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# ROLE OF VARIOUS SPECIES OF MYCOPLASMA AND BACTERIA IN TURKEY'S SINUSITIS WITH DESCRIPTION OF PATHOLOGICAL PICTURE

(With 5 Tables, One Figure and 15 Photos)

By

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## دور الميكوبلازما والبكتيريا في التهاب الجيوب الانفية للرومي مع وصف الصورة الباثولوجية

***فادية عبد الحميد ، دينا الشافعي ، نجاح عبد الدايم ، ليلى طنطاوي***

الغرض من العمل المقدم كان معرفة الاسباب المحتملة المسئولة عن التهاب الجيوب الأنفية في طيور متعددة الأعمار مع نفوق أجنة البيض المخصب بمزرعة رومي بمحافظة المنيا بصعيد مصر. أوضح الفحص الاكلينيكي ودراسة الافات التشريحية لبعض الطيور النافقة والاختبارات السيرولوجية المصحوبة بطرق العزل التقليدية مع استخدام البيولوجيا الجزيئية المتمثلة في اجراء اختبار انزيم البلمرة المتسلسل التفاعلي ( PCR ) أن ميكروب الميكوبلازما جاليسيبتكم ( *Mycoplasma gallisepticum* )– اما منفردا أو مقترنا بميكروبات ممرضة أخرى (باستيرلا مالتوسيدا والايشيريشيا كولاي ( *Pasteurella multocida – Escherichia coli* ) – كان هو الميكروب السائد المسئول عن الأعراض التنفسية بالقطيع الرومي. من ناحية اخرى مهد تواجد ميكروب الميكوبلازما جاليسيبتكم في الجهاز التنفسي لغزو ميكروب الباستيريلا للجهاز التنفسي والانتشار مما زاد من حدة الأعراض التنفسية بالطيور المصابة والذي ايضا ساعد على زيادة معدل افراز الميكوبلازما عبر البيض التي قلصت معدل الفقس مع نفوق الأجنة. هذا وقد تم انجاز عدوى تجريبية لبعض بيض الرومي المخصب الخالي من الميكروبات الممرضة باحدى عترات الميكوبلازما المعزولة من بيض القطيع المصاب وتم دراسة الافات التشريحية للأجنة والهستوباثولوجيا. أظهرت العدوى التجريبية ان نفوق ٢٨ % من البيض المحقون بعد ثمانية أيام من حقن العدوى وكان الورم الاوديمي المسهب مع ظهور أعراض التقزم خاصة قصر الارجل مع الزيادة الواضحة باغلفة الأجنة. ولوحظ أيضا زيادة سمك اغلفة الأجنة بزيادة ايام التحضين (تقدم الاصابة). هذا وقد تم سرد نتائج دراسة الهستوباثولوجي للأجنة المحقونة تجريبيا في الايام المختلفة بإسهاب.

## SUMMERY

The current work was carried out to clear-up the probable causes responsible for signs of infraorbital sinusitis with embryonic deaths of the fertile eggs in a multiple-age turkey's farm located in El-Minia Governorate, Upper Egypt. Clinical and necropsy examinations, and serological testing in association with conventional culturing procedures and molecular diagnosis (PCR) indicated that *Mycoplasma gallisepticum (MG)*, either alone or coupled with other pathogens (*Pasteurella multocida* and *Escherichia coli*) was the predominant etiologic agent responsible for the respiratory problems including infraorbital sinusitis of the infected turkey flocks. The presence of *MG* infection in upper and lower respiratory tracts facilitates the portal entry of *Pasteurella multocida* infection to invade the lower respiratory tract and distributed systematically. This may increased the severity of the respiratory signs and increased the shedding rate of the in-ovo transmission of *MG* infection, which reduced the hatchability rate with dramatic embryonic death of the fertile eggs. Experimentally, in-ovo infection in fertile turkey's eggs with an *MG*-isolate isolated from the examined fertile eggs and organs of the naturally infected flock was carried out. Gross lesions and the histopathological alterations of the inoculated embryos were monitored during the period of experiment. Eight-days post inoculation, 28 % of the inoculated fertile eggs were succumbed. Diffuse edematous swellings and signs of dwarfisms particularly shortening legs (micromelia), in association with hyper-thickening of the membranes were obviously remarked in the inoculated embryos. The thickening of membranes was increased by increasing the days of incubation. Histopathological examinations of the experimentally inoculated embryos with *MG* (on the 3$^{rd}$, 6$^{th}$ and 8$^{th}$ days post-inoculation) were described in detail.



***Key words:** Turkey infectious sinusitis, embryonic death, Mycoplasma gallisepticum, Pasteurella multocida, In-ovo experimental infection*


## INTRODUCTION

*Mycoplasma gallisepticum* was frequently incriminated as an important pathogen affecting chicken, turkeys and other avian species causing a considerable level of economic losses on farms. These losses included chronic respiratory disease, reduced feed conversion, and decreased egg production (Ali and Youssef, 2003; Bradbury, 2006,

Büyüktanir *et al.,* 2008 and Nicholas *et al.,* 2009). Moreover, *MG* is a serious infection transmitted horizontally and vertically, which plays an outstanding role in decreasing the hatchability rates of the fertile eggs of chicken and turkeys with embryonic death (Lin and Kleven, 1982; Abd-El-Rahman, 1995; OIE, 2004; Alessandri *et al.,* 2005 and Bradbury, 2006). Respiratory problem is a complex problem, where different microorganisms coupled with the sublevel of hygienic measures are simultaneously interacted (OIE, 2008). However, *MG* considers an outstanding cause of respiratory problems and infectious sinusitis on chickens and turkeys farms particularly in endemic areas (Abdelhameed, 2000, Nascimento *et al.,* 2005; Abdelhameed, 2006; Bradbury, 2006, and Büyüktanir *et al.,* 2008).

In Egypt, Sokkar *et al.* (1986) reported that 32 % and 30 % of the bacteriologically examined samples (tracheas, sinuses and air sacs) of turkeys located in different areas of Upper Egypt were culturally positive to *M. gallisepticum* and *M. meleagridis,* respectively. Their results also indicated that *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella gallinarun pullorum* were also isolated with Mycoplasmas. Nine years later, Abd-El-Rahman (1995) studied the incidence of Mycoplasma infections in different turkey's flock of different ages and revealed that *MG*, *M. meleagridis* and *M. synoviae*, and *M. Iowa* were serologically determined. The author concluded that *MG* was the predominant detected mycoplasmas (90 %) of the examined turkeys' flocks. These results may refer to the spread of *M. gallisepticum* among turkey's farms rather than *M. meleagridis*. Conversely, the offered results by Eissa *et al.* (2000) diminish the significant role of *MG* infection as a cause of infraorbital sinusitis in turkey and increase the role of *M. synoviae*. They indicated that the isolation rate of *M. gallisepticum* and *M. synoviae* from the aspirated exudates of diseased turkeys was 6.66 % and 13.33 %, respectively.

A multiple-age turkey farm located in El-Minia Governorate (May 2007 to Feb. 2008), showed signs of respiratory problem with gradual increasing in the population morbidity rate of the affected turkeys. Moreover, some of the affected birds showed various stages of swelling of the infraorbital sinuses, and others were succumbed. On the other side, the practitioners of the farm noticed that the rate of hatchability of the fertile eggs is significantly reduced with a considerable level of embryonic death. Several visits to the respective farm were carried out to clear-up the probable etiologic agent(s) responsible for the respiratory problem of the affected birds and the

cause(s) of embryonic deaths of the fertile eggs, where Mycoplasma infection either alone or coupled with other pathogens was suspected. On the other hand, an experimental infection with an isolated *Mycoplasma* in turkey's fertile eggs was achieved and the gross lesion and histopathological alterations of the experimentally infected embryos were monitored.

## MATERIALS and METHODS

**Farm:**

A commercial multiple-breed and multiple-age turkey farm located at El-Minia Governorate; Upper Egypt, was visited based on a complaining of respiratory signs including infraorbital sinusitis, with remarkable reduction of the hatchability rate of the fertile eggs. The examined flock contains 250 (male and female) mature birds of different ages; approx. 30% of them were at the beginning of egg-production, used for production of fertile eggs. At the end of the examined flock, there were 63 young growing birds (6 – 9 weeks of age) kept as a separate unit separated from the others by wire separation. All birds bred on litter system and fed on a commercial ration. The eggs were collected manually, candled for fertility, incubated and hatched in a separate hatching unite on farm. Vaccination schedule was carried out according to vaccines' manufacturers and no vaccination attempted against Mycoplasma infection. The level of sanitary measures of the examined flock was sublevel.

**Samples collection and sampling procedures:**

**I – Mycoplasma:**

**I.A - Detection of Mycoplasma:**

**I.A.1 – Serological detection:**

One percentage of the examined flock turkeys was selected based on the appearance and/or disappearance of clinical signs. Of these selected birds, 10 turkeys showed signs of infraorbital sinusitis, 5 birds showed respiratory sings in form of nasal discharges and coughing, and the remainders (n = 10) were apparently healthy. Blood sera of the selected birds were collected and serologically tested by serum plate agglutination test (SPAT) using a commercial antigens (NobilisReg., Intervet International B.V. Boxmeer Holland®).

**I.A.2 – Culturing detection:**

**I.A.2.a –Tracheal Swabs:**

Tracheas of the serologically tested birds (n = 25) were swabbed and cultured into Frey's liquid medium (FM) (Frey *et al.*, 1968), swine serum was replaced by normal horse serum, and incubated until changing color of the indicator (phenol red, from red to orange or yellow). The positive samples were kept at – 20°C. The apparently negative samples (uncolored samples) were subjected to 3 serial blind passages at 5 – 7 days intervals (Abdelhameed, 2006). The sample considered negative after three blind passages.

**I.A.2.b –Exudates of infraorbital lesions:**

In addition to the serologically tested adult turkeys (n = 10), seven birds of the growing (6 - 9 weeks of age) turkeys with signs of infraorbital sinusitis were also selected and their lesions were incised and swabbed. The swabs were cultured into FM containing a much amount of inhibitors and incubated until a color change of the indicator from red to orange or yellow. The positive samples were stored at – 20°C.

**I.A.2.c – Fertile and Infertile Eggs:**

Twenty-five eggs of the infected flock were randomly selected from the hatchery room on farm. The collected eggs were primarily candled and categorized into infertile and fertile, and subjected to Mycoplasma culturing examinations (Abdelhameed, 2006). The positive Mycoplasma samples were stored at – 20 °C.

**I.A.2.d - Tracheas, lungs, air sacs of recently succumbed birds:**

Seven of the recently succumbed birds during the period of investigation were necropsied and portions of their respiratory tracts were aseptically excised. Culturing examination of the collected specimens was done as described by Abdelhameed (2000). The positive samples were stored at –20 °C.

**I.B – Identification of Mycoplasma:**

The frozen positive broth samples were thawed and platted onto FM agar and incubated with regularly examined. Agar blocks of the positives plate with the characteristic shape of Mycoplasma colonies were picked-up and thereafter cloned. The purified strains of Mycoplasma were subjected to digitonin-tolerance test. The digitonin-sensitive isolates were biochemically tested (glucose fermentation and arginine deamination). The isolated Mycoplasma strains were molecularly identified using polymerase chain reaction (PCR). Based on

the results of SPAT test and biochemical profile of the purified isolated Mycoplasma strains, PCR procedure was trended to molecular detecting of MG infection of the examined samples.

**I.B.a – PCR Procedure:**

PCR—assay carried out according to the protocol illustrated by Kempf *et al.* (1993). Ten-isolated of Mycoplasma strains (4 isolates from eggs, 3 from infraorbital lesions, 2 isolates from respiratory tracts and one from the apparently healthy bird) randomly selected and cultured onto PPLO broth medium, incubated at 37°C for five days. Post-incubation, 1 ml of each broth was pipetted, drawn in an epindorff tube, and centrifuged for 20 minutes at 14000 xg. The pellet washed 3 times in a phosphate buffer saline. The washed pellet was re-suspended in a mixture consisted of an equal amount of 100 µl solution A (10 mM Tris HCL pH, 8.3 containing 100mM KCL and 2.5 mM $MgCl_2$) and 100 µl solution B (10 mM Tris HCL, pH 8.3 containing 2.5 mM $MgCl_2$, 1 % Tween 20, 1 % Triton X-100 and 120 µg/ml of proteinase K). The suspension was incubated at 60 °C for one h., and then incubated at 95°C for 10 minutes.

Small-subunit rDNA of *MG* was amplified using a forward primer RANG1 which corresponds, to nucleotide positions 173 to 195 (5'–TAA CTA TCG CAT GAG AAT AAC-3') of *Escherichia coli* 16S rRNA and a reverse primer RANG2 corresponding to the complement of positions 502 to 481 (5'–GTT ACT TAT TCA AAT GGT ACA G – 3'). The PCR—assay was performed with a thermostable taq DNA polymerase (Boehringer Mannheim) in an automated DNA thermal cycler. The reaction mix (total volume 45 µl) was 10 mM Tris HCL pH 8.3, 2.5 mM MgCl2, 50 mM KCl, 0.01 % gelatin, 0.2 mM each dNTP, 50 pM each primer, 2.5 nM tetra-methyl-ammonium chloride. The sample to be analyzed (5 µl) was added last. PCR was performed on a Perkin Elmer Gene Amp™ PCR system. Following initial 1-minute incubation at 90 °C, thermal cycling (40 cycles) proceeded with each segment of one cycle being: 95 °C for 15 second, 60 °C for 20 second, and 75 °C for 15 second. At final step, an additional cycle (95 °C for 15 second, 60 °C for 45 second and 75 °C for 5 minutes) was included (Kempf *et al.*, 1993). After amplification, the amplified products (10µl) were subjected to electrophoresis through 2% agarose gel and stained by ethidium bromid. Thereafter DNA was visible under UV lamp and the visible lanes sized by DNA ladder molecular marker conceded as positive isolates.

**II – Bacteria:**

**Bacterial sampling and culturing procedures:**

At the same time of Mycoplasma examination, the collected samples were also cultured onto 10 % sheep blood agar, serum dextrose agar and MacConkey agar plates. The cultured plates were aerobically incubated at 37 °C for 24 – 48 hours and examined. The suspected colonies were picked-up, purified and morphologically and biochemically identified by conventional procedures according to the protocols illustrated by Quinn *et al.* (1994) and Hirsh and Zee (1999). The biochemically identified strains were thereafter stored for further investigation.

**III – In-ovo experimental infection with an *MG*-isolate:**

Thirty-five fertile turkey's eggs were commercially purchased and five of them were primarily tested for the presence of Mycoplasmal and bacterial infections by conventional culturing procedures. The purchased eggs were carefully incubated for one week and all precautions for incubation and management of fertile eggs were taken as described by Grimes (2002). One strain of *Mycoplasma gallisepticum* isolated from the culturally tested eggs of the infected flock was cultured into PPLO broth medium (Difco) without inhibitors and ten-fold serial dilution was achieved. Mycoplasma colonies were counted at dilution of $10^9$ and 0.3 ml of the later dilution was inoculated into yolk sac of each egg (n = 25) on the $8^{th}$ day post incubation. The remainder eggs were kept as control, inoculated with uncultured broth. The inoculated eggs were incubated with daily candling and determining the viability of the embryos as described by Grimes (2002). Dead eggs within 24 hours post inoculation (PI) were discarded due to an accidental or an unknown cause. On the $3^{rd}$, $6^{th}$, and $8^{th}$ days PI, the alterations of embryos and their membranes were grossly monitored and histopathologically examined. On the other hand, *MG* was reisolated at the end of experiment as described by (Abdelhameed, 2006).

**IIV - Histopathological examinations of the inoculated embryos:**

Histopathological examination of the experimentally infected turkey's embryo CAM of the inoculated eggs were fixed in 10% formole saline solution, then dehydrated, cleared and embedded in paraffin wax and sectioned at 4 µ and stained by Harris hematoxylin (Bancroft and Steven, 1998).

# RESULTS

**Clinical and necropsy findings:**

Forty-seven (18.80 %) of the adult birds of the examined turkey flock (n = 250) were found with signs of infraorbital sinusitis in various forms. The acute form was characterized clinically by remarkable edematous swelling with cutaneous pinkish discoloration (Photo 1 & 2) of the infected bird. The swelling was diffused progressed including the orbit (Photo 2) and was filled with odorless pussy material in the most cases. In others, the pussy material contained caseated particles. The involved eye was lacrimated discharging profuse watery discharge with congestion of the conjunctiva (Photo 2). The chronic form, the paranasal swelling was dried (inspissiated). On other hand, 11 of 63 (17.46 %) of the growing birds (6 – 9 weeks in age) showed infraorbital lesion with signs of labored breathing. Tracheal rales and coughing with nasal discharge were the prominent signs of the examined flock. The nasal passages and tracheas filled with catarrhal exudates with pneumonitis. Slightly enlargement of liver with perihepatitis, oophoritis were the predominant necropsy findings of the succumbed adults birds. Whereas, the examined dead growing birds (n = 7) showed pneumonitis in addition to tracheal lesions. Diffuse edemas with hepatic necroses were the characteristic findings of the examined embryos.

**Serological examination of Mycoplasma infection:**

SPAT indicated that 22 out of 25 the tested serum samples were serologically positive to *MG* infection (Table 1).

**Cultural detection of Mycoplasma:**

Results of the isolation rate of Mycoplasma from cultured samples (tracheal swabs, aspirated infraorbital lesions, eggs and organs) of the examined flocks were tabulated on Table 2. The isolated Mycoplasma strains were biochemically positive to glucose-fermentation test and negative to arginine hydrolysis test (Table 3) referring to the isolated Mycoplasma trend to *MG*.

**Molecular detection (PCR) of the isolated Mycoplasma:**

All tested strains of the isolated Mycoplasma showed the characteristic PCR product at 330 bps (Photo 4) for *M. gallisepticum* as described by Kempf *et al.* (1993).

**Bacterial isolates:**

Isolation of the various types of bacteria from the bacteriologically examined samples were summarized on Table 4, which revealed that *Pasteurella multocida* was the frequently predominant

isolate followed by *Escherichia coli*, *Staphylococcus aureus* and coagulase negative staphylococci. The frequent distributions of the bacterial isolates were illustrated in Table 5 and Fig. 1.

**Experimental inoculation:**

Eight days post *MG*-inoculation (PI), 7 of the inoculated fertile eggs were succumbed. Hyper-thickening membranes (Photo 8 & 9) with remarkable congestion of the blood vessels (Photo 10) were obvious in association with generalized edema with slightly hepatic necrosis of embryo (Photo 5). Embryonic dwarfing particularly shortening legs, micromelia (Photo 5) and curling appearance were also obvious (Photo 6 and 7).

Histologically, on the 3rd day *MG*-PI the CAM exhibited mild hyperplasia and hypertrophy of the ectodermal cells in comparison with the control (Photo 11). The changes progressed on the 6th day *MG*-PI to pronounced congestion and hemorrhage with hyperplasia of the endodermal cells (Photo 12). Moreover, vacuolar degeneration with necrosis and cellular debris were seen in the ectodermal cells (Photo 13). Mesoderm was suffered from edema with heterophils and mononuclear cells infiltration (Photo 14). On the 8th day *MG*-PI, severe congested blood vessels with hyperplasia and hypertrophy of the ectodermal layer were demonstrated (Photo 15).

**Table 1:** Serological screening of Mycoplasma infection by serum plate agglutination test (*MG*-antigen):

| Birds/sera | Number of tested birds | Number of positive birds MG | Percentage of seropositive birds MG |
|---|---|---|---|
| Birds with infraorbital lesions | 10 | 10 | 100 |
| Birds with respiratory signs | 5 | 4 | 80 |
| Apparently healthy birds | 10 | 8 | 80 |
| Total | 25 | 22 | 88 |

**Table 2:** Isolation rate of *Mycoplasma* from the culturally examined samples:

| Samples | Number of examined samples | Number of positive samples | *Mycoplasma* isolation rate |
|---|---|---|---|
| *I – Exudates of Infraorbital sinusitis (IOS)* | | | |
| Exudates of IOS of adult birds | 10 | 8 | 80. 00 % |
| Exudates of IOS of growing birds | 7 | 7 | 100.00 % |
| Total | 17 | 15 | 88.23 % |
| *II - Tracheal swabs (TS)* | | | |
| TS of birds with IOS (adult birds) | 10 | 9 | 90.00 % |
| TS of birds with respiratory signs | 5 | 3 | 60.00 % |
| TS of apparently healthy birds | 10 | 5 | 50.00 % |
| Total | 25 | 17 | 68.00 % |
| *III – Eggs** | | | |
| Fertile eggs | 16 | 5 | 31.25 % |
| Eggs with dead embryos | 8 | 4 | 50.00 % |
| Infertile eggs | 1 | 0 | 00.00 % |
| Total | 25 | 9 | 36.00 % |
| *IV - Organs of recently dead birds* | | | |
| Tracheas (lower third) | 7 | 5 | 71.43 % |
| Lungs and air sacs | 7 | 2 | 28.57 % |
| Total | 14 | 7 | 50.00 % |

*: The control eggs (n = 5) were bacterial and Mycoplasma free. The observed percentage of the fertile eggs is therefore 70 % (16/30), and the percentage of dead embryos and the infertile eggs is consequently 30.00 %.

**Table 3:** Biochemical profile of the isolated Mycoplasma strains:

| Tested Strains | Genus determination (Digitonin sensitive) | Glucose fermentation test | Arginine hydrolysis test |
|---|---|---|---|
| 61 isolates* | 49 (+) | 49 (+) | (-) |

*Twelve isolate were digitonin insensitive, Acholeplasma species. The remained strains are digitonin sensitive.

(+) Positive strains (-): Negative

**Table 4:** Isolated bacteria from the culturally examined samples:

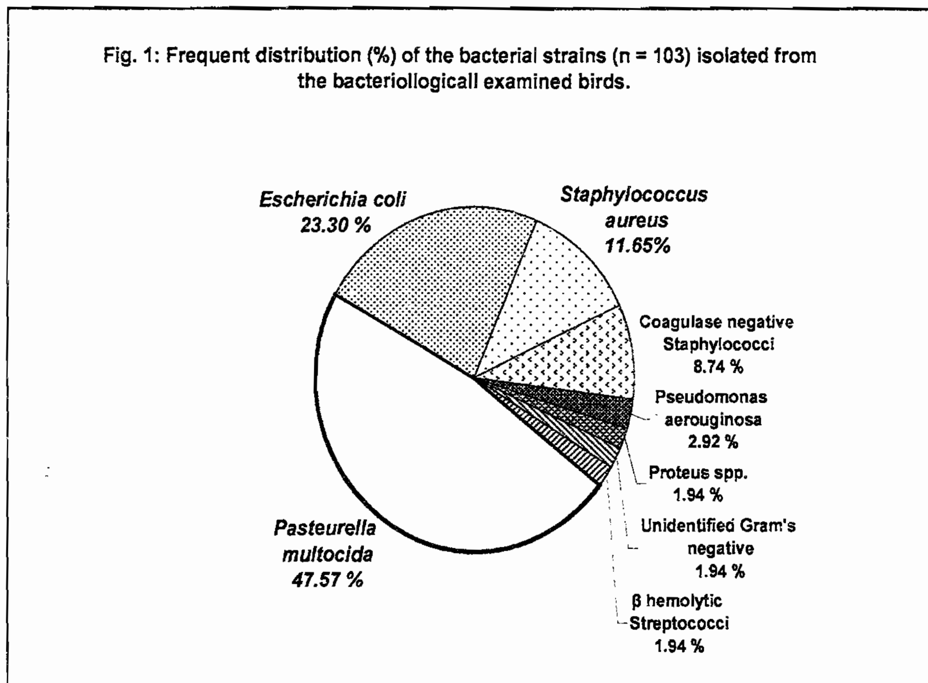
| Samples | Number of examined samples | Number of positive samples | No. of Isolates | Bacterial Species |
|---|---|---|---|---|
| *I – Exudates of Infraorbital sinusitis (IOS)(n = 17 Birds)* | | | | |
| Exudates of IOS of adult birds | 10 | 5 | 24 | *Pasteurella multocida*<br>*Staphylococcus aureus*<br>*Escherichia coli*<br>*Pseudomonas aerouginosa* |
| Exudates of IOS of growing birds | 7 | 3 | 9 | *Pasteurella multocida*<br>Unidentified Gram's negative |
| *II - Tracheal swabs (TS)(n = 25 birds)* | | | | |
| TS of birds with IOS (adult birds) | 10 | 7 | 4 | *Escherichia coli*<br>Proteus spp. |
| TS of birds with respiratory signs | 5 | 4 | 11 | *Pasteurella multocida*<br>Coagulase negative staphylococci |
| TS of apparently healthy birds | 10 | 3 | 5 | Coagulase negative staphylococci<br>Unidentified Gram's negative |
| *III – Eggs (n =25)* | | | | |
| Fertile eggs | 13 | 0 | --<br>-- | --<br>-- |
| Eggs with dead embryos | 8 | 0 | -- | -- |
| Infertile eggs | 4 | 0 | -- | -- |
| Total | 25 | 0 | -- | -- |
| *IV - Organs of recently dead birds (n = 7 birds)* | | | | |
| Tracheas | 7 | 6 | 29 | *Pasteurella multocida*<br>*β hemolytic Streptococci*<br>*Pseudomonas aerouginosa*<br>*Escherichia coli*<br>*Proteus spp.* |
| Lungs and air sacs | 7 | 6 | 21 | *Pasteurella multocida*<br>*Coagulase negative staphylococci* |

N.B. On-hundred-three bacterial isolates were recovered from the bacteriologically examined samples. The isolation rate of the bacterial isolates was tabulated in Table 5.

**Table 5:** Frequent isolation (%) of the bacterial strains (n = 103) isolated from the different examined samples.

| Isolated Bacterial species | Frequent Nr. of isolates | % to all isolates |
|---|---|---|
| *Pasteurella multocida* | 49 | 47.57 |
| *Escherichia coli* | 24 | 23.30 |
| *Staphylococcus aureus* | 12 | 11.65 |
| Coagulase negative Staphylococci | 9 | 8.74 |
| *Pseudomonas aerouginosa* | 3 | 2.92 |
| Proteus spp. | 2 | 1.94 |
| Unidentified Gram's negative | 2 | 1.94 |
| *β hemolytic Streptococci* | 2 | 1.94 |

**Fig. 1: Frequent distribution (%) of the bacterial strains (n = 103) isolated from the bacteriollogicall examined birds.**



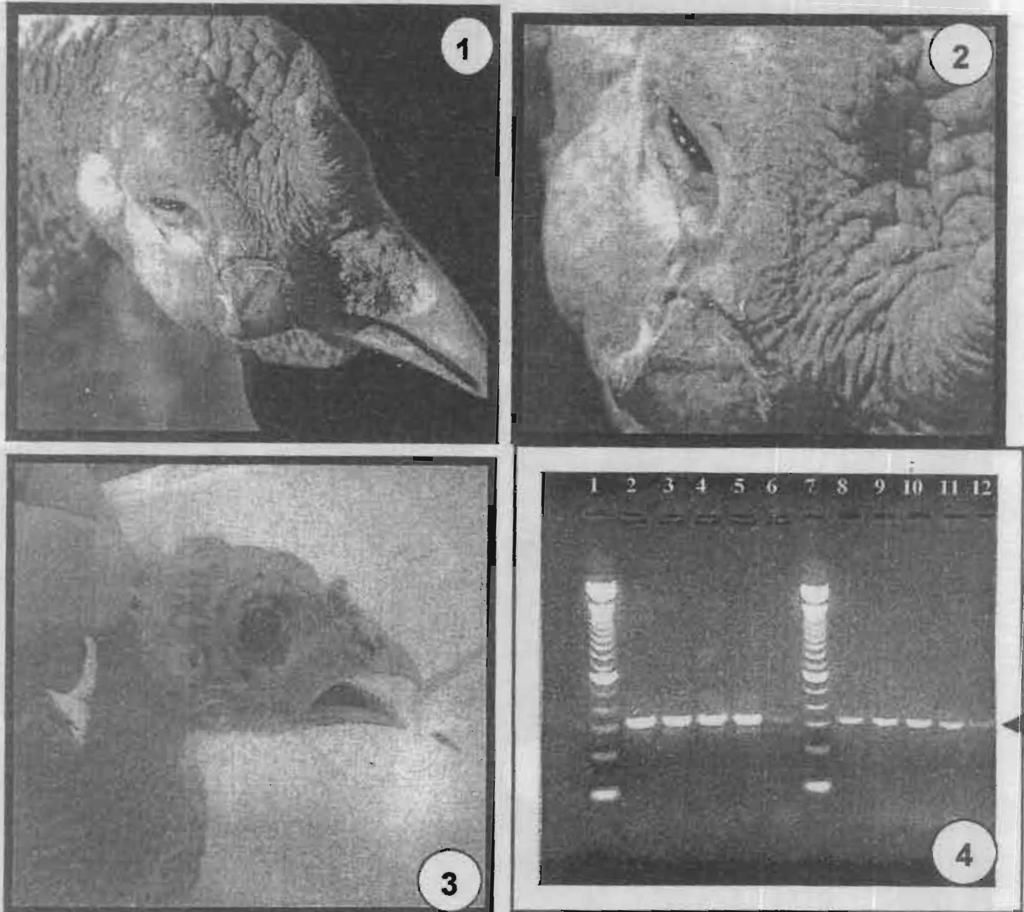



**Photo 1:** Characteristic signs of infraorbital sinusitis (advanced case) in adult bird.

**Photo 2:** Close-up photograph focusing on the infraorbital lesion showing the diffuse edematous swelling occupied the lateral side of the face including the orbit with cutaneous pinkish discoloration.

**Photo 3:** an eight-week-old-poult showing typical lesion of infraorbital sinusitis with labored breathing.

**Photo 4:** Agrose electrophoresis gel showing PCR amplification reaction product;

- Lanes 1 & 7: Molecular marker, 100 bp.
- Lanes 2 – 6 and 8 – 12, field isolates of Mycoplasma.
- The Black arrow refers to the specific band at 330 bp of *Mycoplasma gallisepticum*.

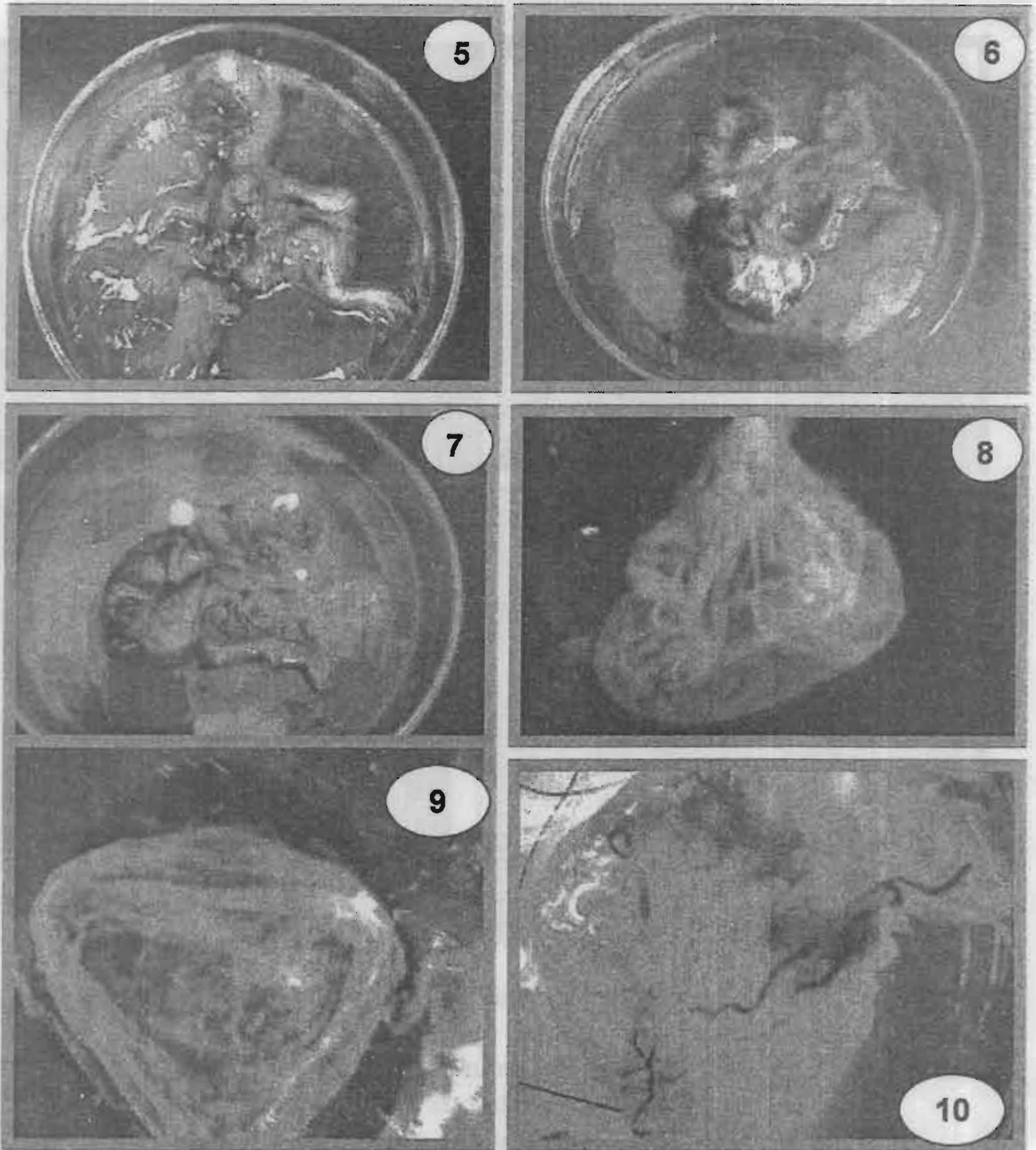



**Photo 5:** Generalized edema with signs of dwarfing (shortening legs, micromelia) and slightly liver necrosis

**Photos 6 & 7:** Curling appearance of the embryos

**Photo 8:** The beginning of thickening of the membrane.

**Photo 9:** Increase the thickness of the membrane with distribution of fine caseous material and hemorrhages.

**Photo 10:** Remarkable thickening of the membrane, which is duskier, with severe congestion of the blood vessels.

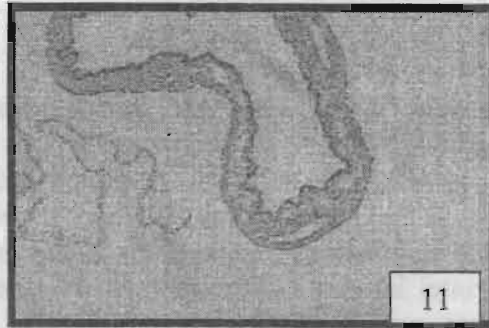


**Fig. 11:** Normal thickening of the membrane (Chorioallantoic membrane CAM) of control embryo

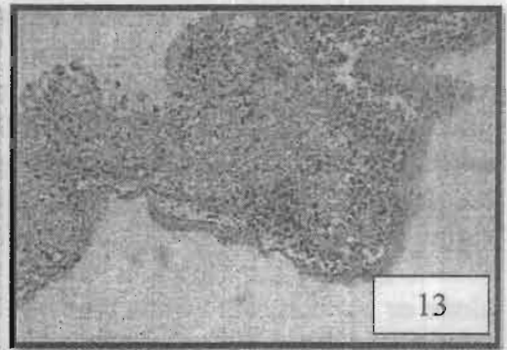
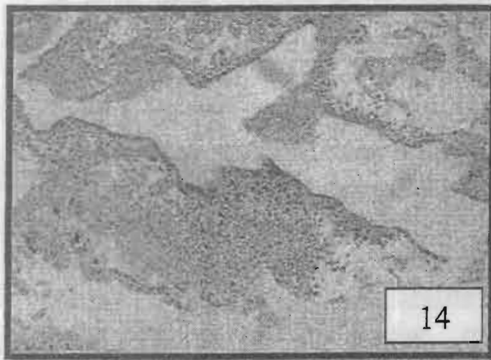


**Photo 12:** The CAM exhibited mild hyperplasia and hypertrophy of the ectodermal cells.

**Photo 13:** Pronounced congestion and hemorrhage with hyperplasia of the endodermal cells.



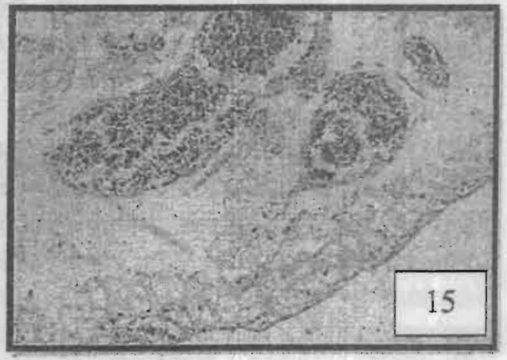


**Photo 14:** Mesoderm shows edema with heterophils and monomorpnuclear cells infiltrations.

**Photo 15:** Severe congested blood vessels with hyperplasia and hypertrophy of the ectodermal layer.

# DISCUSSION

*M. gallisepticum* is an endemic infection among poultry population in different Governorates of Upper Egypt, (Sokkar *et al.*, 1986; Saif-Edin, 1997; Aly, 1998; Abdelhameed, 2000 and Abdelhameed, 2006). In the present work, the higher proportion of the serologically tested birds in association with the higher isolation rate of *MG* from the infraorbital lesions and the cultured samples of the adults and growing birds may refer to the high spread of *MG* throughout the examined flock and the infection was inherent. Turkeys are more susceptible to *MG* infection than chickens and the infection is transmitted during close contact between birds as well as on fomites and aerosol spread of infection occurs over short distances and can be responsible for transmission within a flock (Marios *et al.*, 2002; Ley, 2003, Bradbury, 2006 and OIE, 2008). The spread of *MG* in poultry farms in Upper Egypt may ascribe to various reasons including lack of biosecurity, insufficient hygienic measures and the omission of the significant role of *MG*-vaccination (Abdelhameed, 2000 and 2006).

The higher isolation rate of *Mycoplasma gallisepticum* either alone or synchronized with other microorganisms from the infraorbital sinusitis of the bacteriologically examined turkeys may indicates that the isolated mycoplasma is a primary cause of such lesion. Büyüktanir, et al. (2008) corroborated that *Mycoplasma gallisepticum* was a primary etiologic agent of chronic respiratory disease in chicken and of infectious sinusitis of turkey causing important economic losses. Conversely, the published results by Eissa *et al.* (2000) may give a signal that *Mycoplasma synoviae* is a prominent cause responsible for turkey's paranasal sinusitis rather than *Mycoplasma gallisepticum*.

From an immunological point of view, *Mycoplasma gallisepticum* is frequently incriminated as a notorious etiologic agent responsible for immunosuppression of the infected birds and induced a significant pathological changes including extensive deciliation of the upper respiratory tract (Ganapathy and Bradbury, 2003; Lam, 2003 and Nascimento *et al.*, 2005). These alterations may hinder the defense mechanism of the respiratory system, which induce a favorable microenvironment suitable for invading of different microorganisms to deep respiratory tissue. In addition to these notoriousness effects of *MG* infection, Dingfelder *et al.* (1991) emphasized that the lower respiratory tract was the most prominent infection site of *Mycoplasma gallisepticum* in turkeys. These may interpret the isolation of *Pasteurella multocida*,

either alone or coupled with *Escherichia coli*, in association with *Mycoplasma gallisepticum* from the upper (infraorbital sinuses and tracheas) and the lower respiratory (lungs) tracts of the examined turkeys.

Isolation of *Pasteurella multocida* and *Escherichia coli* in association with *Mycoplasma gallisepticum* infection from the respiratory tracts and infraorbital sinus of the examined turkeys may refers to synergistic action between them. Synergistic action between *Mycoplasma gallisepticum* and *Escherichia coli* (either alone or associated with other pathogens) as a major etiologic agents responsible for swollen head syndrome in turkeys, chickens and ostriches is naturally and experimentally reported (Sokker *et al.,* 1986; Ali and Yossef, 2003; KeBin, 2003 and Moustafa, 2005).

The *In-ovo* shedding of *MG* in turkeys is varies; egg transmission is more frequent in birds infected during laying than in birds infected before they mature and the infected birds may carry *MG* for life, and can remain as a hidden form (asymptomatic) until they are stressed (Ley, 2003). On the current work, the presence of *Pasteurella multocida* either alone or coupled with *Escherichia coli* may act as marvelous stressing factors for *Mycoplasma gallisepticum* infection of the afflicted turkey flock leading to vertical shedding of *MG*. This may give an account to the highest isolation rate of *M. gallisepticum* from the examined fertile eggs. Similarly, Reinhardt *et al.* (2005) emphasized that viral infections reactivated *Mycoplasma gallisepticum* infection of the asymptomatic infected birds.

Vertical transmission of *M. gallisepticum* in turkeys was reported by Nicholas *et al.* (2009). The frequent isolation of *Mycoplasma gallisepticum* from the examined fertile eggs and eggs with dead embryos of the naturally infected turkey's flock suggests that the isolated microbe might responsible for the occurrence of embryonic deaths and reduced the hatchability rate of the fertile eggs. Such suggestion was supported experimentally by inoculation of *MG* into fertile eggs causing embryonic death within 8 days post inoculation in some eggs and pathological alterations of the non-succumbed embryos. Hoa *et al.* (2000) concluded that *Mycoplasma gallisepticum* significantly reduced the laying performance and dramatically decreased the egg-hatchability rate (reduction rate, 86.07 %) of the infected birds in comparison with the uninfected birds (5.33 %).

Histopathologically, Lam and DaMassa (2003) and Ley (2003) elucidated that *Mycoplasma gallisepticum* could induce marked

thickening of the mucous membrane of the affected tissues due to infiltration with mononuclear cells and hyperplasia of the mucous gland and the prominent feature of *MG* was a lymphoproliferative response at the site of infection. Such pathological features may interpret the remarkable thickening of the embryonic membranes and the presence of massive infiltration of lymphocytes of the chorioallantoic membranes of the experimentally inoculated turkeys' embryos with a field isolate of *MG*. The lymphotactic features of *Mycoplasma gallisepticum* in embryonic membranes may ascribe to the capability of this microbe to motivate the embryos to secrete lymphotactin, which responsible for attraction and accumulation of lymphocytes to the sites of infection (Lam and DaMassa, 2003).

It is conclude that *M. gallisepticum*, either alone or coupled with others, appears to be the predominant cause of turkey's infectious sinusitis and respiratory distress. *Pasteurella multocida* and/or *Escherichia coli* infection may increase the rate of in-ovo transmission of *MG* causing reduced hatchability with embryonic deaths. Antibiotic therapy may be temporarily useful in controlling of infections (Reinhardt *et al.,* 2005). Otherwise, vaccination with one of the modified live *MG* vaccines is recommended in endemic areas (Ley, 2003 and Nicholas *et al.,* 2009)

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