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# Genetic analysis on some microbial causes of mortalities in turkeys in Behira, Egypt

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## ABSTRACT

Respiratory signs, swelling of infraorbital sinus and variable percentages of mortalities were observed in some turkey flocks in Behira, Egypt. This study aimed to investigate Avian influenza virus (AIV), Escherichia coli (*E. coli*), Salmonella, Avian metapneumovirus (aMPV), Mycoplasma gallisepticum (MG) and Mycoplasma meleagridis (MM) in suspected cases, followed by the genetic characterization of the detected microorganisms. AIV subtype H5N8 was detected from one backyard case that showed 100% mortality and was genetically classified under clade 2.3.4.4.b. Fourteen turkey farms that showed mortality percentages ranged between 7%-23% were tested for other pathogens, All of them were positive for *E. coli* isolation and negative for Salmonella isolation. Iuta and Iss virulence genes were present in 100% of the tested *E. coli* isolates, while eaeA virulence gene was detected in 30% of these isolates. Antibiotic profiling of *E. coli* isolates revealed high sensitivity to Gentamicin, Erythromycin and Ciprofloxacin and high resistance with Tetracycline, Amoxicillin and Trimethoprim+Sulphadiazine. AMPV was detected in one farm. MG and MM were detected in 11 and 6 farms, respectively. Genetic analysis of mgc2 gene of 3 MG strains showed higher similarity with published Egyptian stains, ts-11 and 6/85 vaccinal strains. Also genetic sequencing of the 16s rRNA gene of 3 MM strains showed 100% similarity with recently published Egyptian strains. MM isolation was successfully done in pure form, followed by PCR confirmation. Further studies will be required to investigate the spreading and pathogenicity of MM in Egypt.

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## INTRODUCTION

Compared to other poultry species, turkeys are less susceptible to disease, have a higher market value with lower death rate **(Asaduzzaman et al. 2017**) they typically differ from other poultry species, especially in terms of weather adaptation and susceptibility to illness (**Anandh et al. 2012 and Jahan et al. 2018).**

*Escherichia coli* is the most prevalent disease-producing bacterium, which can reduce egg and meat production and ultimately cause the death of the turkeys (**Sackey et al. 2001 and Hafez 2005**).

*E. coli* can affect on the turkey production system by decreasing feed efficiency, slowing growth, increasing morbidity and mortality, and predisposing the turkeys to further infections **(Schmidt et al. 1988).**

Infections with Escherichia coli and *Salmonella* spp. have detrimental effects on turkey farming leading to decreased egg output, reduced hatchability, and increased death rates **(Kar et al. 2017)** *E. coli* and *Salmonella spp.* have been identified as the main causes of problems in turkeys (**Poppe et al. 2005 and Yeh et al. 2018).**

Avian colibacillosis caused by *E. coli* can result in symptoms such as cellulitis, coliseptic-emia, swollen head syndrome, synovitis, salpingitis, coligranuloma, osteomyelitis, omphalitis, peritonitis, and panophthalmitis. These symptoms, commonly cause mortality in turkeys (**Barnes and Gross 1997; Blanco et al. 1998** and **De Oliveira et al. 2020).**

*Salmonella* Infections in poultry can decrease hatchability, fertility and growth, while increasing mortality rates **(Andino and Hanning 2015).**

Poult enteritis and mortality syndrome (PEMS) is a condition of highs morbidity and mortality with an interaction between several known and might be unknown agents considered as a significant problem affecting turkeys and continues to cause severe economic losses worldwide**.** Several causative agents are implicated as the main causes of PEMS include enteroviruses (turkey corona virus (TCoV), turkey astroviruses (TAstV), reoviruses and adenoviruses), as well as bacteria such as *E. coli* and *Salmonella* **(Jindal et al. 2009 and Spackman et al. 2010).**

Turkeys are more susceptible to avian influenza viruses (AIV) compared to other domestic poultry species (**Karunakaran et al. 1983; Halvorson et al. 1985 and Pillai et al. 2010).**

The avian metapneumovirus (aMPV) is considered an important emerging pathogen. Just like other avian viruses. Turkeys and chickens are natural hosts for aMPV. Subtypes A and B cause disease in both chickens and turkeys, while aMPV-C primarily infects turkeys **(Yu et al. 2019).**

Co-infections of aMPV and other pathogens such as bacteria can lead to more severe clinical symptoms and increased mortalities (**Sid et al. 2015**). Field aMPV infection has been directly linked to colibacillosis-related mortality in turkeys (**Giovanardi et al. 2014) and** mortality rates of up to 6% within the first week of life (**Carver et al. 2000).**

Mycoplasma is egg transmitted bacteria can cause chronic respiratory disease in poultry, particulary when flocks are stressed or other respiratory pathogens are present. This disease is characterised by symptoms such as conjunctivitis, sneezing, and **sinusitis**, especially in turkeys. It can result in significant economic losses due to misshaping, and difficulties in marketing, spread of infection and retarded growth, and loss of egg production **(Mourad, 2023).** MG has been previously detected in turkey farms in Egypt through polymerase chain reaction (PCR) using mgc2 gene specific primers. When accompanied with *E. coli* infection it can impair immune response to vaccination **(Awad et al. 2019; Marouf et al. 2022 and Mourad 2023).** MM has been isolated from turkeys that suffering from infectious sinusitis in india **(Elsayed et al. 1981)** and from chickens exhibiting respiratory signs in Tunisia **(Béjaoui-Khiari et al. 2011)** and recently detected by PCR from turkey farms in Alexandia, Egypt **(Mourad, 2023).**

This study aimed to detection of microbial causes of mortalities in Behira, Egypt and genetic characterization of detected pathogens.

## MATERIALS and METHODS

### Ethical approval

Tracheal swabs were collected from diseased turkeys humanely while infraorbital sinus swabs and internal organs were collected from freshly sacrificed turkeys humanely following the regulations of the General Organization for Veterinary Services and Animal Health Research Institute, Giza, Egypt

### The collection of samples

Fifteen total samples involved one sample from pooled tracheal swabs was collected from turkey backyard flock showing 100% mortality and cyanosis of the neck region (Figure 1). This sample was examined for Avian influenza detection. Samples of infraorbital sinus swabs and internal organs (liver, kidney, heart and lung) were collected from freshly sacrificed turkeys from fourteen turkey farms suffering from infraorbital sinus swelling (Figure 1) and respiratory signs. These samples were collected during the period of 2021-2022 from Beheira governorate, with marked mortalities ranging between 7%-23% (Table 1). The internal organs were examined for bacterial isolation while infraorbital sinus swabs were examined for detection of MG, MM and aMPV.

Samples for bacteriological investigation were kept on ice box, then immediately transported to the laboratory. While samples for Avian influenza, aMPV, MG and MM were collected on PBS and transported on ice to the laboratory.

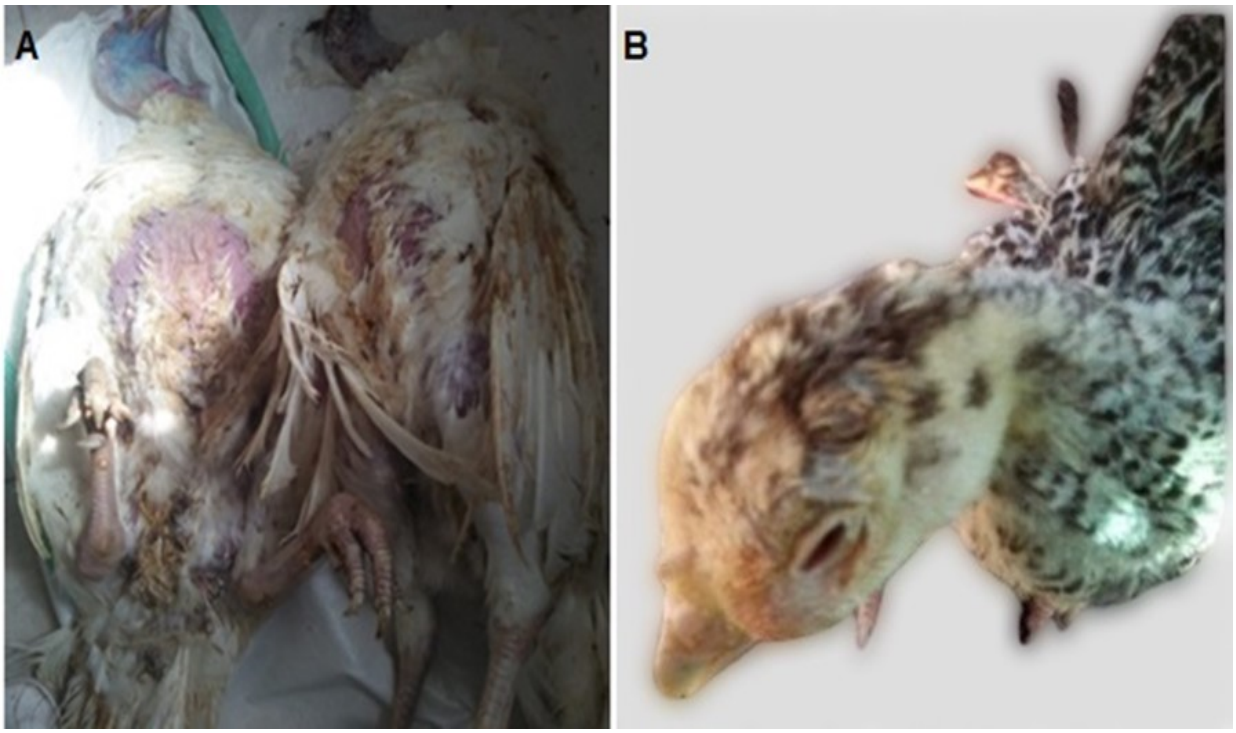


Figure 1: (A) A white turkey showing cyanosis in the head and neck regions with abdominal hemorrhage, (B) A bronze turkey poult showing swelling of the infraorbital sinus

Table 1. History of collected samples from infected turkey flocks.

| Sample No. | Age (day) | Clinical signs | Mortality % | Number | Breed | Type of rearing |
|---|---|---|---|---|---|---|
| 1 | 65 | Facial swelling, cyanosis, dehydration and respiratory distress, small hemorrhages throughout the body | 100% | 35 | White turkey | Backyard |
| 2 | 35 | Swelling of sinuses | 17 | 800 | Bronze turkey | Farm |
| 3 | 24 | Swelling of sinuses and nasal discharges | 18 | 1300 | Bronze turkey | Farm |
| 4 | 30 | Swelling of sinuses and nasal discharges | 12 | 1850 | Bronze turkey | Farm |
| 5 | 45 | Swelling of sinuses and nasal discharges | 14 | 2500 | Bronze turkey | Farm |
| 6 | 23 | Swelling of sinuses and nasal discharges | 17 | 2000 | White turkey | Farm |
| 7 | 18 | Swelling of sinuses and nasal discharges | 23 | 1600 | White turkey | Farm |
| 8 | 33 | Swelling of sinuses and nasal discharges | 12 | 3500 | Bronze turkey | Farm |
| 9 | 45 | Swelling of sinuses and nasal discharges | 15 | 2750 | Bronze turkey | Farm |
| 10 | 22 | respiratory distress, Diarrhea | 9 | 1300 | Bronze turkey | Farm |
| 11 | 40 | Swelling of sinuses and nasal discharges | 18 | 1000 | White turkey | Farm |
| 12 | 25 | Swelling of sinuses and nasal discharges | 10 | 3300 | White turkey | Farm |
| 13 | 24 | respiratory distress, Diarrhea | 7 | 2650 | Bronze turkey | Farm |
| 14 | 30 | Swelling of sinuses and nasal discharges | 17 | 1200 | White turkey | Farm |
| 15 | 22 | Swelling of sinuses and nasal discharges | 11 | 2650 | Bronze turkey | Farm |

## Bacterial isolation and identification

To isolate E. *coli,* MacConkey and Eosin Methylene Blue medium were utilized, furthermore biochemical tests (IMVIC) were performed as mentioned by **Quinn et al. (2002).**

The serological determination of the isolates was carried out using rapid antisera sets obtained from (DENKA SEIKENCo., Japan) as noted by **Kok et al. (1996).**

The detection of Salmonella was performed according to ISO 6579 – 1:2017/ Amd.1: 2020.

## Antimicrobial test

For antimicrobial susceptibility testing of 14 *E. coli* isolates, Agar disc diffusion method on Mueller Hinton Agar plates (HIMEDIA) was employed. Additionally, 10 widely used antibiotic discs were used according to **CLSI (2013)** guidelines.

## PCR detection of certain virulence genes in *E. coli* isolates, MG and MM

DNA extraction was performed on 10 *E. coli* isolates to detect 3 virulence genes and on 14 infraorbital sinus swabs samples to detect MG and MM using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to manufacturer‘s protocol.

PCR was conducted using EmeraldAmp Max PCR Master Mix (Takara, Japan) and specific primers listed in table 2.The thermal profile protocols were carried out as indicated in table 2. The reaction was performed in an Applied biosystems 2720 thermal cycler.

**Detection of aMPV and AI by reverse transcriptase PCR**

RNA extraction from pooled tracheal swabs was done to detect AI and from infraorbital sinus swabs to detect aMPV using Easy pure viral RNA extraction kit (Transgen biotech, China).

Reverse transcriptase-PCR for aMPV was carried out using AgPath-ID one step RT-PCR master mix and specific primers (table 2). The thermal profile protocols were conducted as in table 2. The reaction was performed in an Applied biosystems 2720 thermal cycler.

Real time rt-PCR for AI (H5, H9, N1 and N8 genes) was conducted using AgPath-ID one step RT-PCR master mix and specific primers and probes listed in table 2. The thermal profile protocols were conducted as stated in table 2. The reaction was performed in stepone plus real time PCR **(Applied biosystem).**

**Analysis of the PCR Products**

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gene ruler 100 bp ladder (Fermentas, Germany) was used as a size marker. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed using computer software.

**Genes sequencing**

Sequencing of HA and NA genes of positive AIV sample was performed using the SuperScript III Platinum One-Step RT-qPCR (Invitrogen, California, USA) and primers reported previously **Höper et al. (2009), Hoffmann et al. (2016) and Naguib et al. (2015)**, Additionally PCR product of three positive MG and MM samples were selected for DNA gene sequencing using same PCR primers.

Agarose gel electrophoresis was conducted to separate the gene-specific PCR amplicons. The amplicons were excised and purified from gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Cycle Sequencing was performed directly on the purified PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). The purified reaction products were then sequenced on an ABI 3500 XL Genetic Analyzer (Life Technologies, California, USA) using DyeEx® kit (Qiagen, Hilden, Germany).

**Genetic and phylogenetic characterization**

The acquired sequences were assembled, alligned and analyzed using Bioedit software. To determine the identity with other strains, Nucleotide sequences were submitted to BLAST searches[https:/ blast.ncbi.nlm.nih.gov/Blast.cgi] on NCBI platform.

Phylogenetic tree for the nucleotide sequences was constructed using Egyptian strains as well as other reference strains and vaccinal strains obtained from Genbank. The bootstrap approach with 1000 repeats based on maximum likelihood and Bayesian (BIC) analysis **(Sagulenko et al. 2018)** was employed to determine the topological correctness of the phylogenetic tree utilizing MEGA version 6 software (**Tamura et al. 2013**).The CLUSTAL-W tool and the MegAlign module of DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI, USA) were utilized to align the nucleotide sequences **(DNAStar 2001)**.

Table 2. Primers sequences, target genes, amplicon sizes and thermal profile of PCR

| Target agent | Target gene | Primers sequences | Product size (bp) | Reverse transcription | Primary denaturation | Amplification 35 cycles | | | Final extension | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Denaturation | Annealing | Extension | | |
| *E. coli* | *Iss* | F: ATGTTATTTTCTGCCGCTCTG<br>R: CTATTGTGAGCAATATACCC | 266 | | | | 54 ˚C 30 sec | | | Yaguchi et al., 2007 |
| | *iutA* | F: GGCTGGACATGGGAACTGG<br>R: CGTCGGGAACGGG-TAGAATCG | 300 | | | | 63 ˚C 30 sec | | | |
| | *eaeA* | F: ATGCTTAGTGCTGGTTTAGG<br>R: GCCTTCATCATTTCGCTTTC | 248 | - | | | 51 ˚C 30 sec | | 72 ˚C 10 min | Bisi-Johnson et al., 2011 |
| MG | *Mgc2* | F: CGC AAT TTG GTC CTA ATC CCC AACA<br>R: TAA ACC CAC CTC CAG CTT TAT TTCC | 300 | | | | 55 ˚C 30 sec | | | Lynsyansky et al., 2005 |
| MM | 16s rRNA | F: CGA GCGAAG TTT TTC GGAAC<br>R: GGTACC GTCAGG ATA AATGC | 422 | | | | 56 ˚C 30 sec | | | Lierz et al., 2008 |
| AMPV | *M gene* | F: GATGACTACAGCAAACTA-GAG<br>R: CTTCAGGACATATCTCGTAC | 468 | | | | 51 ˚C 30 sec | | | Shin et al., 2000 |
| *AI* | *HA* | *H5*<br>F: ACA TAT GAC TAC CCA CAR TAT TCA G<br>R: AGA CCA GCT AYC ATG ATT GC<br>Probe: FAM- ACA GTG GCG AGT TCC CTA GCA- TAMRA | - | 50 ˚C 30 min | 94 ˚C 5 min | 94 ˚C 30 sec | 54 ˚C 30 sec | 72 ˚C 30 sec | - | Slomoka et al., 2007 |
| | | *H9*<br>F: GGAA-GAATTAATTATTATTGGTCGGT AC<br>R: GCCACCTTTTTCAG-TCTGACATT<br>Probe: JOE-AACCAGGCCAGACATTGCGAG-TAAGATCC-TAMRA | - | | | | 54 ˚C 30 sec | | | Ben Shabat et al., 2010 |
| | *NA* | *N1*<br>F:TAYAACTCAAGGTTTGAGTC TGTYGCTTG<br>R: ATGTTRTTCCTCCAACTCTT-GATRGTGTC<br>Probe: FAM-TCAGCRAG-TGCYTGCCATGATGGCA- TAM-RA | - | | | | 55 ˚C 30 sec | | | Li et al., 2013 |
| | | *N8*<br>F: TCC ATG YTT TTG GGT TGA RAT GAT<br>R: GCT CCA TCR TGC CAY GAC CA<br>Probe: FAM-TCH AGY AGC TCC ATT GTR ATG TGT GGA GT-TAMRA | - | | | | 55 ˚C 30 sec | | | Hoffmann et al., 2016 |

### Isolation of Mycoplasma meleagridis

Positive MM samples were subjected to bacterial isolation and identification by inoculating them into Frey's broth. They were then incubated at 37°C with 5 to 10% $Co_2$ and humidity for 72 hours following the standard procedures of Sabry and Ahmed (1975). After incubation a loopful from each sample, was streaked on pleuropneumonia-like organisms (PPLO) agar with supplements (Glucose 10%, swine serum, yeast extract, cystine hydrochloride, nicotinamide adenine dinucleotide, 0.1% phenol red, Thalium acetate and penicillin G) **(WOAH, 2021)** using the drop technique. The agar plates were incubated at 37°C with 5 to 10% $Co_2$ with humidity for 3 days. The colonies were examined under a dissecting microscope for the characteristic fried egg appearance of Mycoplasma.To obtain a pure culture, one fried egg-shaped colony was selected and inserted into a broth medium along with the agar block. Purified isolates were stored at -20°C in the form of agar blocks.

## RESULTS

### *E. coli* isolation, serotyping and antibiotic resistance profile

*E. coli* was detected in all examined cases representing 100% of the samples. *Salmonella spp*. was not detected.

Serotyping of 10 randomly tested *E. coli* isolates revealed the presence of 6 serotypes as O78 (2 isolates), O141 (1 isolates), O2 (2 isolates), O11 (1 isolates), O21 (1 isolate) and O1 (1 isolate), Two isolates were untypeable.

Antimicrobial resistance testing was performed on the 14 identified *E.coli* isolates. The isolates showed high se -nsitivity to Gentamicin, Erythromycin and Ciprofloxacin (92.8%). Other tested antibiotics showed variable levels of sensitivity (Table 3).

### Molecular analysis of *E. coli* virulence genes

PCR analysis was conducted on 10 *E. coli* isolates to detect the iss, iutA and eaeA virulence genes. All of examined isolates tested positive for iss gene and iutA gene with a detection rate of 100% for both genes. The eaeA was detected in 30% of the isolates (Table 4).

Table 3. Antimicrobial pattern of *E.coli* isolates.

| Antimicrobial agents | Concentration. of disc | Symbol | (14 isolates) Sensitive Number | Resistant Number |
|---|---|---|---|---|
| Amoxicillin | 25 ug | AMX | 1 | 13 |
| Colistin sulfate | 30 ug | CT | 2 | 12 |
| Ciprofloxacin | 5 ug | CIP | 13 | 1 |
| Enrofloxacin | 5 ug | ENR | 10 | 4 |
| Erythromycin | 15 ug | E | 13 | 1 |
| Gentamicin | 30 ug | CN | 13 | 1 |
| Lincomycin | 2 ug | L | 9 | 5 |
| Norfloxacin | 10ug | NOR | 11 | 3 |
| Tetracycline | 30 ug | TE | 0 | 14 |
| Trime-thoprim+Sulphadiazine | 25ug | SXT | 1 | 13 |

Table 4. Results of PCR amplifications of different genes used with *E. coli* serogroups

| Sample | Serotype | Gene | | |
|---|---|---|---|---|
| | | ***Iss*** | ***IutA*** | ***EaeA*** |
| 1 | O78 | + | + | + |
| 2 | Untypeable | + | + | - |
| 3 | O141 | + | + | - |
| 4 | O2 | + | + | - |
| 5 | O2 | + | + | - |
| 6 | O21 | + | + | - |
| 7 | O11 | + | + | - |
| 8 | Untypeable | + | + | - |
| 9 | O1 | + | + | + |
| 10 | O78 | + | + | + |

## Molecular detection of AI, aMPV, MG and MM

AI (H5N8) and aMPV were detected in one sample while MM was detected in 6 samples and MG was detected in 11 sample by PCR .Mixed infection of *E. coli*, aMPV, MG and MM recorded in one sample while a mixed infection of E coli, MG and MM recorded in 5 samples, Additionally mixed infection of *E. coli* and MG only was recorded in 5 samples, and a single infection of *E. coli* was recorded in 3 samples only (Table 5).

Table 5. Results of *E. coli* and *Salmonella* isolation and PCR detection of aMPV, MG and MM.

| Sample no. | AI | *E. coli* | *Salmonella* | AMPV | MG | MM |
|---|---|---|---|---|---|---|
| 1 | +ve for H5 and N8 and -ve for H9 and N1 | NT* | NT | NT | NT | NT |
| 2 | NT | +ve | -ve | -ve | -ve | -ve |
| 3 | NT | +ve | -ve | -ve | +ve | +ve |
| 4 | NT | +ve | -ve | -ve | +ve | -ve |
| 5 | NT | +ve | -ve | -ve | +ve | -ve |
| 6 | NT | +ve | -ve | -ve | +ve | +ve |
| 7 | NT | +ve | -ve | +ve | +ve | +ve |
| 8 | NT | +ve | -ve | -ve | +ve | -ve |
| 9 | NT | +ve | -ve | -ve | +ve | +ve |
| 10 | NT | +ve | -ve | -ve | -ve | -ve |
| 11 | NT | +ve | -ve | -ve | +ve | +ve |
| 12 | NT | +ve | -ve | -ve | +ve | -ve |
| 13 | NT | +ve | -ve | -ve | -ve | -ve |
| 14 | NT | +ve | -ve | -ve | +ve | +ve |
| 15 | NT | +ve | -ve | -ve | +ve | -ve |

*NT: Not tested

**Genetic analysis**

Positive H5N8 AI sample was subjected to partial gene sequencing for HA and NA genes, This sequence was uploaded to the NCBI platform with name (A-Turkey-Egypt-DAM22-2022) and accession numbers OR502903 and OR502904 for HA and NA genes respectively. HA gene sequencing revealed that this virus is highly pathogenic, as it has numerous basic amino acid pattern PLREKRRKR/GLF at the HA cleavage site, The HA protein's receptor binding pocket of this strain contained the amino acids N182, G221, Q222, and G224 (H5 numbering), which may indicate a predilection for avian-like 2,3-sialic acid receptor binding. Like all Egyptian strains isolated in 2020–2021, our strain had a specific mutation (175L and 236D, 522A, 140A) in the HA gene, while the NA gene had mutations V106I, V201I, I213V, A245S, T265A, and T295M. No oseltamivir-resistant related substitutional amino acid mutations were detected in the NA. The HA and NA genes under study had amino acid and nucleotide identities of 98.3%-96-1% and 98.1%-95.9% to H5N8 viruses reported in Egypt from 2016 to 2021, respectively. It appears to have 80% similarity to Egyptian H5N1 (clade 2.2.1.1) and 97.6% similarity to new H5N1clade (2.3.4.4b). The HA gene has an amino acid identity of 86.9% to the rHVT-H5 vaccine (A/mute_swan/Hungary/4999/2006) and 75.5% to the activated vaccine (A-chicken-Hidalgo-28159-232-1994-H5N2) (Figure 2).

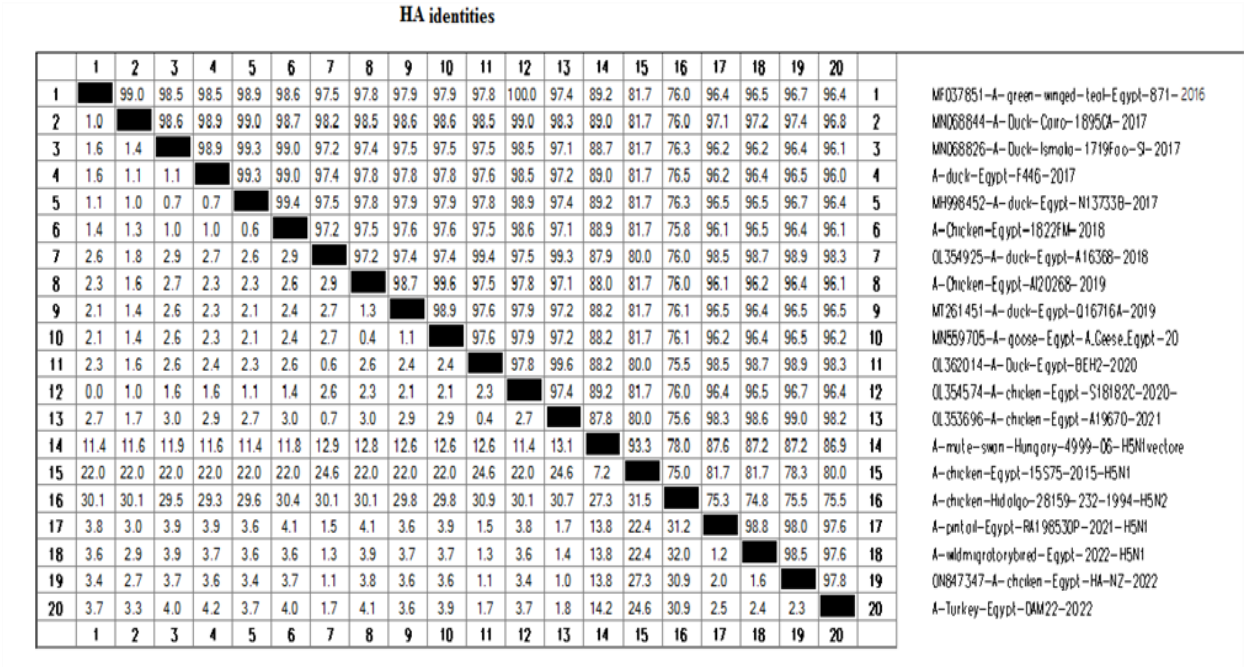


Figure 2. Identity percentage for nucleotide sequences of AI H5N8 strain A-Turkey-Egypt-DAM22-2022 and other related strains.

Phylogenetic analysis of The HA and NA genes of the Egyptian strain (A-Turkey-Egypt -DAM22-2022) revealed that this strain was grouped in clade 2.3.4.4b of subgroup II-B (Figure 3).

Three positive MG samples were selected for partial mgc2 gene sequencing of 225 base pair, these sequences were uploaded to NCBI platform with names Dam1, Dam2 and Dam3 and accession numbers OR757485, OR757486 and OR757487 respectively. They

were compared with other Egyptian strains and vaccine strains such as F strain, S6 strain, 6/85 strain and ts-11 strain. The identity percent between our strains and other Egyptian strains ranged from 96.5% to 100%. The highest identity percent with vaccinal strains was found with the ts-11 strain (96.1%-97.4%) while the lowest percent was with the 6/85 (81.9%) (Figure 4).

Phylogenetic analysis of these three strains in the study revealed that two of our strains (Dam1 and Dam2) were closely related with strains Eis5-C-10, Man-Reh.2-Mg-CK-EG016, Nouh-C-15 and Egypt-QA-53-2022, with accession numbers HQ591359, KY404987, KT992784 and OP660886 respectively.Dam3 strain was clustered with strain Heb.2-Mg-CK-EG017 and strain EGY-ORABI-Raheel-2020, with accession numbers MG676447 and MW679029 respectively (Figure 5).

For partial 16S rRNA gene sequencing of 330 base pair, three positive MM samples were chosen. These sequences named Dam4, Dam5 and Dam6 were uploaded to NCBI platform with accession numbers OR759962, OR759963 and OR759964 respectively. A comparison was made with other published Mycoplasma meleagridis strains and other Mycoplasma strains. It was discovered that these sequences were identical among themselves and with other published Egyptian Mycoplasma meleagridis strains such as Maryout -1, Maryout-2 and Maryout-3 which have the accession numbers MW700296, MW700297 and MW700298 respectively (Figure 6). Phylogenetic further analysis of our strains indicated that they are clustered together with other Mycoplasma meleagridis strains like strain 17529 and strain NBRC-14852, as well as other Egyptian M-meleagridis strains, while being separated from other Mycoplasma species (Figure 7).

**Isolation of Mycoplasma meleagridis**

Three positive samples which tested positive for both MG and MM were chosen for Mycoplasma meleagridis isolation. This isolation process resulted in numerous colonies with a distinctive fried egg appearance. Fifteen single colonies were selected and subsequently tested for MM and MG by PCR. Among of them three colonies tested positive for MM, while tested negative for MG, These colonies were reisolated, Producing colonies with the characteristic fried egg appearance once again (Figure 8).

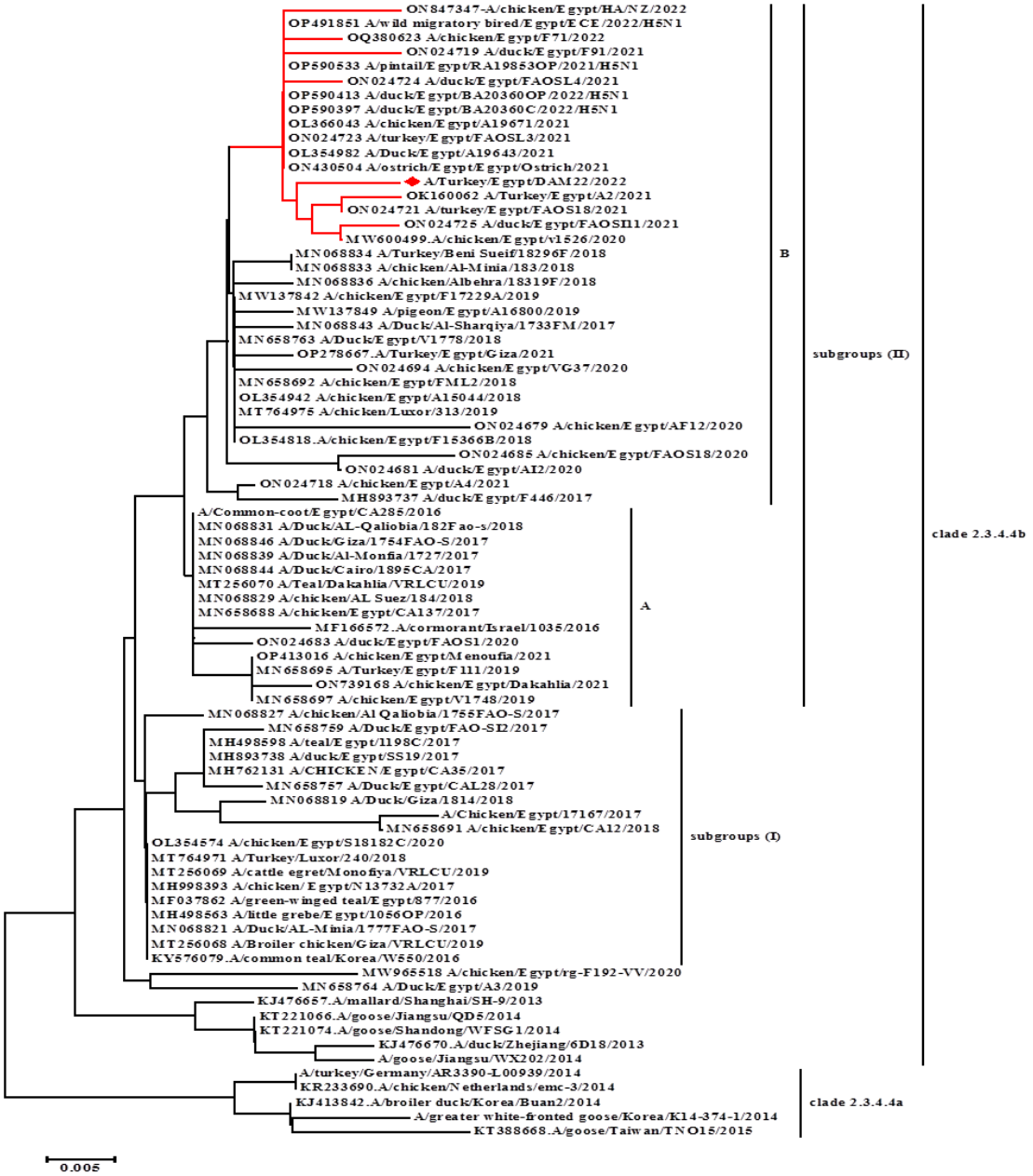


**Figure 3 .**Phylogenetic tree for the nucleotide sequence of Partial HA gene of Avian influenza H5N8 strain A-Turkey-Egypt-DAM22-2022 (labeled with black rhomboid) with other related strains using MEGA 6 software. Tree was constructed by the maximum-likelihood analysis using the distance-based method with bootstrap for1000 replicates.

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 99.6 | 98.3 | 98.3 | 98.3 | 98.7 | 100.0 | 97.4 | 93.5 | 95.3 | 100.0 | 100.0 | 100.0 | 100.0 | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 1 |
| 2 | 0.4 | | 97.8 | 97.8 | 97.8 | 98.3 | 99.6 | 97.0 | 93.1 | 94.8 | 99.6 | 99.6 | 99.6 | 99.6 | 95.7 | 97.4 | 89.2 | 96.1 | 95.7 | 81.9 | 2 |
| 3 | 1.8 | 2.2 | | 100.0 | 100.0 | 99.6 | 98.3 | 95.7 | 94.4 | 93.5 | 98.3 | 98.3 | 98.3 | 98.3 | 97.0 | 99.6 | 90.5 | 97.4 | 97.0 | 81.9 | 3 |
| 4 | 1.8 | 2.2 | 0.0 | | 100.0 | 99.6 | 98.3 | 95.7 | 94.4 | 93.5 | 98.3 | 98.3 | 98.3 | 98.3 | 97.0 | 99.6 | 90.5 | 97.4 | 97.0 | 81.9 | 4 |
| 5 | 1.8 | 2.2 | 0.0 | 0.0 | | 99.6 | 98.3 | 95.7 | 94.4 | 93.5 | 98.3 | 98.3 | 98.3 | 98.3 | 97.0 | 99.6 | 90.5 | 97.4 | 97.0 | 81.9 | 5 |
| 6 | 1.3 | 1.8 | 0.4 | 0.4 | 0.4 | | 98.7 | 96.1 | 94.8 | 94.0 | 98.7 | 98.7 | 98.7 | 98.7 | 97.4 | 99.1 | 90.9 | 97.8 | 97.4 | 82.3 | 6 |
| 7 | 0.0 | 0.4 | 1.8 | 1.8 | 1.8 | 1.3 | | 97.4 | 93.5 | 95.3 | 100.0 | 100.0 | 100.0 | 100.0 | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 7 |
| 8 | 2.7 | 3.2 | 4.6 | 4.6 | 4.6 | 4.1 | 2.7 | | 93.5 | 95.3 | 97.4 | 97.4 | 97.4 | 97.4 | 96.1 | 95.3 | 89.2 | 94.8 | 96.1 | 80.2 | 8 |
| 9 | 6.9 | 7.4 | 6.0 | 6.0 | 6.0 | 5.5 | 6.9 | 6.9 | | 96.6 | 93.5 | 93.5 | 93.5 | 93.5 | 94.8 | 94.0 | 87.9 | 93.5 | 94.8 | 78.9 | 9 |
| 10 | 5.0 | 5.5 | 6.9 | 6.9 | 6.9 | 6.4 | 5.0 | 5.0 | 3.6 | | 95.3 | 95.3 | 95.3 | 95.3 | 94.0 | 93.1 | 87.1 | 92.7 | 94.0 | 78.9 | 10 |
| 11 | 0.0 | 0.4 | 1.8 | 1.8 | 1.8 | 1.3 | 0.0 | 2.7 | 6.9 | 5.0 | | 100.0 | 100.0 | 100.0 | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 11 |
| 12 | 0.0 | 0.4 | 1.8 | 1.8 | 1.8 | 1.3 | 0.0 | 2.7 | 6.9 | 5.0 | 0.0 | | 100.0 | 100.0 | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 12 |
| 13 | 0.0 | 0.4 | 1.8 | 1.8 | 1.8 | 1.3 | 0.0 | 2.7 | 6.9 | 5.0 | 0.0 | 0.0 | | 100.0 | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 13 |
| 14 | 0.0 | 0.4 | 1.8 | 1.8 | 1.8 | 1.3 | 0.0 | 2.7 | 6.9 | 5.0 | 0.0 | 0.0 | 0.0 | | 96.1 | 97.8 | 89.7 | 96.6 | 96.1 | 81.9 | 14 |
| 15 | 4.1 | 4.6 | 3.2 | 3.2 | 3.2 | 2.7 | 4.1 | 4.1 | 5.5 | 6.4 | 4.1 | 4.1 | 4.1 | 4.1 | | 96.6 | 92.2 | 97.8 | 100.0 | 82.3 | 15 |
| 16 | 2.2 | 2.7 | 0.4 | 0.4 | 0.4 | 0.9 | 2.2 | 5.0 | 6.4 | 7.4 | 2.2 | 2.2 | 2.2 | 2.2 | 3.6 | | 90.1 | 97.0 | 96.6 | 81.5 | 16 |
| 17 | 9.9 | 10.4 | 8.9 | 8.9 | 8.9 | 8.4 | 9.9 | 10.4 | 11.9 | 13.0 | 9.9 | 9.9 | 9.9 | 9.9 | 6.9 | 9.4 | | 92.7 | 92.2 | 77.6 | 17 |
| 18 | 3.6 | 4.1 | 2.7 | 2.7 | 2.7 | 2.3 | 3.6 | 5.5 | 6.9 | 7.9 | 3.6 | 3.6 | 3.6 | 3.6 | 2.2 | 3.2 | 6.4 | | 97.8 | 84.1 | 18 |
| 19 | 4.1 | 4.6 | 3.2 | 3.2 | 3.2 | 2.7 | 4.1 | 4.1 | 5.5 | 6.4 | 4.1 | 4.1 | 4.1 | 4.1 | 0.0 | 3.6 | 6.9 | 2.2 | | 82.3 | 19 |
| 20 | 18.7 | 18.7 | 18.7 | 18.7 | 18.7 | 18.1 | 18.7 | 21.0 | 22.8 | 22.8 | 18.7 | 18.7 | 18.7 | 18.7 | 18.1 | 19.3 | 22.8 | 15.9 | 18.1 | | 20 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | |

1 OR757485-MG-Dam1
2 OR757486-MG-Dam2
3 OR757487-MG-Dam3
4 MG676447-MG-Heb.2-Mg-CK-EG017
5 MW679029-MG-EGY-ORABI-Raheel-2020
6 MG820791-MG-EGY1
7 KP691072-MG-Eid1.mg-TK-EG014
8 MW713809-MG-Maryout-3
9 MW713805-MG-Amria-A
10 MW713803-MG-Hawaria
11 HQ591359-MG-Eis5-C-10
12 KY404987-MG-Man-Reh.2-Mg-CK-EG016
13 KT992784-MG-Nouh-C-15
14 OP660886-MG-Egypt-QA-53-2022
15 KY421065-MG-Eis10-17
16 HQ591357-MG-Eis6-T-10
17 JQ770176-MG-F
18 JQ770175-MG-ts-11
19 JQ770177-MG-S6
20 KP318741-MG-6-85

Figure 4. Identity percentage for nucleotide sequences of three selected turkey MG strains and other related strains.

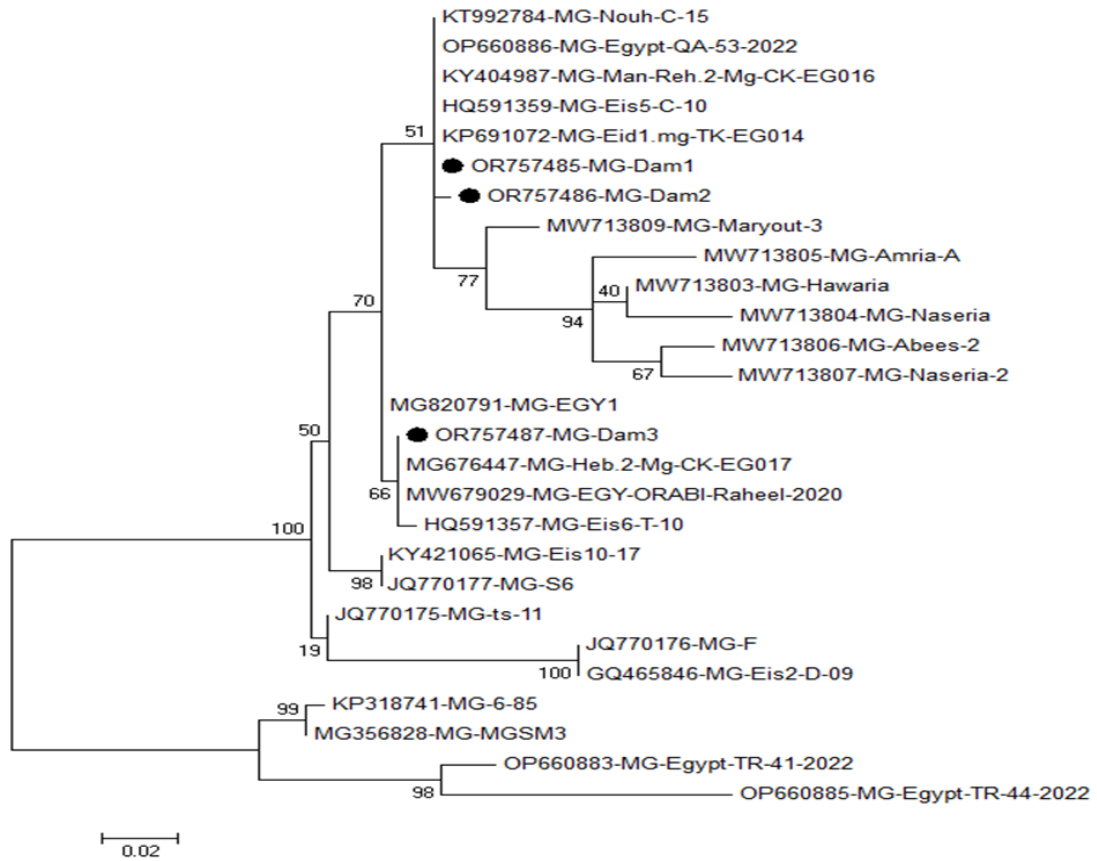


Figure 5. The phylogenetic tree of the nucleotide sequence of mgc2 gene from three MG strains (identified with black circles) alongside other related strains. Tree was constructed using MEGA 6 software through the maximum-likelihood analysis using the based on the distance method with bootstrap for 1000 replicates.

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 100.0 | 100.0 | 99.7 | 99.7 | 100.0 | 75.5 | 75.8 | 76.1 | 91.2 | 85.8 | 85.2 | 85.5 | 85.2 | 100.0 | 100.0 | 100.0 | 100.0 | 91.5 | 92.4 | 1 | OR759962-M-meleagridis-Dam4 |
| 2 | 0.0 | | 100.0 | 99.7 | 99.7 | 100.0 | 75.5 | 75.8 | 76.1 | 91.2 | 85.8 | 85.2 | 85.5 | 85.2 | 100.0 | 100.0 | 100.0 | 100.0 | 91.5 | 92.4 | 2 | OR759963-M-meleagridis-Dam5 |
| 3 | 0.0 | 0.0 | | 99.7 | 99.7 | 100.0 | 75.5 | 75.8 | 76.1 | 91.2 | 85.8 | 85.2 | 85.5 | 85.2 | 100.0 | 100.0 | 100.0 | 100.0 | 91.5 | 92.4 | 3 | OR759964-M-meleagridis-Dam6 |
| 4 | 0.0 | 0.0 | 0.0 | | 99.7 | 99.7 | 75.2 | 75.5 | 75.8 | 90.9 | 85.5 | 84.9 | 85.2 | 84.9 | 99.7 | 99.7 | 99.7 | 99.7 | 91.2 | 92.1 | 4 | AB680683-M-meleagridis-NBRC-14852 |
| 5 | 0.3 | 0.3 | 0.3 | 0.0 | | 99.7 | 75.2 | 75.5 | 75.8 | 90.9 | 85.5 | 84.9 | 85.2 | 84.9 | 99.7 | 99.7 | 99.7 | 99.7 | 91.2 | 92.1 | 5 | U04649-M-meleagridis-17529 |
| 6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | | 75.5 | 75.8 | 76.1 | 91.2 | 85.8 | 85.2 | 85.5 | 85.2 | 100.0 | 100.0 | 100.0 | 100.0 | 91.5 | 92.4 | 6 | MW700292-M-meleagridis-Amria-K |
| 7 | 27.1 | 27.1 | 27.1 | 27.2 | 27.6 | 27.1 | | 98.5 | 98.5 | 71.6 | 74.0 | 74.0 | 74.0 | 73.7 | 75.5 | 75.5 | 75.5 | 75.5 | 71.6 | 74.0 | 7 | MW647914-M-gallisepticum-AAAQB4 |
| 8 | 27.4 | 27.4 | 27.4 | 27.5 | 27.8 | 27.4 | 0.3 | | 99.7 | 71.9 | 73.7 | 73.7 | 73.7 | 73.4 | 75.8 | 75.8 | 75.8 | 75.8 | 71.9 | 74.0 | 8 | MT363785-M-gallisepticum-NI-3 |
| 9 | 27.4 | 27.4 | 27.4 | 27.5 | 27.8 | 27.4 | 0.0 | 0.0 | | 72.2 | 74.0 | 74.0 | 74.0 | 73.7 | 76.1 | 76.1 | 76.1 | 76.1 | 72.2 | 74.0 | 9 | MH986184-M-gallisepticum-Diwanyiah-IQ-N |
| 10 | 8.9 | 8.9 | 8.9 | 9.0 | 9.3 | 8.9 | 34.4 | 34.6 | 34.6 | | 85.8 | 83.1 | 83.4 | 83.1 | 91.2 | 91.2 | 91.2 | 91.2 | 88.8 | 90.3 | 10 | JN935884-M-gallinarum-ATCC-15319 |
| 11 | 14.2 | 14.2 | 14.2 | 14.2 | 14.6 | 14.2 | 30.5 | 30.9 | 30.9 | 15.0 | | 89.4 | 89.1 | 88.8 | 85.8 | 85.8 | 85.8 | 85.8 | 83.7 | 85.2 | 11 | NR_171433-M-hafezi-M26 |
| 12 | 15.6 | 15.6 | 15.6 | 15.6 | 15.9 | 15.6 | 31.2 | 31.5 | 31.5 | 19.3 | 11.1 | | 99.7 | 99.4 | 85.2 | 85.2 | 85.2 | 85.2 | 81.3 | 80.4 | 12 | MH539117-M-synoviae-B458-15-1 |
| 13 | 15.6 | 15.6 | 15.6 | 15.6 | 15.9 | 15.6 | 30.8 | 31.2 | 31.2 | 19.3 | 11.1 | 0.0 | | 99.7 | 85.5 | 85.5 | 85.5 | 85.5 | 81.6 | 80.7 | 13 | MH539051-M-synoviae-B2777-15A-7-2 |
| 14 | 15.9 | 15.9 | 15.9 | 16.0 | 16.3 | 15.9 | 31.3 | 31.7 | 31.7 | 19.7 | 11.5 | 0.3 | 0.3 | | 85.2 | 85.2 | 85.2 | 85.2 | 81.3 | 80.4 | 14 | MG846121-M-synoviae-EB44 |
| 15 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 27.1 | 27.4 | 27.4 | 8.9 | 14.2 | 15.6 | 15.6 | 15.9 | | 100.0 | 100.0 | 100.0 | 91.5 | 92.4 | 15 | MW700298-M-meleagridis-Maryout-3 |
| 16 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 27.1 | 27.4 | 27.4 | 8.9 | 14.2 | 15.6 | 15.6 | 15.9 | 0.0 | | 100.0 | 100.0 | 91.5 | 92.4 | 16 | MW700297-M-meleagridis-Maryout-2 |
| 17 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 27.1 | 27.4 | 27.4 | 8.9 | 14.2 | 15.6 | 15.6 | 15.9 | 0.0 | 0.0 | | 100.0 | 91.5 | 92.4 | 17 | MW700296-M-meleagridis-Maryout-1 |
| 18 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 27.1 | 27.4 | 27.4 | 8.9 | 14.2 | 15.6 | 15.6 | 15.9 | 0.0 | 0.0 | 0.0 | | 91.5 | 92.4 | 18 | MW700295-M-meleagridis-North-coast |
| 19 | 8.6 | 8.6 | 8.6 | 8.6 | 9.0 | 8.6 | 32.6 | 32.9 | 32.9 | 11.3 | 16.3 | 20.2 | 20.2 | 20.7 | 8.6 | 8.6 | 8.6 | 8.6 | | 92.1 | 19 | EU859979-M-columbinum-FG295 |
| 20 | 7.8 | 7.8 | 7.8 | 7.9 | 8.2 | 7.8 | 29.8 | 30.5 | 30.1 | 9.6 | 14.5 | 21.7 | 21.7 | 22.1 | 7.8 | 7.8 | 7.8 | 7.8 | 7.5 | | 20 | MH539111-M-iners |

Figure 6. Identity percentage for nucleotide sequences of three selected MM strains and other related strains.

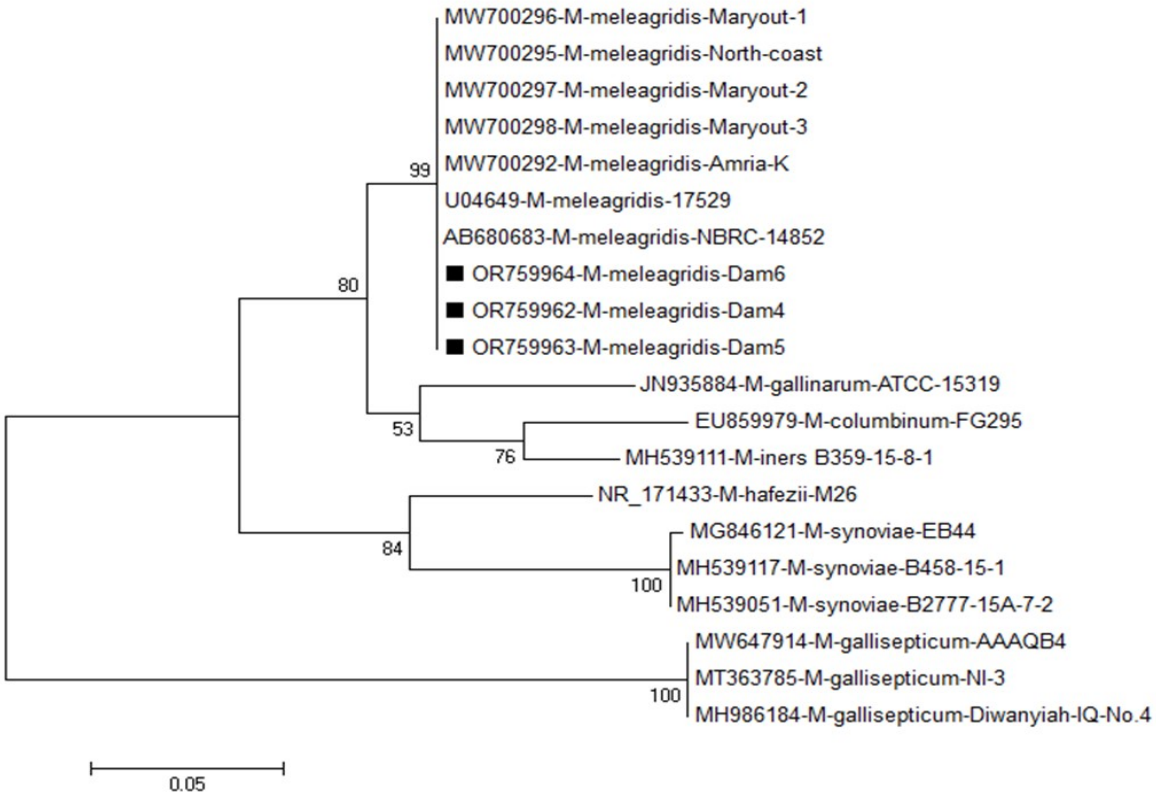


Figure 7. The phylogenetic tree for the nucleotide sequence of the partial 16s rRNA gene of three MM strains (labeled with black rectangulars) along with other mycoplasma strains using MEGA 6 software. The tree was constructed using maximum-likelihood analysis through the distance-based method with bootstrap for1000 replicates.

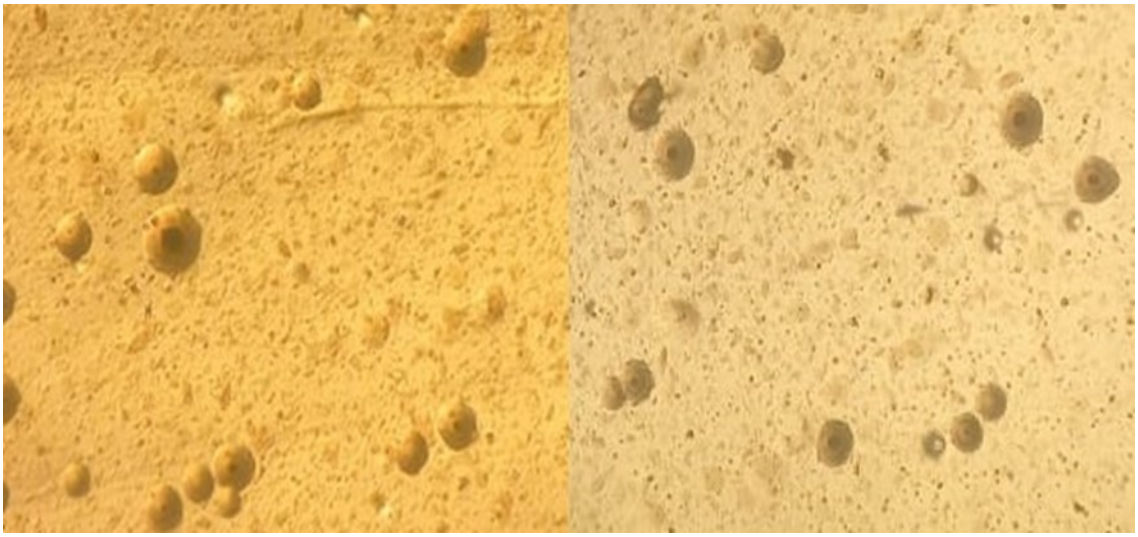

Figure 8. Microscopical appearance of M. meleagridis with characteristic fried egg appearance with depressed center colonies.

## DISCUSSION

Many bacterial and viral pathogens can cause mortalities in turkey flocks leading severe economic losses. In this study E. coli, *Salmonella*, AI, aMPV, MG and MM were investigated among diseased turkey flocks in Behira governorate in Egypt.

One of the most significant bacterial causes of financial loss in the global turkey industry is colibacillosis (**Hafez 2005)** by *E.coli*, which was detected in high rates as 100% during our investigation this contrasts with the finding of **Kundu et al. (2021)** who reported a lower rate of E. coli isolation from fecal and cloacal swabs collected from turkey farms at percentage 62.22% as positive *E.coli* isolates. Another study of **Zhao et al. (2001)** found an even lower isolation rate of only 11.9% *E. coli* infection in a commercial turkey flocks.

Regarding *Salmonella* isolation, all our results were negative to disagreeing with **Jahantigh et al. (2015)** who registered incidence of 14.8% of Salmonella *sp*.

Our results of *E.coli* serotypes was agree with **Nolan et al. (2013) and Giovanardi et al., (2013)** as they implicated serotypes O1, O2, and O78 as the most often associated with poultry diseases including turkeys and often represent the majority of isolated *E.coli* serogroups indicating the importance of monitoring and controlling these pathogens in turkey flocks to prevent economic losses. These serogroups have been detected in outbreaks dating back to the 1960s (**Altekruse et al. 2002**), Additionally **AbdelRahman et al. (2020)** reported the presence of several serotypes mainly O78, O28, O29, O168, O125, O158, and O115 in imported turkey poults.

Our findings are in line with earlier studies (**Yang et al. 2004; Zhao et al. 2005; Gosling et al. 2012 and Russo et al. 2012)** that found varying degrees of antibiotic resistance in *E. coli* strains isolated from poultry. The highest rate of resistance was found in Tetracycline at percentage 100% followed by Amoxicillin and Trimethoprim+Sulphadiazine at percentage 92.8% for each. This agree with the finding of **Kundu et al. (2021)** who reported 100% resistance for tetracycline, Amoxicillin and Trimethoprim, separately. However, **AbdelRahman et al. (2020)** reported the highest rate of resistance found to cefotaxime (60.5%), tetracycline (44.7%) and Sulpha trimethoprim (42.1%).The greatest sensitivity reported in our investigation was 92.8% for Gentamicin ,Ciprofloxacin and Erythromycin, This agree with **Kundu et al. 2021** in case of gentamicin, where 100% of examined isolates were sensitive ,However in case of Erythromycin, all isolates were resistant (100%),

which completely contradict our results.

During our study the iss gene was present at 100% of examined isolates which is similar to 93% reported by **Cunha et al. (2014)** in isolates from turkeys with colibacillosis. Lower rates were reported by **de Oliveira et al. (2020)** as 66% in systemic isolates and **Al-Kandari and Woodward (2019)** found a rate of 55% during the analysis of *E. coli* from cecal swabs.

In our investigation the Iuta gene was found at high rate of 100%, compared to 64% found by **Carli et al. (2015)**. A lower rate of 46% was found by **de Oliveira et al. (2020)**.

The aMPV was detected in one sample out of fourteen samples in this study, The low incidence may be due to its shedding from infected birds only a few days after infection without latency **(Kaboudi and Lachheb, 2021**). Previous studies reported APMV subtype A and B from turkey farms from Egypt by rt-PCR and partial fusion gene sequencing **(Arafa and Fahmy 2009 and Abdel-Azeem et al. 2014)**

In this work AI subtype H5N8 recorded in one sample that taken from backyard turkey flock suffering from 100% mortality. When subjected to partial HA sequencing, it showed higher identity with AI H5N8 clade 2.3.4.4b and lower identity with vaccinal strains (rHVT -H5 and inactivated H5N2 vaccines). However previous research has shown that a single immunization with the vectoremune-H5N1 vaccine is successful in preventing HPAIV H5N8 (2.3.4.4b) transmission among vaccinated chicken populations **Palya et al. (2018)**. Previous study detected AI H5N8 from two vaccinated turkey flocks in Egypt and it also belonged to clade 2.3.4.4b **Salaheldin et al. (2022).** Although AI H5N8 was the dominant AI strain in Egypt during 2019-2021 **Hagag et al. (2022)**, AI H5N1 clade 2.3.4.4b emerged in Egypt during 2023 **Mosaad et al. (2023).**

Additionally, this study found that MG was detected in 11 out of 14 samples (78%) using PCR directly from the infraorbital sinus swabs without prior isolation. All cases of MG detection were accompanied by *E. coli* infection. Previous experimental coinfection of MG and *E. coli* has been shown to cause more severe tissue lesions compared to single infection, with increased of inflammatory chemokines and immuosuppression **(Wu et al. 2019 and Awad et al. 2019)**. Other studies in Egypt have also detected MG in turkey with higher incidence, ranging from 70% to 83% in examined samples (**Marouf et al. 2022 and Mourad 2023)**. Phylogenetic analysis of 3 strains in this study revealed they are clustered in two branches and showed high identity with other Egyptian strains and ts-11 and 6/85 vaccinal strains. This results agreed with recent studies (**Marouf et al. 2022 and Qoraa et al. 2023).**

Furthermore this study detected MM in six samples all of which accompanied by *E. coli* and MG infections. MM was recently detected in seven turkey flocks in Alexandria, Egypt by **Mourad (2023).** This study is the first to isolate of MM in Egypt from about thirty years as the only record of its isolation was done by El-**Ebeedy et al. (1984)** who isolate MM from turkey samples in Upper Egypt. Phylogenetic analysis showed that the three stains in this study clustered with other Egyptian strains and had 100% identity with them suggesting that one strain may be circulating in different Egyptian governorates causing clinical signs and mortality. These results drew attention to the newly detected organism and necessitated the application of control measures to prevent its spread, this includes testing of imported one -day-old turkey poults for MM using validated serological, bacteriological and molecular tests. These tests should be conducted on a representative sample.

## CONCLUSSION

This study discusses some viral and bacterial causes of mortality in turkey flocks in Behira, Egypt. Both the AI H5N8 virus and aMPV were detected in one flock, *E. coli*, MG and MM were detected in 14, 11 and 6 flocks, respectively. *E. coli* was isolated with six different serotypes: O1, O2, O11, O21, O78 and O141. These strains showed high resistant to Tetracycline, Amoxicillin and Trimethoprim+Sulphadiazine. Genetic analysis of MG and MM exhibited a high identity percentage with other published Egyp-

tian strains. Surveillance with analysis of detected pathogens should be done for diseased turkeys from different areas in Egypt. This will help monitor bacterial or viral evolution and implement control measures to stop the spread of diseases. It is also important to select proper vaccines to protect healthy flocks and prevent economic losses.

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