal discharge. Clinical manifestations are usually slow to develop and the disease has a long course. There is no doubt that presence of other pathogens (such as *Escherichia coli*), increased environmental ammonia, high levels of dust, poor nutrition, immunosuppressive agents and social stresses associated with intensive management play an important role in *MG* infection ***(1, 2)***. In fact, one important feature of *MG* infection is that it can persist in the bird during all live, even in the presence of the humoral antibodies ***(3)***. *MG* cytadhesin membrane surface protein (*mgc*2) is an important virulence factor as it provides the pathogen for resisting host defenses, selective antibiotic therapy and establishing chronic infection ***(4)***.

Avian pathogenic *Escherichia coli* (APEC) are responsible for extraintestinal diseases, called colibacillosis, which is considered as one of the principal causes of morbidity and mortality in poultry with high economic losses. It induces a variety of diseases like airsacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis and yolk sac infections. The most common form is infection of the respiratory tract frequently followed by septicemiae ***(5)***. The most important virulence genes of APEC are *F1*-fimbriae (*fim*C), which allow the bacteria to adhere to the epithelial cells of the respiratory organs, a temperature-sensitive hemagglutinin (*tsh*) that responsible for colonization, multiplication and entering the blood stream resulting in septicaemiae, massive lesions in multiple internal organs and sudden death of the bird, iron repressible protein (irp_2) that

responsible for the ability of the bacteria to acquire iron, aerobactin (*iuc*D) which is iron uptake hydroxamate siderophore and increased serum survival (*iss*) protein which is important for the resistance to the bactericidal effects of serum and enables APEC to multiply quickly in their hosts *(6)*.

The undisputed success of detection assays based on the polymerase chain reaction (PCR) has been largely due to its rapidity, sensitivity and specificity in comparison to many conventional diagnostic tools *(7)*. Fluorescent monitoring of DNA amplification is the basis of real-time PCR, from which target DNA concentration can be determined from the fractional cycle at which a threshold amount of amplicon DNA is produced. Absolute quantification can be achieved using a standard curve constructed by amplifying known amounts of target DNA *(8)*.

Therefore, the aim of this work is to apply a comparison between the traditional methods, conventional PCR and real time PCR in diagnosis of single *MG* infection and combined with *E. coli* in respiratory manifested chickens, in addition, to evaluate the role of *MG* and *E. coli* virulence genes in occurrence of respiratory diseases in chickens.

MATERIAL AND METHODS

I.Chickens and sampling: one hundred and two chickens with history of respiratory manifestation were obtained from El-sharkia and El-dakahlia Governorates. The chickens were separated according to breed (Cub, Sasso and Hubbard), age (1-21 days, 22-33 days and 34 days till marketing age) and sampling season was (summer, autumn, winter and spring). Two hundreds and four samples (102 lungs and 102 tracheas) were collected from the diseased chickens which were either alive or freshly died.

II.Isolation and identification of the causative agents

A) *Mycoplasma gallisepticum*

PPLO agar medium was used for the specific isolation of Mycoplasma *(9)*, which was differentiated from Acholeplasma using Digitonin test *(10)*. Biochemical identification of Mycoplasma depended mainly on glucose fermentation and arginine deamination tests *(11)*.

B) *Escherichia coli*

Identification of *E. coli* was done by isolation of the microorganism on MacConkey's agar plates, streaking of the presumptive growths that picked from each plate on EMB. Typical colonies of *E. coli* sulyected for were IMVC and TSI biochemical tests *(12)*.

Biotyping of *E. coli* using rapid API 20E kits (Biomerieux, France)

Rapid API 20E (RapiD 20E ™) was used for identification *E. coli* in four hours. Contents of the kit are 25 rapid 20E strips, 25 incubation boxes, 25 result sheets, 1 package insert in addition to API® suspension medium (2 ml NaCl 0.85%), IND (indole) or JAMES reagent and VP1 + VP2 (acetoin production) reagents.

O-serogrouping of *E. coli* isolates *(13)*

Single colony of *E. coli* isolates was picked, suspended in 3 ml physiological saline, boiled for an hour, centrifuged at 900 xg for 20 minutes then the supernatant was discarded while the precipitate was resuspended in 0.5 ml physiological saline and used as antigenic suspension. On a clean partitioned glass slide with a glass pencil, one drop of each polyvalent sera and physiological saline were placed then a drop of antigenic suspension was added. The reagents were mixed by titling the glass slide for 1 minute to observe the agglutination pattern, in positive result, apply the same steps with each monovalent serum consisting of the polyvalent one.

III. PCR for identification of *M. gallisepticum* and *E. coli*:

1. Conventional PCR

A) Regarding *M. gallisepticum*

1. Preparation of crude cell lysate *(14)*

Two milliliters of *MG* aliquot cultures consisting of approximately 10^9 CFU were pelleted by centrifugation at 12.000 xg for 5 minutes, washed twice with PBS, and resuspended with 25 ml of PBS. The pellet was

then boiled for 10 minutes, quickly placed on ice for 5 minutes and centrifuged. The supernatant containing DNA was collected and stored at 4°C for further PCR application.

2. Primer selection (Sigma)

a) Primers for16S rRNA gene (15)

Forward: 5'-TAACTATCGCATGAGAATAAC-3'
Reverse:5'-GTTACTTATTCAAATGGTACAG-3'

b) Primers for *mgc*2 gene (16)

Forward: 5`-GCTTTGTGTTCTCGGGTGCTA-3`
Reverse: 5`-CGGTGGAAAACCAGCTCTTG-3`

3. PCR amplification and cycling protocol (Fermentas)

DNA samples were amplified in a total of 50 µl of the following reaction mixture: 25µl DreamTaq ™ Green Master Mix (2X), 1µl of each primers, 5µl template DNA and completed to 50 µl by water, nuclease-free. PCR cycling program was performed in thermal cycler (PTC-100 ™ programmable thermal cycler, Peltier-Effect cycling, MJ, RESEARCH, INC., UK) as in Table 1.

Table 1: PCR cycling program for *M. gallisepticum* 16S rRNA and *mgc*2 genes.

Amplified DNA	Initial denaturation	Actual cycles °C/second	Final extension	Amplified product size (bp)
M. gallisepticum 16S rRNA (14)	90°C for 1 minute	40 cycles of : Denaturation: 95/15 Annealing: 60/20 Extension: 75/15	75°C for 5 minutes	330
M. gallisepticum *mgc*2 (15)	94°C for 3 minutes	40 cycles of : Denaturation: 94/20 Annealing: 55/40 Extension: 72/60	72°C for 5 minutes	824

B) Regarding *E. coli*

1. Preparation of crude cell lysate *(17)*

E. coli isolates were grown in nutrient broth at 37°C overnight in shaking incubator. The enriched medium in a 1.5 ml eppendorf tube was centrifugated at 1500 rpm for 10 minutes then the supernatant was discarded. Tris EDTA (TE) buffer was added to the pellet and the tube was freezed at -80°C for 10 minutes then boiled for 5 minutes. The tube was centrifugated at 1500 rpm for 10 minutes and supernatant was taken as a source of DNA.

2. Primer selection (Sigma)

Oligonucleotide primers for *iuc*D and *fim*C virulence genes of APEC were used in a uniplex PCR *(5)* while *iuc*D, *iss*, *irp*2 and *tsh* virulence genes were used in a multiplex PCR *(18)* as shown in Table 2.

Table 2: Oligonucleotide primers for virulence associated genes of APEC

Virulence gene	Primer	Product size(bp)
*iuc*D	5`ACAAAAAGTTCTATCGCTTCC3` 5` CCTGATCCAGATGATGCTC3`	714
*fim*C	5` GGGTAGAAAATGCCGATGGTG 3` 5` CGTCATTTTGGGGGTAAGTGC 3`	497
iss	5` ATCACATAGGATTCTGCCG 3` 5` CAGCGGAGTATAGATGCCA 3`	309
*irp*2	5` AAGGATTCGCTGTTACCGGAC 3` 5` AACTCCTGATACAGGTGGC 3`	413
tsh	5` ACTATTCTCTGCAGGAAGTC 3` 5` CTTCCGATGTTCTGAACGT 3`	824

3. PCR amplification and cycling protocol (Fermentas)

DNA samples were amplified in a total of 50 µl of the following reaction mixture: 25µl DreamTaq ™ Green Master Mix (2X), 0.2µl of each primers, 5µl template DNA and completed to 50 µl by water, nuclease-free. PCR cycling program was performed for *iuc*D and *fim*C virulence genes of APEC in a uniplex PCR in thermal cycler (PTC-100™ programmable thermal cycler Peltier-Effect cycling, MJ, RESEARCH, INC., UK) as following *(5):* initial denaturation at 94°C for 5 minutes then 35 cycles consisting of (denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds) for *iuc*D virulence gene while for *fim*C gene: initial denaturation at 94°C for 5 minutes then 30 cycles consisting of (denaturation at 94°C for 60 seconds, annealing at 59°C for 60 seconds and extension at 72°C for 60 seconds) followed by final extension at 72°C for 5 minutes.

Multiplex PCR cycling program was performed as following *(18)*: initial denaturation at 94°C for 3 minutes then 25 cycles consisting of (denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 68°C for 3 minute) followed by final extension at 72°C for 10 minutes.

Detection of PCR products *(19)*

Eight µl of each amplicon were loaded on 1.5% agarose gel containing 0.5 µg of ethidium bromide. A 100 bp DNA ladder was used as a molecular weight standard (Pharmacia). The samples were electrophoresed at 90 V for 90 minutes on a mini horizontal electrophoresis unit (BIO-RAD, USA); the gel was visualized under UV transilluminator (Spectroylyne Model TR-312 A) and photographed.

2. Real time PCR

I. Oligonucleotide primers (Sigma)

For *mgc*2 genome of *M. gallisepticum (19)*

Forward:5`-AACACCAGAGGCGAAGGCGAGG-3`

Reverse:5`-ACGGATTTGCAACTGTTTGTATTGG-3`

For *iuc*D virulence gene of *E. coli (5)*

Forward: 5`ACAAAAAGTTCTATCGCTTCC3`

Reverse: 5` CCTGATCCAGATGATGCTC3

II. Real time PCR amplification and cycling protocol (KAPA BIOSYSTEM): It was applied in the following steps:

1.QPCR reaction setup: DNA samples were amplified in a total of 25 µl of the following reaction mixture: 12.5 µl SYPR® Green (1X), 1 µl of each primer, 2 µl template DNA and 8.5 µl water, nuclease-free. The samples were transferred to each well of a PCR plate.

2. Running of the QPCR: It was applied in 40 cycles according to the following program: enzyme activation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds then annealing and extension at 60°C for 30 seconds.

3.Analysis of the results using the standard curve method *(20):*

The standard curve method is based on using a DNA sample of known concentration to construct a standard curve. Once the standard curve has been generated, it can then be used as a reference standard for the extrapolation of quantitative information regarding the unknown concentration.

RESULTS

I.Prevalence of *M. gallisepticum*, *E. coli* and combined infection in respiratory manifested broiler chickens

Out of 204 samples collected from respiratory manifested broiler chickens, 28 *M. gallisepticum*, 100 *E. coli* and 18 combined isolates were detected with percentage of 13.7%, 49% and 8.8% respectively. The prevalence rates of *M. gallisepticum*, *E. coli* and combined infection were 9.8%, 55.9% and 9.8% in lung while they were 17.6%, 42.2% and 7.8% in trachea respectively. Regarding to different breeds, the prevalence of *E. coli* was 62.5% in cub breed while no *M. gallisepticum* nor combined infection were recovered from this breed at all. However, the prevalence rates of *M. gallisepticum*, *E. coli*, and combined infection were 12.5%, 66.67% and 20.8% in Sasso breed respectively while they were 27.4%, 26.2% and 9.5% in Hubbard breed respectively. Regarding to different ages, the prevalence rates were zero%, 47.56% and 2.4% in 1-21 days old age,

33.87%, 30.6% and 11.76% in 22-33 days old age and 11.67%, 70% and 15% from 34 days old age till marketing for *M. gallisepticum, E. coli* and combined infection respectively. Regarding to different seasons, the prevalence rates were 6.5%, 76.1% and 2.2% in summer, 14.1%, 32.8% and 1.6% in autumn, 26.3%, 31.6% and 36.9% in winter and 10.7%, 57.1% and 3.6% in spring for *M. gallisepticum, E. coli* and combined infection respectively. Lastly, the prevalence rates of *M. gallisepticum, E. coli* and combined infection were 4.7%, 63.3% and 3.1% respectively in El-sharkia Governorate, while they were 28.9%, 25% and 18.4% respectively in El-dakahlya Governorate.

II. Isolation and identification of *M. gallisepticum*

1) Traditional methods

On PPLO agar medium, the microorganism appeared as fried egg when examined by stereomicroscope (10-40X) which was differentiated from Acholeplasma using digitonin test. Mycoplasma was sensitive to digitonin and showed marked inhibition zone surrounding the disc. Biochemical characterization of the isolates revealed that Mycoplasma was positive for glucose fermentation test (yellow or orange yellow colour) and negative for arginine deamination test (pink colour).

2.PCR for identification of Mycoplasma isolates

a- Conventional PCR

PCR results showed that 16S rRNA and *mgc*2 genes were detected in all examined samples and gave characteristic bands at 330 and 824 bp respectively (Photos 1, 2).

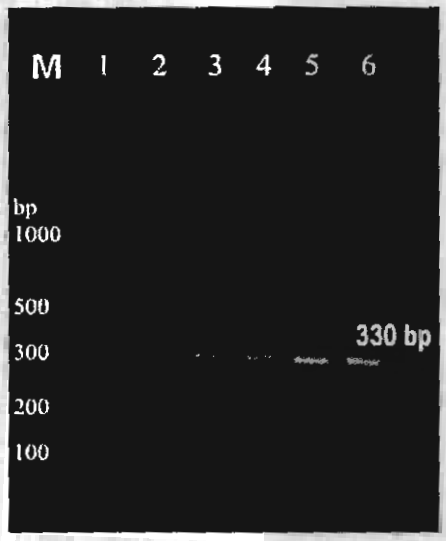


Photo 1. Agarose gel electrophoresis of *MG* DNA product (16S rRNA gene).
M: 100 bp DNA ladder "Marker".
Lane 1-6: *MG* 16S rRNA gene.

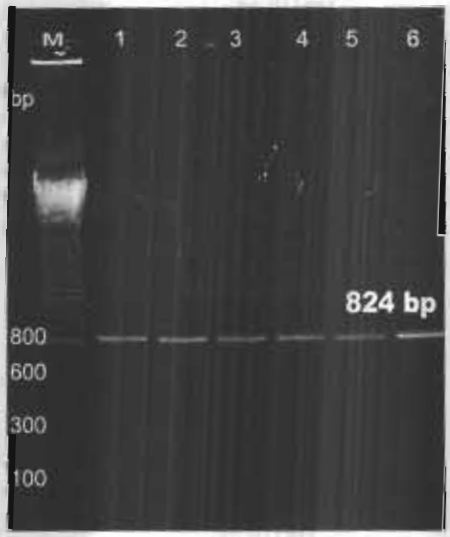


Photo 2. Agarose gel electrophoresis of *MG* DNA product (*mgc*2 gene).
M: 100 bp DNA ladder "Marker".
Lane 1-6: *MG mgc*2 gene.

b.Real time PCR: *M. gallisepticum* field isolates that were identified by the conventional PCR were confirmed by the quantitative real time PCR. The results of RT-PCR are shown in Figures 1, 2 and Table 3.

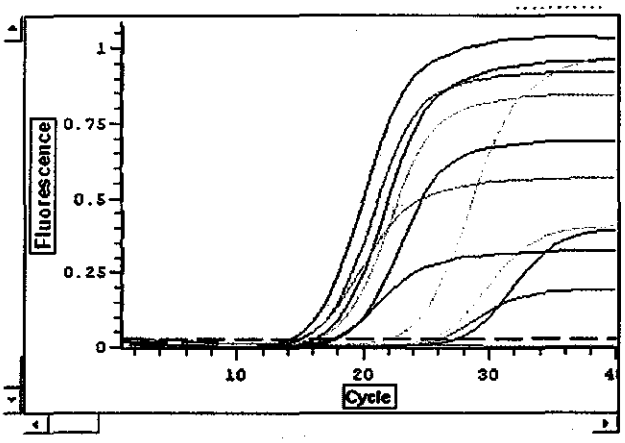


Fig. 1. Amplification curves of RT-PCR for detection of *MG*.

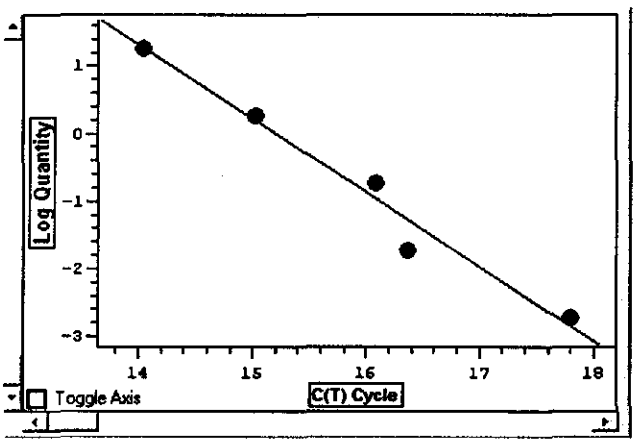


Fig. 2. Linear regression of cycle threshold versus DNA concentration for serial logarithimic dilutions of *MG* isolates.

Table 3. Text report of *MG* isolates RT- PCR and calculations of DNA concentration per sample.

Wells

Well	Dye	Content	Description	Efficiency	C(t)	ng
A1	SBG1	Standard		92.51%	14.04	18.5
A2	SBG1	Standard		94.97%	15.03	1.85
A3	SBG1	Standard		107.72%	16.08	0.185
A4	SBG1	Standard		99.85%	16.36	0.0185
A5	SBG1	Standard		115.82%	17.80	0.00185
A6	SBG1	Blank		N/A	N/A	N/A
B1	SBG1	Sample	sample 1 MG field	87.85%	25.30	8.141e-012
B2	SBG1	Sample	sample 2 MG field	77.95%	27.28	5.627e-014
B3	SBG1	Sample	sample 3 MG field	58.95%	21.76	6.331e-008
B4	SBG1	Sample	sample 4 MG field	92.10%	14.87	2.254
B5	SBG1	Sample	sample 5 MG field	94.41%	17.42	0.003578
B6	SBG1	Sample	sample 6 MG field	80.98%	26.06	1.196e-012

III. Isolation and identification of *E. coli*

1.Traditional methods A- Colonial appearance: *E. coli* appeared as pink colonies (lactose fermenter) on MacConkey's agar medium and gave a characteristic greenish metallic sheen on EMB agar medium.

B. Microscopical examination: it appeared as G-ve, medium sized bacilli, non spore forming and arranged single, pairs and in groups when stained with Gram's stain and examined under OIL (100X).

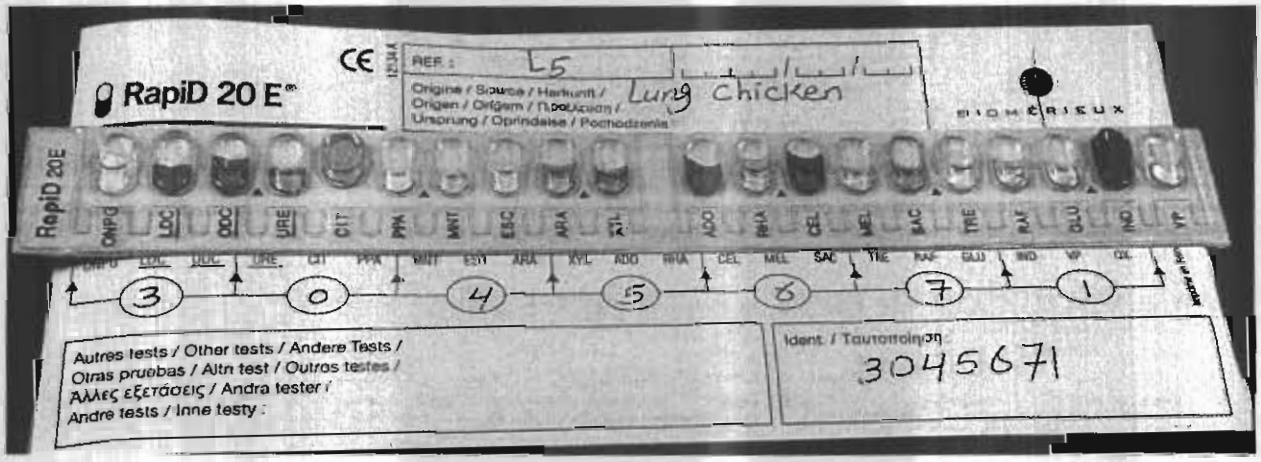
C. Biochemical reactions: the examined isolates were indole positive (red ring), methyl red positive (red colour), voges proskauer negative (copper like colour), citrate negative (green colour) and TSI yellow butt/yellow slant H_2S^{-ve}+gas production.

D. Results of rapid API 20 E kits: it revealed different seven digit profile numbers which were identified through rapid API 20 E analytical profile index (Ref. 20 790) as shown in Table 4 and Photo 3.

Table 4. Results of rapid API 20E.

Seven digit Profile No.	Identification (id %)
7045251	*Escherichia coli* 1 (99.9)
3045671	*Escherichia coli* 1 (99.6)
3045271	*Escherichia coli* 1 (99.5)
3047351	*Escherichia coli* 1 (99.4)
7045271	*Escherichia coli* 1 (99.4)
7045671	*Escherichia coli* 1 (98)
5045271	*Escherichia coli* 1 (95.4)
3041251	*Escherichia coli* 1 (73.9)
	Serratia odorifera 2 (25)

id % (Identification percentage):
99.9% or more: Excellent identification.
99% or more: Very good identification,
90% or more: Good identification.
80% or more: Acceptable identification.



1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2
ONPG	LDC	ODC	URE	CIT	PPA	MNT	ESC	ARA	XYL	ADO	RHA	CEL	MEL	SAC	TRE	RAF	GLU	IND	VP
+	+	-	-	-	-	-	-	+	+	-	+	-	+	+	+	+	+	+	-
	3			0			4			5			6			7		1	

Photo 3: Biochemical identification of *E. coli* field isolate using API 20E kits showing very good *E. coli* identification (id %: 99.6).

E. Serotyping of *E. coli* field isolates: the results showed that O78 was the most predominant serotype with a percentage of 33.33% followed by O27 (22.22%), while the remained isolates were serotyped as O86a, O115, O29, O125 and O146 one isolate for each serotype while 3 isolates were untypable.

F. PCR for detection and identification of some virulence genes in different APEC serotypes

1. Uniplex PCR for detection of *iuc*D and *fim*C virulence genes in different APEC serotypes

The recorded results revealed that *iuc*D virulence gene was detected in O27, O78, and O86a but not in O115 field isolates giving an amplicon size of 714 bp while *fim*C virulence gene was detected in all examined isolates giving an amplicon size of 497 bp (Photos 4, 5).

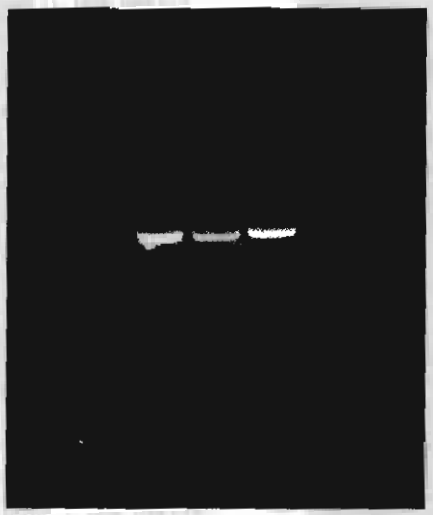


Photo 4. Agarose gel electrophoresis of *E. coli* DNA product (*iuc*D gene).
M: 100 bp DNA ladder "Marker"
Lane 1: *E. coli* O 27 field isolate
Lane 2: *E. coli* O 78 field isolate
Lane 3: *E. coli* O 86a field isolate
Lane 4: *E. coli* O 115 field isolate (-ve for *iuc*D virulence gene

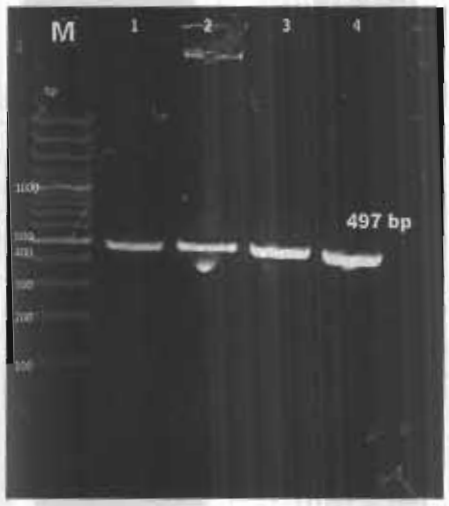


Photo 5. Agarose gel electrophoresis of *E. coli* DNA product (*fim*C gene).
M: 100 bp DNA ladder "Marker"
Lane 1: *E. coli* O 27 field isolate
Lane 2: *E. coli* O 78 field isolate
Lane 3: *E. coli* O 86a field isolate
Lane 4: *E. coli* O 115 field isolate

2. Multiplex PCR for detection of the most important virulence genes in some APEC serovars field isolates

Mutiplex PCR results showed that O27 serogroup had the four virulence genes (*iuc*D, *irp*2, *iss* and *tsh*), O78 and O86a serogroups had two virulence genes (*iuc*D and *iss*) while O115 serogroup had iss virulence gene only. The PCR products of the obtained virulence genes gave characteristic bands at 714, 413, 309 and 824 bp for *iuc*D, *irp*2, *iss* and *tsh* virulence genes respectively (Photo 6).

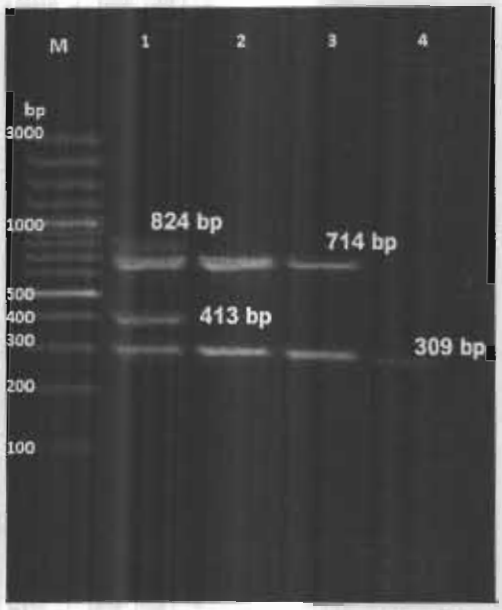


Photo 6: Agarose gel electrophoresis of *E. coli* DNA product virulence genes in a multiplex PCR.

M: 100 bp DNA ladder "Marker".
Lane (1): *E. coli* O 27 field isolate had four virulence genes (*tsh*, *iuc*D, irp_2 and *iss*).
Lane (2): *E. coli* O 78 field isolate had both *iuc*D and *iss* virulence genes.
Lane (3): *E. coli* O 86a field isolate had both *iuc*D and *iss* virulence genes.
Lane (4): *E. coli* O 115 field isolate had *iss* virulence gene only.

3.RT-PCR for quantitative detection of *iuc*D in different serovars of APEC (O27, O78, O86a and O115) (Table 5 and Figures 3, 4).

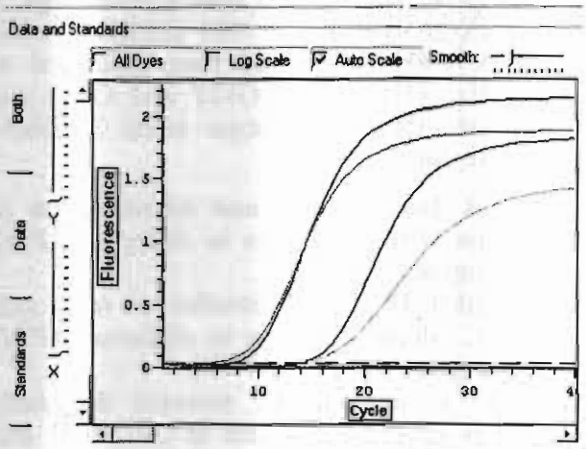


Fig. 3.Amplification curves of real time PCR for detection of *iuc*D in different serovars of APEC field isolates.

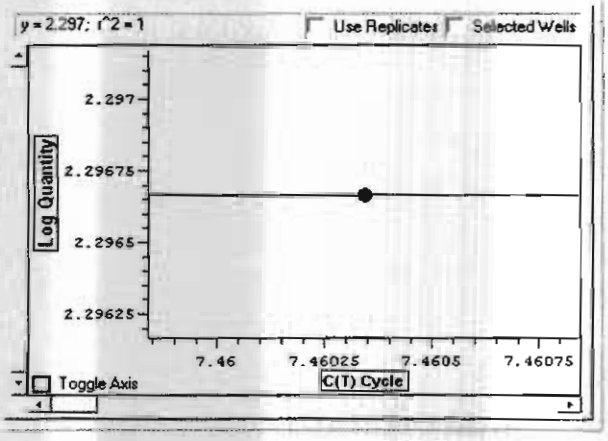


Fig. 4. Linear regression of cycle threshold versus DNA concentration for the standard APEC O27.

Table 5. Text report of different serotypes of APEC field isolates real time PCR and calculations of DNA concentration per sample.

Wells

Well	Dye	Content	Description	Efficiency	C(t)	ng
A1	SBG1	Standard	027	127.48%	7.46	198
A2	SBG1	Sample	078	42.50%	5.12	18.7
A3	SBG1	Sample	086	128.11%	13.97	6.02
A4	SBG1	Sample	0115	77.32%	13.73	1
A5	SBG1	Blank		N/A	N/A	N/A

DISCUSSION

Mycoplasma gallisepticum infection is commonly known as chronic respiratory disease in chickens. It predisposes the birds to other infections such as *E. coli* through the inhibition of immune functions causing aggravation of the disease leading to further economic losses ***(21)***. Concerning to the prevalence of *M. gallisepticum* and/or *E. coli* in respiratory organs of respiratory manifested chickens, it was found that *E. coli* was highly detected with a percentage of 49% ***(22)*** followed by *MG* (13.7%) ***(23, 34)*** and lastly combined infection (8.8%) ***(3)***.

Regarding to the prevalence of *M. gallisepticum* and/or *E. coli* in different breeds, ages and seasons of respiratory manifested chickens collected from El-sharkia and El-dakahlia Governorates , it was recorded that higher prevalence of *MG* was detected in Hubbard breed (26.2%), while *E. coli* and combined infection were detected mostly in Sasso breed with percentage of 66.67% and 20.8% respectively. These findings were resultant of our work and there is no similar work has been reported before. Prevalence of single and combined *MG* infection was detected in middle age (33.87% and 15% respectively); while *E. coli* was highly recorded in old age (70%) ***(25, 26)***. The spread of single and combined *MG* infection was detected in winter with percentages of 26.3% and 36.9% respectively ***(27)***, while *E. coli* was highly recorded in summer season (76.1%) ***(28)***. Finally, the results showed higher incidence of single and combined *MG* infection in El-dakahlia Governorate (28.9% and 18.4% respectively), while *E. coli* was highly recorded in El-sharkia Governorate (63.3%) ***(22)***. From the previously listed findings, it was noted that *MG* and/or *E. coli* infections were accused of CRD in naturally infected chickens as clear signs and post mortem lesions of the disease appeared obviously on the bird in all cases.

Results of traditional methods for isolation and identification of Mycoplasma (isolation on PPLO agar medium, digitonin test and biochemical characterization of the isolates) ***(9, 14, 29)*** and *E. coli* (isolation on MacConkey's and eosine methylene blue agar media, microscopical examination and biochemical tests) were reported ***(12, 30)***.

Rapid API 20 E revealed different seven digit profile numbers and the identification percentages were 99.9%, 99.6%, 99.5%, 99.4%, 98% and 95.4%. It was found that id % for an isolate was 73.9% *E. coli* and 25% *Serratia odorifera* indicating simple similarities in some biochemical reactions of such isolates but the final identification was in favor of *E. coli* ***(31)***.

Serotyping of 18 *E. coli* field isolates showed that O78 was the most predominant serotype (33.33%) followed by O27 (22.22%) while the remained isolates were serotyped as O86a, O115, O29, O125 and O146 one isolate for each serotype while 3 isolates were untypable ***(13, 32, 33)***.

Conventional PCR results proved that all examined *MG* isolates had 16S rRNA and *mgc2* genes which gave a characteristic bands at 330 and 824 bp when visualized under UV transilluminator ***(15, 16)***.

Uniplex PCR on O27, O78, O86a and O115 APEC field isolates revealed that *iuc*D virulence gene was detected in O27, O78, and O86a not O115 giving a product size of 714 bp while *fim*C virulence gene was detected in all examined isolates giving an amplicon size of 497 bp ***(5)***.

Multiplex PCR on four APEC field isolates (O27, O78, O86a and O115) showed that O27 serogroup had the four virulence genes (*iuc*D, *irp2*, *iss* and *tsh*), O78 and O86a serogroups had two virulence genes (*iuc*D and *iss*) while O115 serogroup had *iss* virulence gene only ***(13)***. PCR products of *iuc*D, *irp2*, *iss* and *tsh* virulence genes gave characteristic bands at 714, 413, 309 and 824 bp respectively ***(17, 18)***.

M. gallisepticum field isolates that were identified by the conventional PCR were confirmed by the quantitative real time PCR and the results revealed that all tested *MG* isolates were positive for *mgc2* gene which were detected through amplification curves and calculations of DNA concentration per sample ***(34)***.

Quantitative real time PCR proved that APEC field isolates of serogroups O27, O78, O86a and O115 had *iuc*D virulence gene, so, it can detect and quantify *iuc*D virulence gene of *E. coli* O115 which was negative when detected by conventional PCR (faint product of 1ng) indicating that real time PCR is sensitive, specific and could detect DNA with very low concentration *(35)*. These results ensured that *iuc*D gene act as an important factor in pathogenesis and virulence APEC *(5)*.

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الملخـــص العربـــي

دراسات متقدمة على تشخيص العدوى الأحادية بالميكوبلازما جاليسيبتكم والمقترنة بالميكروب القولوني في الدواجن

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تعد الميكوبلازما جاليسيبتكم من أكثر الميكروبات المتنقلة بين الدجاج إلى حد كبير كما انه يهيأ الطائر لعدوى أخرى مثل الايكولاى مما يترتب عليه خسائر كبيرة في الأداء والاقتصاد لجميع قطاعات صناعة الدواجن. استهدفت الدراسة تقييم دور جينات الضراوة لـ الميكوبلازما جاليسيبتكم و الايكولاى في حدوث الأمراض التنفسية في الدواجن باستخدام التقنيات المتقدمة. أوضحت الدراسة أن العدوى الأحادية بالميكوبلازما جاليسيبتكم والمختلطة بالايكولاى كلاهما يلعب دور هام في حدوث أمراض الجهاز التنفسي المزمنة في الدواجن. تم عزل وتصنيف الميكوبلازما جاليسيبتكم بالطرق المختلفة كالعزل على الأوساط البيئية, عمل اختبار الديجيتونن للتفرقة بين جنسي الميكوبلازما والاكوليبلازما, عمل الاختبارات البيوكيميائية على العترات المعزولة وأخيراً تم التصنيف النهائي للميكروب كميكوبلازما جاليسيبتكم بعد إجراء اختباري تفاعل إنزيم البلمرة المتسلسل و تفاعل إنزيم البلمرة المتسلسل حقيقي الوقت على العترات المعزولة. أيضاً تم عزل وتصنيف ميكروب الايكولاى بالطرق المختلفة كالعزل على الأوساط البيئية المختلفة وعمل الاختبارات البيوكيميائية واختبار ملف فهرس تحليلي ٢٠ السريع لتصنيف الميكروبات المعوية خلال أربع ساعات فقط وعمل اختبار السيرولوجي على العترات المعزولة. أوضحت النتائج أن العترة O78 كانت الأكثر شيوعا بنسبة ٣٣,٣٣٪ يليها O27 بنسبة ٢٢,٢٢٪ وقد ظهرت أيضاً أنماطاً أخرى مثل O86a, O115, O29, O125, O146. تم إجراء تفاعل إنزيم البلمرة المتسلسل للكشف عن الجينين 16S rRNA ،*mgc*2 في عترات الميكوبلازما الحقلية والمعزولة من الدجاج المصاب بأعراض تنفسية وأوضحت النتائج أن العترات الحقلية جميعها تحوى الجينين 16S rRNA ،*mgc*2 واللذان أعطيا منطقتين مميزتين عند الوزن الجزيئى ٣٣٠ و ٨٢٤ قاعدة مزدوجة.

تم إجراء تفاعل إنزيم البلمرة المتسلسل على بعض أنماط الايكولاى المعزولة من الدجاج المصاب بأعراض تنفسية مثل O27, O78, O86a, O115 وأظهرت النتائج أن العترات جميعها تحوى جين الضراوة *iuc*D عدا نمط O115 بينما جميعها تحوى جين الضراوة *fim*C. تم إجراء تفاعل انزيم البلمرة المتسلسل المتعدد على تلك الأنماط السابق ذكرها لمعرفة إذا كانت تحوى جينات الضراوة *tsh*, *irp2*, *iss*, *iuc*D وبينت النتائج أن نمط O27 يحوى الجينات الأربع بينما نمطي O78 and O86a بهم جينين فقط وهم *iuc*D, *iss* أما نمط O115 فيحوى جين واحد فقط وهو *iss*. قد حقق تفاعل إنزيم البلمرة المتسلسل حقيقي الوقت مزيج من الحساسية والنوعية وسرعة الأداء فقد اثبت وجود جين الضراوة *iuc*D في نمط O115 والذي وجد بكمية قليلة جدا فشل تفاعل إنزيم البلمرة المتسلسل التقليدي في الكشف عنه.