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Pathological changes associated with *Mycoplasma*, *Pasteurella* and *E. coli* infections in respiratory manifested turkeys

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Abstract
This study throws a spot of light on some bacterial agents as one of the causes of the respiratory diseases in different aged turkeys in some turkey farms at Al-Sharkia Governorate. These agents include; *Mycoplasma*, *Pasteurella* and *E. coli* species as well as *Staph.* and *Klebsiella* species. The prevalence rate declared 29.06%, 16.27%, 9.3%, 11.6%, 5.8% of *E. coli*, *Mycoplasma*, *P. multocida*, *Staph.* and *Klebsiella* isolates, respectively of either single or mixed infection. Biochemical identification revealed the detection of *M. gallisepticum*, *M. synoviae*, *M. gallinarum S. aureus* and coagulase negative *Staph*. "CNS" isolates. *E. coli*, *P. multocida* and *K. pneumoniae* were serologically identified revealing *E. coli* serogroups; O78, O1 and O2, *P. multocida* Type A and *K. pneumoniae* K1, K2. Molecular studying using PCR technique detected the specific genes for these isolates. In addition, *M. gallinarum* isolate was sequenced using the *16SrRNA* gene and submitted on the Genbank. *MG*, *MS* and *E. coli* showed sensitivity to ciprofloxacin, while *K. pneumoniae* and *Staph* spp. revealed sensitivity to gentamycin and chloramphenicol. Clinical signs, postmortem and pathological examination revealed commonly prevalent but not characteristic respiratory disorders in both trachea and lung represented in detached cilia, glandular hypertrophy, mucosal metaplasia with leucocytic cells infiltration and hemosiderosis, while lung showed lymhofollicular reaction, thickening of interalveolar septa, fibrosis, hemorrhage and caseous necrosis beside leucocytic cells infiltration of mainly heterophiles. Full description to incidence and prevalence of these lesions accompanied with each pathogen were provided.

Keywords: *Turkeys, Mycoplasma, Pasteurella, E. coli, Pathology, PCR, Antibiotics sensitivity.*

Introduction

Turkey is a large native bird in Egypt. Its meat is highly nutritious and is a popular protein source consumed around the world. Respiratory diseases cause heavy economic losses in poultry industry due to increased mortality rates, alter bird's health resulting in high condemnation rates at slaughter and increased medication costs. They may be induced by various viral and bacterial agents, either alone or in combination **(Marien *et al*., 2005)**.

Poultry respiratory diseases are known to be caused by many pathogens including; *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, *M. iowae*, *P. multocida*, *Klebsiella* spp., *Staph.* spp., and *E. coli* with associated significant economic losses to the industry **(Sid *et al*., 2015).**

Mycoplasmosis is one of the most important poultry diseases causing significant economic losses in many countries. Most of these losses are related directly or indirectly to *M. gallisepticum* (*MG*) with or without complicating factors **(Levisohn and Kleven, 2000)**. *MG* can cause severe chronic respiratory disease (CRD) when present in concern with other poultry pathogens including *E. coli* **(Stipkovits *et al*., 2012)**.

M. gallinarum belongs to the hominis phylogenetic group within the *Mollicutes* class. The

ubiquitous nature of *M. gallinarum* in poultry as well as mammals has been reported by many investigators **(Wan *et al*., 2010)**. In poultry, *M. gallinarum* is commonly thought to be unable to cause disease by itself but can cause the disease when associated with viral diseases or vaccines.

Pneumonia and airsacculitis are common pathological findings in turkeys with fowl cholera; however, morphological descriptions of *P. multocida* induced, acute experimental pneumonia and airsacculitis in the turkey are lacking **(Quinn *et al*., 2011)**.

Avian colibacillosis could start as a respiratory infection then became generalized. Systemic infection by avian pathogenic *E. coli* (APEC) needs respiratory organs colonization and subsequent bacteremia **(Guabiraba and Schouler, 2015)**. Serotyping is still the most frequently used typing method for diagnostic purposes. O1, O2 and O78 are reported as the main serotypes in poultry **(Vandekerchove *et al*., 2004)**.

Post mortem examinations in association with conventional culturing procedures coupled by recent molecular techniques had encountered *MG*, either alone or together with other bacterial population as *P. multocida* and *E. coli* as the main etiologic agents accounted for respiratory problems in infected turkey flocks in Egypt **(Abdelhameed *et al*., 2009**).

Grossly, bilateral lung consolidation with numerous miliary caseous nodules with signs of gasping or dyspnea were noticed in staphylococcal infected turkeys while, pathology revealed the lungs with multifocal bronchopneumonia characterized by flooding of the parabronchi by degenerating heterophils and serofibrinous exudate often containing bacterial colonies. Multi nucleated giant cells, resulting in formation of heterophilic granulomas. The interstitium was congested and infiltrated by a mixture of heterophils, macrophages, and lymphocytes **(Linares and Wigle, 2001).** Avian pasteurellosis in turkey showed vascular disturbances as passive hyperaemia and congestion with hemorrhages in lungs. The lungs showed hemorrhages, followed by necrosis and fibrinous pleuro-pneumonia with extensive exudation of fibrin is common. The lesions are mainly associated with heterophilic infiltrations. In chronic forms, suppurative lesions may be widely distributed **(Christensen and Bisgaard, 2000).**

Antimicrobials in poultry are considered a double weapon as they are an appreciated tool in controlling the clinical disease and to maintain healthy birds, but they also have been incriminated in dissemination of antibiotic resistant bacteria **(Gosh and LaPara, 2007)**. This can pose a risk not only to poultry but also to humans either by direct contact or indirectly via the food production chain **(WHO, 2011)**.

This study aimed to declare the role of some bacterial agents to cause respiratory disease and their lesions in turkeys as a single or mixed infection and thus their control.

Materials and Methods

Sampling:

Eighty six different samples including; 34 swabs and 52 tissues were collected under complete aseptic conditions for bacteriological, molecular and pathological examinations. Swabs for bacteriological examination were collected from nostrils and tracheas from living diseased turkeys suffering respiratory signs. Tissues including; 26 tracheas and 26 lungs of freshly dead or sacrificed diseased birds were also collected. Samples were collected from three breeds of turkeys; Hybrid Grade Maker, Hybride XL and Native Balady housed in four farms at Al- Sharkia Governorate of average ages of 5wk to one year.

Isolation and identification of bacterial agents:

Collected samples were cultured according to **Frey *et al*., 1968** for the isolation of *Mycoplasma* spp.

Mycoplasma isolates identification was carried out by digitonin sensitivity test as described by **Erno and Stipkovits (1973)** and **Razin *et al*. (1998).**

Biochemical characterization of purified *Mycoplasma* isolates including; glucose fermentation, arginine deamination and film & spot formation tests were done according to **Fabricant and Freundt (1967)** and **Watson *et al*. (1988).**

All samples were cultured onto nutrient broth, incubated aerobically then streaked on blood agar, MacConkey agar, EMB agar, Mannitol salt agar, paired parker media. Members of Enterobacteraceae were identified biochemically

by IMVC tests, TSI, urea production, lysine decarboxylase tests according to **Quinn *et al.* (2011)**. *Pasteurella* isolates were identified according to **Cruickshank *et al.* (1975)** and **Holt *et al.* (1994)**. *Staph* colonies were identified according to **Quinn *et al.* (2002)**.

Serologic identification of *P. multocida*, *E. coli* and *Klebsiella* isolates:
The suspected *P. multocida, E. coli* and *Klebsiella* isolates were sent to Food Analysis Center - Faculty of Veterinary Medicine - Benha University for the serologic identification according to **Kok *et al.* (1996)** and **Carter (1984),** respectively.

Molecular identification of the bacterial isolates:
DNA extraction:
Mycoplasma isolates DNA was extracted using Thermo genomic DNA extraction kit, Cat. No. k0721, Lithuania, while the bacterial isolates DNA extraction was performed using the QI-Aamp DNA Mini kit (Qiagen, Germany, GmbH) according the manufacturer's recommendations.

Oligonucleotide Primers:
Mycoplasma primers were prepared by "Macrogen Company", South Korea, while other bacterial primers were supplied from "Metabion", Germany as shown in **table (1)**.

Cycling reaction:
PCR reactions of *Mycoplasma* isolates were performed in a "Gradient Thermal cycler 1000S" (Bio – RAD, USA).The reaction mixture (total volume of 50µl) was 25µl Dream Taq Green PCR Master Mix (Thermo Scientific Company, Lithuania), 5µl target DNA, 2µl of each primers (containing 10pmole/µl) and the mixture was completed by RNAse DNAse free sterile distilled water to 50µl. Primers of other bacterial isolates were utilized in a 25µl reaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an "Applied biosystem 2720 thermal cycler".

Table (1). Primers used for identification of different bacterial isolates

Bacterial isolates	Target gene	Product sequence (5'-3')	Product size (bp)	References
***Mycoplasma* spp.**	***16SrRNA***	GGG AGC AAA CAG GAT TAG ATA CCC T TGC ACC ATC TGT CAC TCT GTT AAC CTC	280	**Van Kuppeveld *et al.*, (1994)**
M. synoviae	***16SrRNA***	GAG AAG CAA AAT AGT GAT ATC A CAG TCG TCT CCG AAG TTA ACA A	214	**OIE, (2008)**
M. gallisepticum	***mgc2***	GCT TTG TGT TCT CGG GTG CTA CGG TGG AAA ACC AGC TCT TG	824	**Ferguson *et al.*, (2005)**
P. multocida	***16SrRNA***	GCT GTA AAC GAA CTC GCC AC ATC CGC TAT TTA CCC AGT GG	460	**Townsend *et al.*, 1998**
E. coli	***phoA***	CGA TTC TGG AAA TGG CAA AAG CGT GAT CAG CGG TGA CTA TGA C	720	**Hu *et al.*, (2011)**
***Klebsiella* spp.**	***gyrA***	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GAT CAC TTC GGT CAG G	441	**Brisse and Verhoef, (2001)**
S. aureus	***clfA***	GCA AAA TCC AGC ACA ACA GGA AAC GA CTT GAT CTC CAG CCA TAA TTG GTG G	638	**Mason *et al.*, (2001)**

PCR cycling protocol of the bacterial isolates:

The thermal profile of *16SrRNA* gene of *Mycoplasma* consisted of 40 cycles of denaturation at 94°C for 1 min., primer annealing at 55°C for 1 min., and primer extension at 72°C for 2 min. **(Van Kuppeveld *et al*., 1994).**

DNA amplification of *mgc2* specific gene of *M. gallisepticum* was performed as the following; 94°C for 3 min., and 40 cycles of 94°C for 20 sec., 58°C for 40 sec., 72°C for 60 sec., and 72°C for 5 min. **(Ferguson *et al*., 2005)**.

Concerning *M. synoviae*, the DNA amplification was performed for 40 cycles as follows; 94°C for 30 sec., 55°C for 30 sec., 72°C for 60 sec., then final extension cycle at 72°C for 5 min. **(OIE, 2008)**.

P. multocida DNA was amplified with an initial denaturation at 95°C for 4 min., followed by 30 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min., extension at 72°C for 1 min., and a final extension at 72°C for 9 min. **(Townsend, *et al*., 1998)**.

E. coli DNA was amplified as followed; initial denaturation at 94°C for 5 min., followed by 35 cycles of 94°C for 30 sec., 55°C for 40 sec., 72°C for 45 sec. and 72°C for 10 min. **(Hu *et al*., 2011)**.

Klebsiella spp. PCR cycling protocol is performed as; 95°C for 5 min. followed by 35 cycles of 94°C for 30 sec. , 55°C for 40 sec., 72°C for 45 sec., 72°C for 10 min. **(Brisse and Verhoef, 2001).**

S. aureus DNA amplification profile was; 94° C for 5 min., followed by 35 cycle of 94°C for 30 sec., 55°C for 45 sec., 72°C for 45 sec., 72° C for 10 min. **(Mason *et al*., 2001)**.

Analysis of the PCR Products:

Amplified bacterial PCR products were electrophoresed on 1.5% agrose gel in Tris acetate EDTA and visualized by UV transilluminator. A 100bp Plus DNA Ladder, Cat. No. BM311-01 (TransGen Boitech, China) was used to determine the amplicon sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Purification and sequencing of Mycoplasma isolate:

The PCR product was sequenced on GATC Company by using ABI 3730x1 DNA sequencer using the forward and reverse primers.

Sequence analysis:

BioEdit sequence alignment editor **(Hall, 1999)** and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic tree was constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW **(Thomposon *et al*., 1994)**. Sequence divergence and identity percent was calculated by MegAlign.

Pathological examinations:

Specimens from tracheas and lungs of the affected turkey birds either sacrificed or freshly dead were collected after PM examination and fixed in 10% buffered neutral formalin. Paraffin sections of 2-3 micron thickness were prepared and stained with hematoxylin and eosin stain then examined microscopically **(Survarna *et al*., 2013)**.

Antibiotic Sensitivity Test:

The antibiotic susceptibility test was performed using agar disc diffusion method against commonly used chemotherapeutic agents **(Koneman *et al.*, 1997).** Interpretation of the results based on the diameter of the inhibition zones was performed according to guidelines of **CLSI (2017)**.

Resluts

Recovery rate of different bacterial isolates:

The recovery rates of *E. coli*, *Mycoplasma spp*. and *P. multocida* were 25/86 (29.06%), 14/86 (16.27%), and 8/86 (9.3%), respectively. In addition, 10/86 (11.6%) were recovered as *Staph* isolates including; *S. aureus* 7/86 (8.13%) and CNS 3/86 (3.48%) while, *Klebsiella* isolates 5/86 (5.8%) were also isolated **(Table 2)**.

Table (2). Prevalence of single and mixed bacterial infections

Bacterial isolates	Samples						Total
	Trachea		Lung		Swab		
	single	mixed	single	mixed	single	mixed	
E. coli	1		2		1		4
E. coli + *Mycoplasma* spp.		4		6		3	13
E. coli + *P. multocida*		1		2		0	3
E. coli + *Staph.* spp.		0		4		1	5
Total	25 (29.06%)						
Mycoplasma spp. + *P. multocida*		0		1		0	1
Mycoplasma spp. + *E. coli*		4		6		3	13
Total	14 (16.27%)						
Staph. spp.	0		3		1		4
Staph. spp. + *P. multocida*		0		1		0	1
Staph. spp. + *E. coli*		0		4		1	5
Total	10 (11.62%)						
P. multocida	1		2		0		3
P. multocida + *Mycoplasma* spp.		0		1		0	1
P. multocida + *Mycoplasma* spp.		0		1		0	1
P. multocida + *E. coli*		1		2		0	3
Total	8 (9.3%)						
Klebsiella spp.	0		3		2		5
Total	5 (5.8%)						

Identification of the bacterial isolates:

All *Mycoplasma* isolates showed the characteristic colonies of "fried egg" appearance with dark raised center were sensitive to digitonin. Biochemical identification of *Mycoplasma spp*. revealed 9 isolates that could ferment glucose but neither hydrolyzed arginine nor make film & spot indicating *M. gallisepticum*, while 4 isolates were positive for glucose fermentation and film & spot formation and negative for arginine deamination indicting *M. synoviae* and 1 isolate couldn't ferment glucose but could hydrolyze arginine and made film & spot indicating *M. gallinarum*.

Serogrouping of *E. coli* isolates according to the somatic "O antigen" revealed that O78, O1 and O2 were the most predominant serogroups. Serological identification of *Pasteurella* isolates revealed that all isolates were identified as *P. multocida* "Type A" while *Klebsiella* spp. were identified as "K1" and "K2", **table (3)**.

Table (3). Identification of the bacterial isolates

Bacterial isolates	Sero-group	No.
***Mycoplasma* spp. (14)**	*M. gallisepticum* *M. synoviae* *M. gallinarum*	9 4 1
***E. coli* (25)**	O78 O1 O2 O113 O158 O91 Untyped	6 4 4 2 3 3 3
***Klebsiella* spp. (5)**	*K. pneumoniae* K1 (HVKP) *K. pneumoniae* K2 (HVKP) *K. pneumoniae* K1 (CKP)	1 1 3
***Pasteurella* spp. (8)**	*P. multocida* Type A	8
***Staphylococcus* spp. (10)**	*S. aureus* CNS	7 3

*HVKP=High virulent *K. pneumoniae* *CKP=Classic *K. pneumoniae* *CNS=Coagulase negative *Staph.*

Molecular identification of the bacterial isolates:

PCR amplification of the *16SrRNA* gene based mycoplasma group specific PCR assay was applied for the tested *Mycoplasma* isolates declared a characteristic fragment at 280bp, as shown in **Fig. (1)**.

Mycoplasma isolates were subjected to further PCR reactions for the detection of *mgc2* and *16SrRNA* genes for the confirmation of *MG* and *MS*, respectively. Results revealed that 9 isolates were positive for the *mgc2* gene of *MG* and 4 isolates were positive for the *16SrRNA* gene of *MS* revealing specific bands at 824bp and 214bp, respectively **(Fig. 2, 3)**.

Conventional PCR results revealed the detection of *pho*A gene in all *E. coli* isolates giving a specific band at 720bp **(Fig. 4)**, also *gyr*A gene was amplified in all examined *Klebsiella* spp. and gave a characteristic band at 441bp **(Fig. 5)**. Additionally, *16SrRNA* gene was identified in *P. multocida* with amplicon size of 460bp and *clf*A gene was found in the tested *S. aureus* isolates with an amplification product of 638bp **(Fig. 6, 7).**

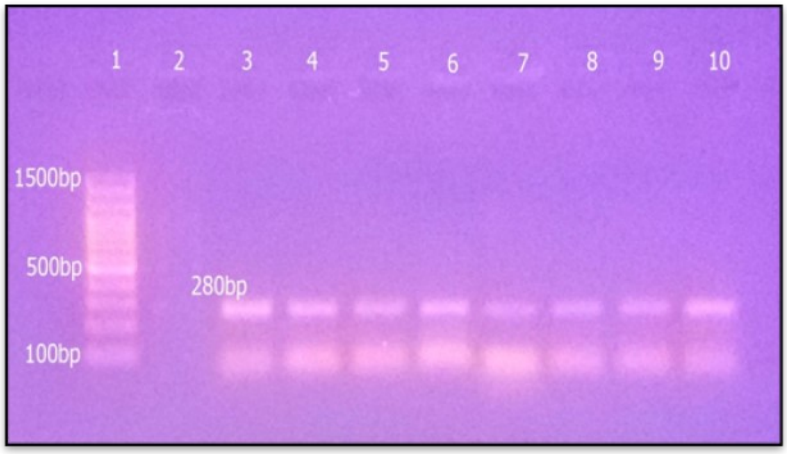


Fig. (1): Electrophoretic pattern of *16SrRNA* gene of *Mycoplasma*. Lane (1): 100bp DNA ladder. Lane (2): control negative. Lane (3): control positive. Lanes (4-10): positive amplifications for target gene at 280bp

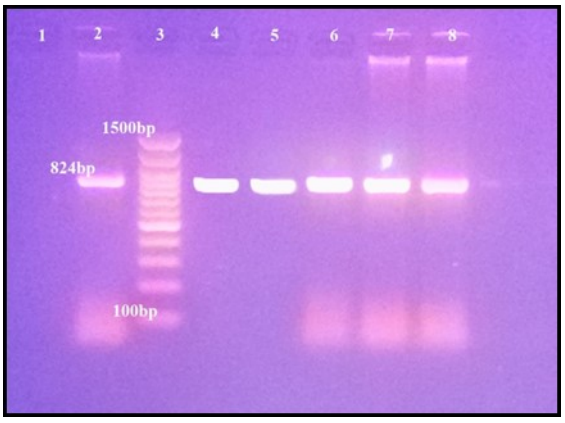


Fig. (2): Electrophoretic pattern of *mgc2* gene of *MG*. Lane (1): negative control. Lane (2): positive control. Lane (3):100bp DNA ladder. Lanes (4-8): positive amplifications for target gene at 824bp.

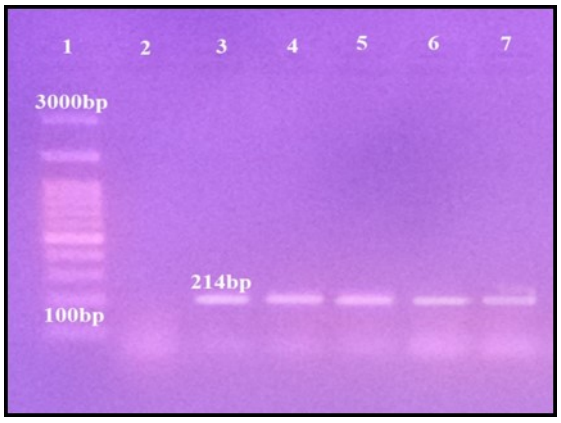


Fig. (3): Electrophoretic pattern of *16SrRNA* gene of *MS.* Lane (1): 100bp DNA ladder. Lane (2): control negative. Lane (3): control positive. Lanes (4-7): positive amplifications for target gene at 214bp.

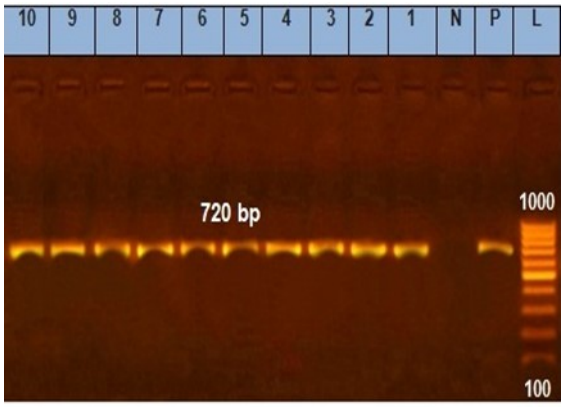


Fig. (4): Electrophoretic pattern of *phoA* gene of *E. coli*. L: 100bp DNA ladder. P: control positive. N: control negative. Lanes (1-10): positive amplifications for target gene at 720bp.

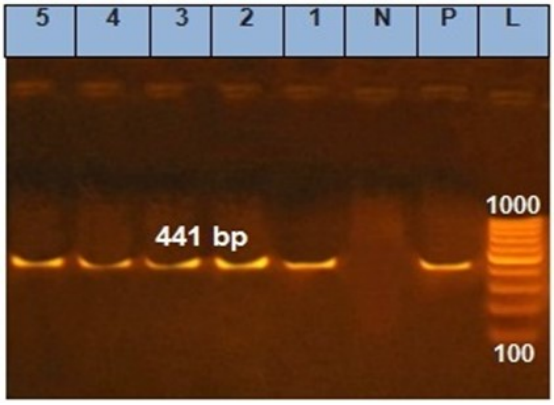


Fig. (5): Electrophoretic pattern of *gyrA* gene of *Klebsiella*. L: 100bp DNA ladder. P: control positive. N: control negative. Lanes (1-5): positive amplification for target gene at 441bp.

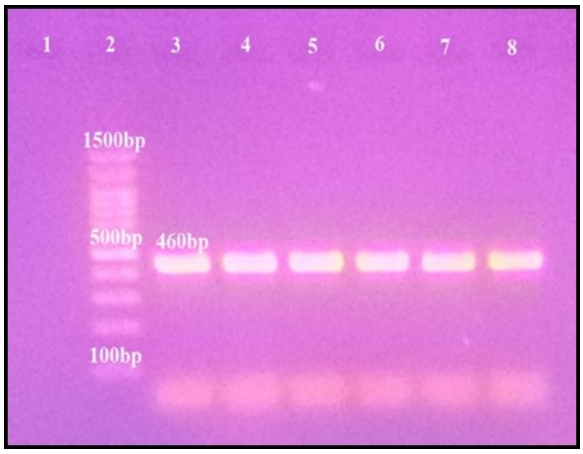


Fig. (6): Electrophoretic pattern of *16SrRNA* gene of *P. multocida*. Lane (1): negative control. Lane (2): 100bp DNA ladder. Lane (3): positive control. Lanes (4-8): positive amplifications for target gene at 460bp.

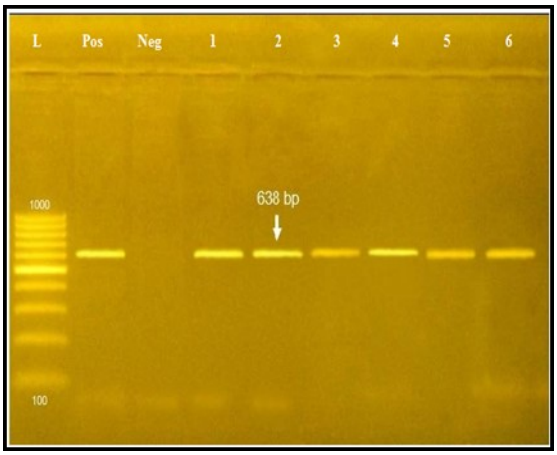


Fig. (7): Electrophoretic pattern of *clfA* gene of *S. aureus*. L: 100bp DNA ladder. Pos.: control positive. Neg.: control negative. Lanes (1-6): positive amplifications for target gene at 638bp.

Sequence results:
A purified *Mycoplasma* PCR product isolated from a tracheal swab of diseased turkey suffering respiratory signs was submitted to sequencing using *Mycoplasma 16SrRNA* gene. Sequencing was conducted in both directions. The original sequence was trimmed to remove the indefinite sequences which usually exist in the beginning of the sequence reaction. The result revealed that it was "*M. gallinarum*" submitted to Genbank database with accession number "MT754562" and designation RT20.

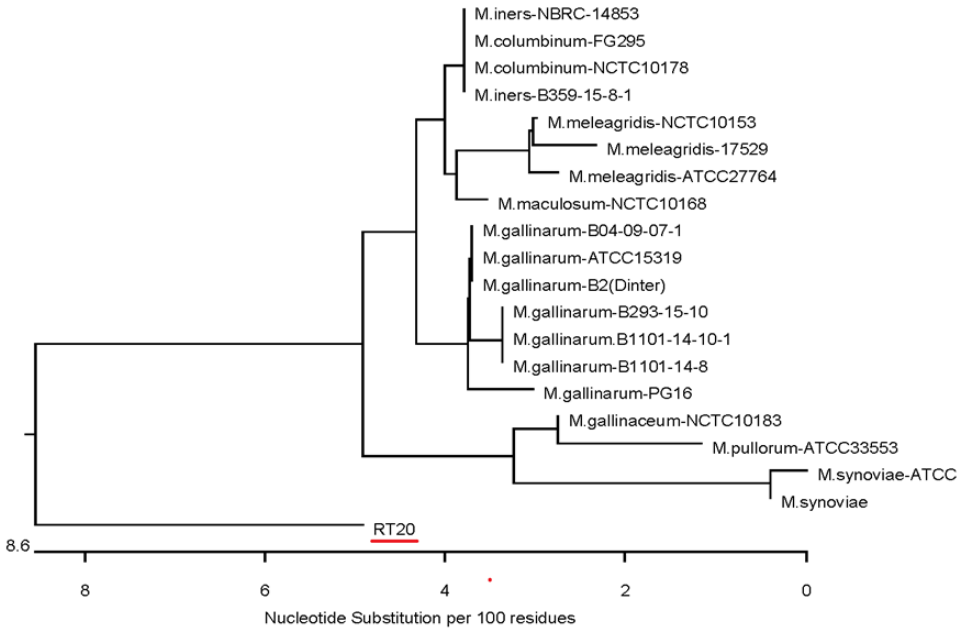
Phylogenetic analysis results:
Nucleotide phylogenetic tree of *16SrRNA* of *M. gallinarum* (RT20) showed similarity ranged from 98.5-100% with reference and field strains as shown in **Fig. (8) & table (4)**.

Table (4). Identity percent of nucleotide sequencing of *16SrRNA* gene of *M. gallinarum* (RT20) compared with reference and field strains

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
1		89.2	89.2	89.2	88.8	88.8	88.8	88.8	88.8	88.8	87.0	88.5	88.5	88.1	87.4	87.7	87.4	85.9	84.8	82.9	1	RT20
2	6.6		100.0	100.0	98.9	99.6	99.6	98.9	98.9	98.9	97.8	98.5	99.3	97.8	96.7	97.4	97.0	95.2	91.1	88.9	2	M.gallinarum-B04-09-07-1
3	6.6	0.0		100.0	98.9	99.6	99.6	98.9	98.9	98.9	97.8	98.5	99.3	97.8	96.7	97.4	97.0	95.2	91.1	88.9	3	M.gallinarum-ATCC15319
4	6.6	0.0	0.0		98.9	99.6	99.6	98.9	98.9	98.9	97.8	98.5	99.3	97.8	96.7	97.4	97.0	95.2	91.1	88.9	4	M.gallinarum-B2(Dinter)
5	6.6	1.1	1.1	1.1		98.5	98.5	100.0	99.3	100.0	96.7	99.6	98.2	98.9	95.6	98.5	98.2	94.1	91.4	89.3	5	M.iners-B359-15-8-1
6	7.0	0.4	0.4	0.4	1.5		100.0	98.5	98.5	98.5	98.2	98.2	98.9	97.4	96.3	97.8	96.7	94.8	90.7	88.5	6	M.gallinarum-B293-15-10
7	7.0	0.4	0.4	0.4	1.5	0.0		98.5	98.5	98.5	98.2	98.2	98.9	97.4	96.3	97.8	96.7	94.8	90.7	88.5	7	M.gallinarum.B1101-14-10-1
8	6.6	1.1	1.1	1.1	0.0	1.5	1.5		99.3	100.0	96.7	99.6	98.2	98.9	95.6	98.5	98.2	94.1	91.4	89.3	8	M.columbinum-NCTC10178
9	7.4	1.1	1.1	1.1	0.7	1.5	1.5	0.7		99.3	96.7	98.9	98.2	98.9	95.6	98.5	98.2	94.1	90.7	88.1	9	M.maculosum-NCTC10168
10	6.6	1.1	1.1	1.1	0.0	1.5	1.5	0.0	0.7		96.7	99.6	98.2	98.9	95.6	98.5	98.2	94.1	91.4	89.3	10	M.iners-NBRC-14853
11	7.0	0.4	0.4	0.4	1.5	0.0	0.0	1.5	1.5	1.5		96.3	97.0	95.6	94.5	95.9	94.8	93.0	88.8	86.7	11	M.gallinarum-B1101-14-8
12	6.7	1.1	1.1	1.1	0.0	1.5	1.5	0.0	0.7	0.0	1.5		97.8	98.5	95.2	98.2	97.8	93.7	91.1	88.9	12	M.columbinum-FG295
13	7.5	0.7	0.7	0.7	1.9	1.1	1.1	1.9	1.9	1.9	1.1	1.9		97.0	95.9	96.7	97.8	94.5	90.3	88.1	13	M.gallinarum-PG16
14	7.9	2.3	2.3	2.3	1.1	2.6	2.6	1.1	1.1	1.1	2.6	1.1	3.0		94.5	99.6	99.3	93.0	90.0	87.8	14	M.meleagridis-NCTC10153
15	7.9	2.6	2.6	2.6	3.8	3.0	3.0	3.8	3.8	3.8	3.0	3.8	3.4	5.0		94.1	93.7	98.5	95.2	93.0	15	M.gallinaceum-NCTC10183
16	8.3	2.6	2.6	2.6	1.5	2.3	2.3	1.5	1.5	1.5	2.3	1.5	3.4	0.4	5.4		98.9	92.6	89.6	87.4	16	M.meleagridis-ATCC27764
17	8.7	3.0	3.0	3.0	1.9	3.4	3.4	1.9	1.9	1.9	3.4	1.9	2.3	0.7	5.8	1.1		92.3	89.2	87.0	17	M.meleagridis-17529
18	9.6	4.2	4.2	4.2	5.4	4.6	4.6	5.4	5.4	5.4	4.6	5.4	5.0	6.6	1.5	7.0	7.4		93.7	91.5	18	M.pullorum-ATCC33553
19	9.2	6.3	6.3	6.3	5.4	6.7	6.7	5.4	6.3	5.4	6.7	5.5	7.1	6.7	3.5	7.1	7.5	5.1		97.8	19	M.synoviae-ATCC
20	8.9	5.9	5.9	5.9	5.1	6.3	6.3	5.1	5.9	5.1	6.3	5.1	6.7	6.3	3.1	6.7	7.1	4.7	0.0		20	M.synoviae
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		

Fig. (8): Phylogenetic tree of nucleotide of *16SrRNA* gene of *M. gallinarum* (RT20) compared with reference and field strains



Clinical signs

Infected turkeys showed respiratory disorders represented in swollen head with closed eyes **(Fig. 9A1).** Swollen infra orbital sinuses **(Fig. 9A2)** beside nasal discharge which is catarrhal (frothy) in case of *Mycoplasma* mixed infection and turned to be thick in some cases and even mucopurulent in few other cases especially of those infected with pyogenic pathogens as *E. coli*. Sneezing, depression and tracheal râls were also observed, high morbidity rate (68-79%) were detected, while mortality rate was mild to moderate in inspected farms (9-21%). Decrease feed intake and weight loss were detected with progress of the disease especially in case of *Mycoplasma* infection.

Postmortem examination:

Grossly, the trachea showed severe congestion **(Fig. 9B)** in some cases, catarrhal tracheitis represented in trachea was filled with yellowish exudates in other cases, while examined lungs from positive infected cases appeared in different forms, edematous, firm with cut surface yielded blood tinged exudate and mild to moderate hemorrhage of mostly focal distribution **(Fig. 9C).** Infected lungs also revealed focal to diffuse congestion **(Fig. 9D)**. Some cases revealed lungs with foal caseation **(Fig. 9E)**, which is mostly common in those mixed infection with *Mycoplasma* most of the. The incidence and prevalence of gross lesions based on the etiological agents isolated were summarized in lesion score **table (2).**

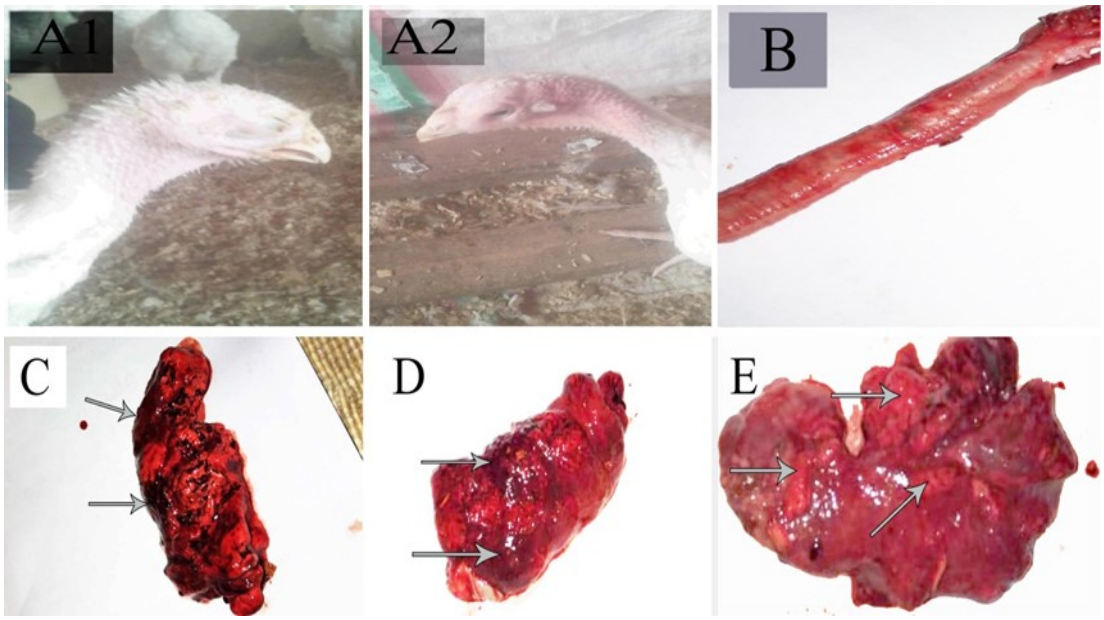


Figure (9): (A1): Infected turkey with closed eyes due to swollen head. **(A2):** Infected turkey showed swollen sinuses. **(B):** Congested trachea. **(C):** Infected lung revealed severe hemorrhage (arrows). **(D):** Infected lung revealed focal congestion (arrows). **(E):** Infected lung revealed multiple miliary caseous nodules (arrows).

Microscopic examination:

The trachea showed detached cilia with partial chondromalacia **(Fig. 10a)**. Focal submucosal hemorrhage with necrosis of some chondrocytes **(Fig. 10b)** was observed. Focal areas of hemosiderosis in the tracheal mucosa **(Fig. 10c)** in some cases were detected. Leucocytic cells infiltrate the tracheal mucosa **(Fig. 10d)** was observed while hyperplasia and metaplasia of mucosal glands with partial chondromalacia of tracheal ring cartilage **(Fig. 10e)** were noticed. Mild cystic dilation of tracheal glands with metaplasia or vacuolation of glandular epithelium **(Fig. 10f)** were also observed. Lungs revealed severe congestion with perivascular fibrosis **(Fig. 11a)**. Thickening of inter alveolar wall (acute interstitial pneumonia, focal to diffuse hemorrhage with focal nodule of lymphocytes) **(Fig. 11b)** which is known as lymhofollicular reaction. Severe perivascular

hemorrhage with thickening of interalveolar wall and hyperplasia of bronchial epithelium **(Fig. 11c)** were observed. Focal area of caseous necrosis and focal proliferation of fibroblasts with mild hemorrhage also observed. Severe heterophilic cells infiltration with giant cells and severe hemorrhage **(Fig. 11d)** were seen.

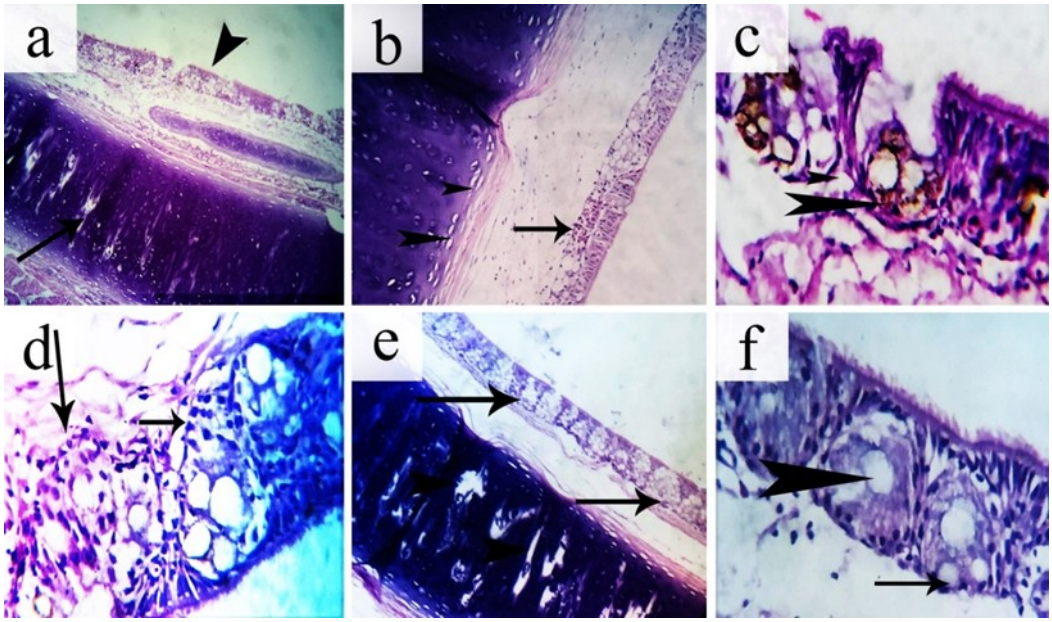


Fig. (10): Photomicrograph of infected tracheas revealed (a): Detached cilia (arrow head) with partial chondromalacia of tracheal ring cartilage (arrow) **(H&E x100)**. **(b):** Focal submucosal hemorrhage (arrow) with necrosis of some chondrocytes (arrow heads) **(H&E x100)**. **(c):** Focal areas of hemosiderosis in the tracheal mucosa (arrow head) **(H&E x200)**. **(d):** Leucocytic cells infiltrate the tracheal mucosa (arrows) **(H&E x200)**. **(e):** Hyperplasia and metaplasia of mucosal glands (arrows) with partial chondromalacia of tracheal ring cartilage (arrow head) **(H&E x100)**. **(f):** Mild cystic dilatation of tracheal gland (arrow head) with metaplasia or vacuolation of glandular epithelium (arrow) **(H&E x200)**.

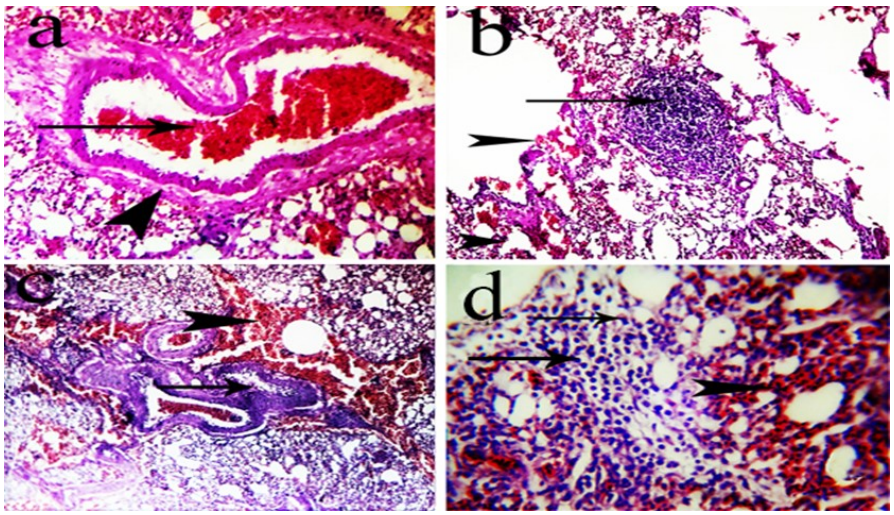


Figure (11): Photomicrograph of different sections of infected lungs revealed (a): Severe congestion (arrow) with perivascular fibrosis (arrow head) **(H&E x200)**. **(b):** Thickening of interalveolar wall, diffuse hemorrhage (arrow head) with focal nodule of lymphocytes (lymphofollicular reaction) (arrow) **(H&E x100)**. **(c):** Severe perivascular hemorrhage (arrow head) with thickening of interalveolar wall and hyperplasia of bronchial epithelium (arrow) **(H&E x100)**. **(d):** Severe heterophilic cells infiltration with giant cells (arrow) and severe hemorrhage (arrow head) **(H&E x200)**.

Lesions score of respiratory disorders with incidence were demonstrated in **table (5).**

Table (5). Lesions score of the respiratory disorders

Affected tissue	Lesion	Type of infection	Lesion severity
Trachea	Catarrhal trachietis	Mostly in *E. coli* or *P. multocida* infection	++
	Congestion	All single and mixed infection	+++
	Detached cilia	All single and mixed infection except *Klebsiella*	++
	Lymphocytic cells infiltration	Mainly in *Mycoplasma* infection	++
	Heterophilic cell infiltration	All bacterial infection	+++
	Hemosiderosis	Mainly in *P. multocida* and *Staphylococcus*	+
	Hypertrophy of mucosal glands	Mainly in *P. multocida* and *Staphylococcus*	++
	Metaplasia of mucosa	All single and mixed infection except *Klebsiella*	+++
Lung	Consolidation	All infection including *Staphylococci*	++
	Caseous nodule formation	All infection including *Staphylococci* and *E. coli* and *Mycoplasma* infection	++
	Congestion	All mixed and single bacterial infection	+++
	Lymphofollicular reaction	Mainly *Mycoplasma* infection and sometimes *E. coli*	++
	Hemorrhage	Mainly *P. multocida* and other mixed infection	++
	Caseous necrosis	Mainly *Mycoplasma* and sometimes *E. coli*	++
	Fibrosis	All mixed infection in chronic stage	++
	Polymorphic cells infiltration	All mixed and single bacterial infection	+++
	Lymphocytic cell infiltration	Mainly in *Mycoplasma* infection sometimes *P. multocida* infection	++
	Acute interstitial pneumonia	All single or mixed infection with *P. multocida* in acute stage	++

Antibiotic Sensitivity:

Phenotypic antibiotic susceptibility profile of all bacterial isolates was performed by disk diffusion methods. Results showed that; *MG* and *MS* were sensitive to ciprofloxacin, lincomycin and doxycycline. *E. coli* were sensitive to colistin, ciprofloxacin and gentamycin. *P. multocida* showed sensitivity to ciprofloxacin, gentamycin and cephalexin. *K. pneumoniae* and *Staph*. spp. were sensitive to gentamycin and chloramphenicol, **table (6)**.

Table (6). Antibiotic sensitivity test results of the bacterial isolates

Antibiotic/ Symbol/ Potency (µg)	*MG* (n=9)		*MS* (n=4)		*E. coli* (n=25)		*P. multocida* (n=8)		*K. pnemonie* (n=5)		*Staph* (n=10)	
	S	R	S	R	S	R	S	R	S	R	S	R
Amoxicillin (AX) 25µg	-	-	-	-	4	21	5	3	0	5	1	9
Ciprofloxacin (CIP) 10µg	8	1	3	1	13	12	6	2	1	4	5	5
Gentamycin (CN) 10µg	3	6	2	2	14	11	6	2	3	2	9	1
Lincomycin (MY) 15µg	7	2	3	1	-	-	-	-	-	-	-	-
Spectinomycin (SH) 100µg	6	3	1	3	-	-	-	-	-	-	-	-
Erythromycin (E) 15µg	2	7	1	3	0	25	4	4	0	5	4	6
Chloramphenicol (C) 30µg	3	6	1	3	8	17	3	5	3	2	7	3
Sulpha+ Trimethoprim (SXT) 25µg	-	-	-	-	10	15	3	5	2	3	7	3
Colistin (CT) 10µg	-	-	-	-	24	1	-	-	-	-	-	-
Doxycycline (DO) 30µg	8	1	3	1	8	17	4	4	2	3	2	8
Cephalexin (CLX) 30µg	-	-	-	-	0	25	6	2	0	5	0	10

n= number of isolates (-) = not used

Discussion

Bacterial infections of the respiratory tract are of a major importance as they cause 30% of mortality per year **(Hossain *et al.,* 2013**). Most studies discussed respiratory diseases in poultry had focused on chickens while there is relatively little data on turkeys **(Argudín *et al*., 2013)**.

Our results proved the isolation of *Mycoplasma* with recovery rate (16.27%) as reported by **Rasoulinezhad *et al*. (2017)** who found that *M. gallisepticum* was isolated from 16.66% of the examined cases with airsacculitis and infra orbital sinusitis in turkey farms in Iran. The results presented by **Eissa *et al.* (2000)** lessened the significant role of *M. gallisepticum* as a cause of infra orbital sinusitis in turkey and increase the role of *M. synoviae*. Whereas the results of our study clarified the role of *M. gallisepticum, M. synoviae* and *M. gallinarumin* in induction of respiratory disease in turkeys.

Our results were in accordance with **Agnes *et al.* (2013)** who had identified APEC, *S. aureus*, and *O. rhinotracheale* as the most frequently diagnosed pathogens in turkeys. Also, **Eid and Samir (2019)** deduced the causative agents implicated in the respiratory problems in turkey flocks in Hefna, AL-Sharkia were *E. coli, Klebsiella* spp. and *Staphylococci* spp.

In our investigation we isolated *Staph* spp. from diseased turkeys with incidence rate 11.62% which was lower than that detected by **Friese *et al.* (2013)** who recorded the prevalence of *S. aureus* in turkey farms of 25.9%.

Under commercial conditions, complicated infections involving multiple etiologies with viruses, mycoplasmas and other bacteria are more commonly observed than single infections. In turkeys, only few studies have been performed to explain the effects of combined

action of viruses, bacteria and mycoplasma **(Marien, 2007)**.

Concerning the mixed infection detected in this study, this was in accordance with the previous studies which identified the respiratory pathogens in turkeys as APEC*, P. multocida* and *Mycoplasma* spp. that can be clinically challenging when two of these pathogens occur concurrently **(Pierson *et al*., 1996)**. Isolation of these microorganisms from the respiratory tracts of turkeys may refer to their ability to act synergistically leading to aggravated condition of chronic respiratory disease "CRD" **(Abdelhameed *et al.*, 2009** and **Norhan *et al*., 2014).**

A study conducted by **Abdelhameed *et al.* (2009)** had detected *M. gallisepticum*, either alone or coupled with *P. multocida* and *E. coli* as the predominant etiologic agents responsible for the respiratory problems in diseased turkey flocks. Others have attributed concurrent infections with *K. pneu-moniae* and *E. coli* to cause respiratory disease and high mortality rates in turkeys **Hinz *et al.* (1992)**.

The detected *E. coli* mixed infection was in agreement with **Martin (2000)** who mentioned that it occured either primary where it acted as the main entity or secondary in which another disease gave a chance for *E. coli* to induce the disease, it is unknown which of these two theories is true.

Our investigation results showed that *E. coli* serogroups; O78, O2, O1 and O91 were the most identified ones as those reported by **Martin (2000)** and **Dziva and Stevens (2008)**.

Identification of *P. multocida* "Type A" was in agreement with other investigations that detected *P. multocida* "Type A" as the major serogroup found in avian host **(Shivachandra *et al.,* 2006)** and **Furian, *et al*. (2014)**.

K. pneumoniae identification from lungs was in line with **Dashe *et al*. (2012)** who mentioned that it could possibly be responsible for severe cases of respiratory distress encountered in poultry.

Regarding the genotypic identification results of *Mycoplasma* isolates, it showed high accuracy and specificity, as confirmed by **Marouf *et al*. (2020)** who identified 86 *MG* and 39 *MS* by PCR. Whereas identification of *E. coli* by targeting its highly conserved gene (*pho*E gene) can successfully act as an alternative to conventional identification methods **(APHA, 2005).**

Our phylogenetic analysis of the partial-genome sequencing of the *16SrRNA* gene results revealed the detection of *M. gallinarum* in the trachea of a diseased turkey which was in accordance with **Volokhov *et al*. (2012)** who used three-target sequence analysis, including the *ITS*, *rpoB*, and *16SrRNA* genes, as a reliable tool for the differentiation of the family *Mycoplasmataceae* based on their phylogenetic relatedness and pairwise sequence similarities. **Beylefled, *et al*. (2018)** identified mycoplasma isolated from South African poultry flocks by full-genome sequencing and phylogenetic analysis of the *16SrRNA* gene and were classified as *M. gallisepticum*, *M. gallinarum*, *M. gallinaceum*, *M. pullorum*, *M. synoviae*, and *M. iners*, as well as one *Acheoplasma laidlawii* strain.

Genetic confirmation of *P. multocida* isolates by targeting the *16SrRNA* gene can act as fast and reliable alternative methods in diagnosis as stated by **Townsend *et al*. (1998)** and **Shivachandra *et al*. (2006)**.

Klebsiella isolates were confirmed using the *gyr*A gene as mentioned by **Younis *et al*. (2016)**. Molecular detection of *S. aureus* isolates was performed by the detection of *clfA* gene; the selection of that gene was based on the studies suggesting that *clfA* gene is present in the chromosome of all *S. aureus* strains and encodes a surface-exposed fibrinogen-binding protein **(Smeltzer *et al.,* 1997)**.

Previous respiratory signs beside the decrease in feed intake and weight loss were described with progress of the disease especially in case of *Mycoplasma* infection were similar to that detected by **Ley (2003)** who explained these findings in case of *Mycoplasma* infection to long course of disease which lasts for months in untreated flocks which also could be attributed to the frequent occurrence of a symptomatic infection **(Levisohn and Kleven, 2000).** Some of previously mentioned lesions were closely related to **Linares and Wigle (2001)** who described bilateral lung consolidation with multiple miliary caseous nodules in case of *Staphylococcus* infection.

Congested catarrhal tracheitis with yellowish

exudates was in harmony with those obtained by **Marien (2007)**. Edematous firm yielded blood cut surface tinged exudate and mild hemorrhage were detected in both of *E. coli* or *P. multocida* infection either mixed or single were in complete accordance with that mentioned by **Lakshman *et al.* (2006).** Lungs with foal caseation were mostly common in case of mixed infection with *Mycoplasma* and *E. coli* species; chronic respiratory disease (CRD) **Marien (2007)**. In severe cases of typical air sac disease, caseous exudates and some degrees of pneumonia were typically reported by **(Ley, 2003**). However, these lesions may occur with other pathogens, and are not pathognomonic for *Mycoplasmosis* **(Razin *et al.*, 1998)** who stated that the clinical picture of *Mycoplasma* infections was more suggestive to be damaged due to the host immune and inflammatory responses rather than the direct toxic effects by *Mycoplasmal* cell components.

Our tracheal lesions in mixed infection with *E. coli* and *Mycoplasma* were in partial accordance with **Marien (2007)** who noticed focal loss of cilia (detached cilia) and/or necrosis of the epithelium, as well as degeneration of mucous glands and with complete accordance with **Lakshman *et al.* (2006)**. *K. pneumoniae* lesions were restricted in the lung giving arise to pneumonia identical to **Cheng *et al.* (2018)** who reported that *K. pneumoniae* usually plays an important role for causing pneumonia in large animal but lower than that in turkey **(Aslan *et al.*, 2002)**. Acute interstitial pneumonia was the most prominent lesion observed in acute respiratory infection in lungs which was closely related to that obtained by **Lakshman *et al.* (2006)** who described the same lesion which characterized by thickening of interalveolar septa with fibrin threads accumulation. Hemorrhages, congestion and pneumonic changes in case of avian *Pasteurellosis* are in line with the findings of **Cynthia (2005)** and **Anitha and Mammen (2013).**

The indiscriminate use of antimicrobials in animal farming is likely to accelerate the development of anti-microbial resistance (AMR), in addition to human health concerns about the presence of antimicrobial residues in meat **(Reig and Toldra, 2008).** Additionally, AMR in poultry pathogens is likely to lead to economic losses, derived from the expenditure on ineffective antimicrobials, as well as the burden of untreated poultry disease **(Nhung *et al.*, 2017),** therefore the emerge of many antibiotic resistant bacterial strains is very important to be concerned**.**

Our study declared the resistance of the most important implicated bacterial respiratory causes to many antibiotics. *M. gallisepticum* and *M. synoviae* showed sensitivity to ciprofloxacin, lincomycin, and doxycycline and represented resistance to gentamycin, erythromycin and chloramphenicol. These were in accordance with those reported by **Gharaibeh and Al-Rashdan (2011)** who stated a statistically significant increase in resistance of *MG* to erythromycin, tilmicosin, tylosin, ciprofloxacin, enrofloxacin, chlortetracycline, doxycycline, and oxytetracycline. This dramatic increase in resistance in a relatively short period of time appears should be concerned. Also **El-shafey *et al.* (2016)** showed the sensitivity of *MS* to doxycycline.

E. coli isolates exhibited absolute resistance to cephalexin and erythromycin (100%), followed by high resistance rate to amoxicillin, chloramphenicol, doxycycline, sulpha+trimethoprim; moderate resistance to ciprofloxacin and gentamycin, meanwhile, least level of resistance was reported to colistin, whereas *P. multocida* exhibited relatively low level of resistance to all tested antibiotics with gentamycin, ciprofloxacin showing high efficacy on most isolates. In contrast *K. pneumoniae* showed high resistance level to all tested antibiotics. It is obvious that *E. coli* and *K. pneumoniae* isolates had demonstrated extensive multidrug resistance patterns whereas *P. multocida* showed lower level of antibiotic resistance this is in accordance with **Nhung *et al.* (2017)** who mentioned that APEC isolates displayed considerably higher levels of AMR, with resistance rate over 80% for ampicillin, amoxicillin, tetracycline across studies. Whereas levels of resistance among *P. multocida* isolates were less than 20% for all antimicrobials. The most identified bacteria species in turkey farms exhibited high levels of multidrug-resistant were *E. coli* and *K. pneumoniae* **(Kim *et al.*, 2005).**

Staph. spp. showed variable resistance levels mostly for cephalexin and amoxicillin and lowest resistance levels to gentamycin, sulpha+trimethoprim and chloramphenicol these results coincide with **El-Adawy *et al.* (2016)** who showed that 18 *S. aureus* isolates from turkey farms were resistant to aminoglycosides and cefoxitin and sensitive to gentamycin, chloramphenicol and sulphonamides.

Conclusion

Mycoplasma, *E. coli*, *P. multocida*, *K. pneumoniae*, and *S. aureus*, either as a single or mixed infection, were the most prominent pathogens incriminated in the respiratory diseases in turkey farms causing low feed intake and weight loss and thus severe economic losses. PCR is a rapid, sensitive and specific assay for the detection of these pathogens. Postmortem and pathological examinations may point to the potential pathogens helping in further diagnosis. The random use of antibiotics must be considered because of the appearance of the multidrug resistant bacterial strains.

Recommendation

Some recommendations must be considered to eliminate or even limit the respiratory diseases in turkeys' farms.

Proper nutrition is important for growth and good health of turkeys.

Strict biosecurity measures, correct management practice and effective vaccination programs should be adopted in turkey farms.

Avoid vertical transmitted diseases as *Mycoplasma* infections.

Application of routine antibiotic sensitivity tests for limiting their random use.

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