Prevalence and Antibiotic Sensitivity of Mycoplasma Spp. Isolated From Chicken

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Abstract

In order to determine the Prevalance of *Mycoplasma* isolated from chicken, A total number of 200 samples collected from birds showing respiratory manifestations and apparently healthy chicken of different ages(two weeks: two months) from different localities(Al-ismailia and Sharkeya Governorates). These samples include 110 samples from respiratory organs(trachea -lung -air sac) ,75 swabs from nasal cleft and 15 samples from fluid of swollen joints. A trail for isolation and identification of different *Mycoplasma* was done using conventional and recent techniques. Primary isolation of the microorganism on PPLO medium, which appeared as fried egg when examined by dissecting microscope yielded118 positive samples with a total incidence rate 59%. The highest recovery rate was from respiratory organs (72.7%) followed by swabs from nasal claft (46.7%) and samples from swollen joints (20%). Application of Digitonin test for differentiation between *Mycoplasma* and *Acholeplasma* . *Mycoplasma* is digitonin positive while Acholeplasma is digitonin negative. The incidence of *Mycoplasma* is 81.3% and the incidence of *Acholeplasma* is 18.6%. Biochemical characterization of the obtained isolates gave 58 isolates suspected to be *M. gallispectum* from different sites of isolation with percentage of 49% and 18 isolates suspected to be *M.gallinarium* with percentage of 15.3% and 3 isolates suspected to be *M.synoviae* with percentage of 2.5% and 7 isolates suspected to be *M.arginini* with percentage of 5.9%. Serological identification of isolates using specific antisera was applied which confirmed the presence of *M. gallispectum* and *M.synoviae* but not other genera (*M.gallinarium and M.arginini*) because of the lack of specific antisera . The minimal inhibitory concentration (MIC) results cleared that the antimicrobials (Doxycycline was followed by Erythromycin and Tilmicosin) were highly active in inhibition of Mycoplasma in vitro, whereas Streptomycin and Lincospectin and Ciprofloxacin were less effective against the tested isolates.

Introduction

Mycoplasmas, belonging to the class Mollicutes, are small free living highly fastidious and slow growing microorganism ***(Nicholas and Ayling, 2003)***. Un like other bacteria ,it lacks arigid cell wall but bounded by a plasma membrane ,what makes it very sensitive to adverse environmental conditions ***(Raviv and Kleven, 2009).*** Avian mycoplasmosis consistitutes one of the major economic problems facing poultry industry allover the world because of it's significant losses which are mainly due to reduced egg production , poor feed conversion and carcass condemination at processing ***(Yoder, 1984 and Cassel et al ., 1985).*** Mycoplasma gallisepticum (MG) and Mycoplasma synoviae(MS) are considered to be the most important of the pathogenic mycoplasmas for chickens , and both occur world-wide ***(OIE 2008).*** They spread vertically through infected eggs and horizontally by close contact ***(Bradbury ,2001).*** Mycoplasma gallisepticum (MG) infection is usually considered as chronic respiratory disease of chickens and infectious sinusitis in turkeys. It is characterized by respiratory rales, coughing, nasal discharges. ***Kleven (1997).*** Mycoplasma synoviae(MS) is an important avian pathogen which can cause both respiratory disease and synovial joint inflammation (synovitis) in poultry which is an acute-to-chronic infectious disease for chickens and turkeys involving primarily the synovial membranes of joints and tendons sheaths. when ***M. synoviae*** combines with other respiratory virus infection , causing significant drop in egg production beside condamination of carcasses due to accumulation of the viscous creamy to grey exudates involving synovial membranes of the tendon sheath, joint, keel bursa and may extend even to muscles and air sacs ***(Kleven, 1997 and Ley et al ., 2003b).*** Mycoplasma gallinarum is considered to be a non - pathogenic commensal for a broad range of hosts. Compared to Mycoplasma gallisepticum , M. gallinarum produces little to no pathology ***(Power and Jordan , 1976)***. Culture techniques are laborious and expensive and require awareness of any recent antibiotic treatement that can inhibit isolation of the organisms. other problems experienced with culture include overgrowth by faster growing mycoplasma species or other bacteria***.(Garcia et al., 1995)***. Antimicrobial use continues to be the most economic method for controlling these infections ,where the disease is still endemic . To achieve successful treatment and prevention of flocks with antimicrobials. it is necessary to examine the sensitivity of mycoplasma species present in the flock ***(Levishon et al., 1981)*** and ***(Pakpinyo and Sasipree Yajan .2007).***

This work was designed to study the prevalence of Mycoplasma spp. in chicken from different respiratory organs , swabs and swollen joint . Identification of isolated strains by biochemical Characterization.

Materials and Methods

1- Samples:

Two hundred samples were collected from birds showing respiratory manifestations and apparently healthy chicken of different ages(two weeks: two months from different localities(Al-ismailia , Sharkeya Governorates). These samples include 110 samples from respiratory organs(trachea - lung -air sac) & 75 swabs from nasal cleft and 15 samples from fluid of swollen joints. as shown in table (1)

2-Digitonin test for Differentiation between *Mycoplasma* and *Acholeplasma*:

Digitonin senstivity test is an indirect indication of sterol requirements in which a loopful of logarithmic broth culture of tested isolate was inoculated on previously dried agar plate by running drop technique . Mycoplasma was digitonin sensitivy and showed marked inhibition zone , while Acholeplasma did not show any inhibition zone.

3-Biochemical characterization:

A) Glucose fermentation test *(Erno and Stipkovits 1973)*

An amount of 0.1ml of the viable Mycoplasma culture was inoculated into 0.9ml of Glucose medium, incubated aerobically at 37°C beside un inoculated control tubes. All tubes were examined daily up to 7 days before final conclusion. No change in color indicates negative reaction while change in color to orange or yellow indicates positive reaction.

B) Argenine deamination test *(Erno and Stipkovits, 1973)*

An amount of 0.1ml of the viable ***Mycoplasma*** culture was inoculated into 0.9ml of test medium, aerobically incubated at 37°C for 7 days along with uninoculated control tubes. No change in color indicates negative reaction while change in color to dark red to violet indicates positive reaction.

C) Film and Spot Formation *(Fabricant and Freundt, 1967)*

The film and spot formation was done by inoculated tested organism with medium and incubated at 37°C in a candle jar for up to 14 days and examined microscopically using reflected light. Production of a film was seen as iridescent or pearly area , usually on areas of heavy growth. The medium sometimes showed some clearing around areas of growth.

4-Broth microdilution minimum inhibitory concentration (MIC) test according to *(Hannan, 2000)*

Antimicrobial agent concentrations ranged from 0.016 to 16 ug/ ml for tested antimicrobials were prepared . The highest dilution of antibiotics

that caused inhibition to the metabolic action of the tested organisms was recorded. The minimum inhibitory concentration (MIC) was determined by the persistence of the original color without changes. MIC results were interpreted according to National Commitee for Clinical Laboratory Standards ***(NCCLS) institute*** and ***CLSI, 2008),*** additionally, MIC50 and MIC90 were calculated using an orderly array method ***(Hamilton-Miller, 1991).***

5-Serological identification:

-Growth inhibition test *(Clyde, 1983):*

The inhibition test is based on a characteristic property of ***Mycoplasma*** as manifested by the finding that incorporation of antiserum into culture medium inhibited growth of the homologous organism. Appropriate agar plates were inoculated by test culture using the running drop technique. Two dilutions (1:10 and 1:100) beside the undiluted test culture were used. Inoculated plates were allowed to dry at room tempareture before applying the discs. Then discs (presaturated with each of the tested antisera and dried was pressed gently on the middle of the inoculated area.

Table(1): *Types and No. of samples*

Sample types	No. of samples
Respiratory organs	110
Swabs	75
Fluid of swollen joints	15
Total	200

Results

1- Primary isolation of Mycoplasma from collected samples

Table (2) *Recovery rate of Mycoplasma isolation from collected samples*

Site of isolation	No. examined	Isolation		Precentage of positive
		+ve	-ve	
Respiratory organs	110	80	30	72.7%
Swabs	75	35	40	46.7%
Swollen joints	15	3	12	20%
Total	200	118	82	59%

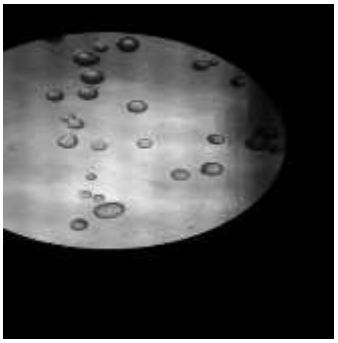

Photo (1) *characterstic morphological apperance of mycoplasma colonies on PPLO agar medium (fried egg apperance) .*

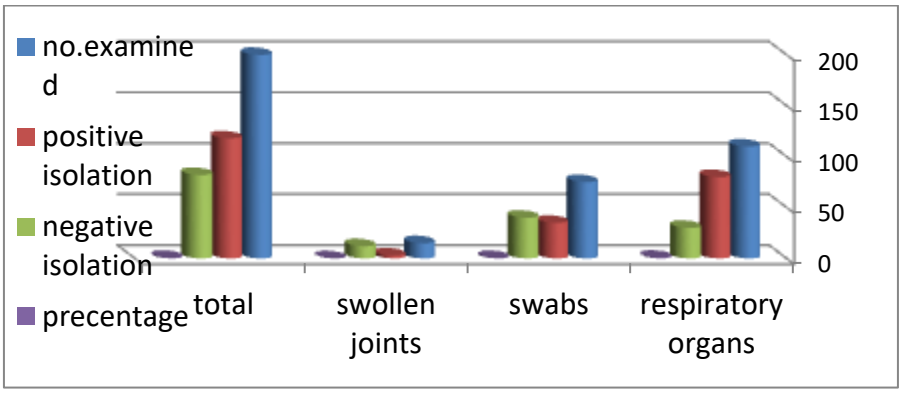

figure (1) *Recovery rate of Mycoplasma isolation from collected samples*

2-Application of digitonin test for characterization of the obtained isolates .

Table (3) Application of digitonin test for the recovered isolates

Site of isolation	No. of positive samples	Digitonin			
		positive		negative	
		No.	%	No.	%
Respiratory organs	80	65	81.2	15	18.7
Swabs	35	28	80	7	20
Swollen joints	3	3	100	0	0
Total	118	96	81.3	22	18.6

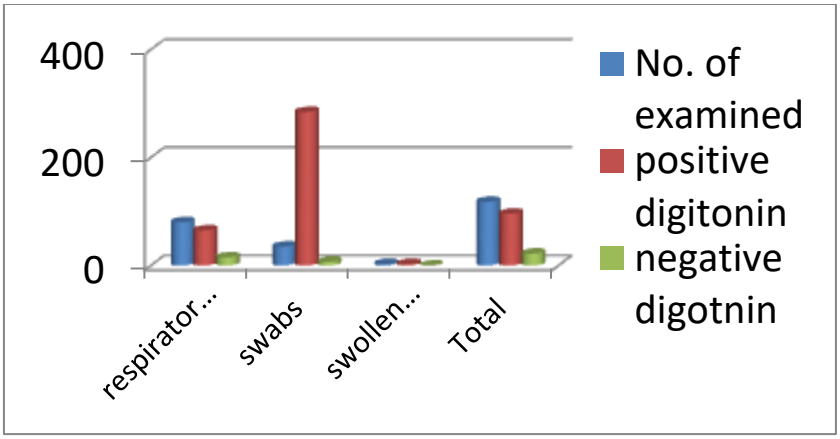

Fig. (2) *Digitonin test for obtained isolates*

3- Biochemical characterization of isolated Mycoplasma:

Table (4) *Biochemical identification of isolates:*

Bio group	No. of isolates (118)	Incidence	Biochemical tests			Suspected type
			Gluco Arginin		Films &spot Formation	
Group I	58	49%	+ve	-ve	-ve	***M. gallispticum***
Group II	3	2.5%	+ve	-ve	Late +ve	***M. synoviae***
Group III	18	15.3%	-ve	+ve	+ve	***M. gallinerum***
Group IV	7	5.9%	-ve	+ve	-ve	***M. arginine***
Group V	32	27%	-	-	-	**Un typed *Mycoplasma***

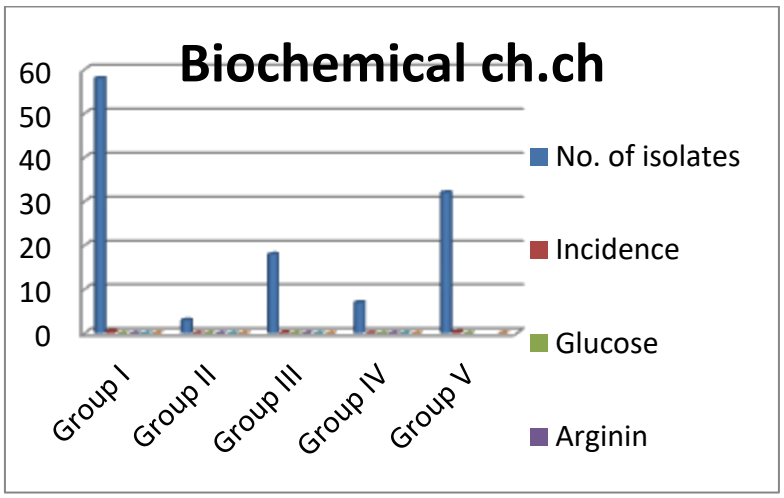

fig. (4) *Biochemical identification of isolates:*

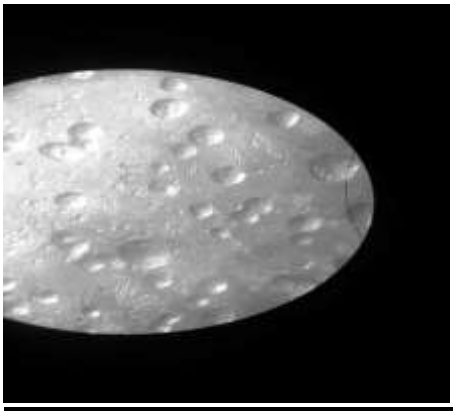

Photo (2) *film and spot formation of Mycoplasma on solid PPLO media.*

4- Serological identification of *Mycoplasma* isolates:
Growth inhibition test (GIT):
Table (5) *Serological identification of* ***Mycoplasma*** *isolates by GIT*

Biotype	No. of positive isolates	Identified Ag
Group I	40/58	MG
Group II	3/3	MS

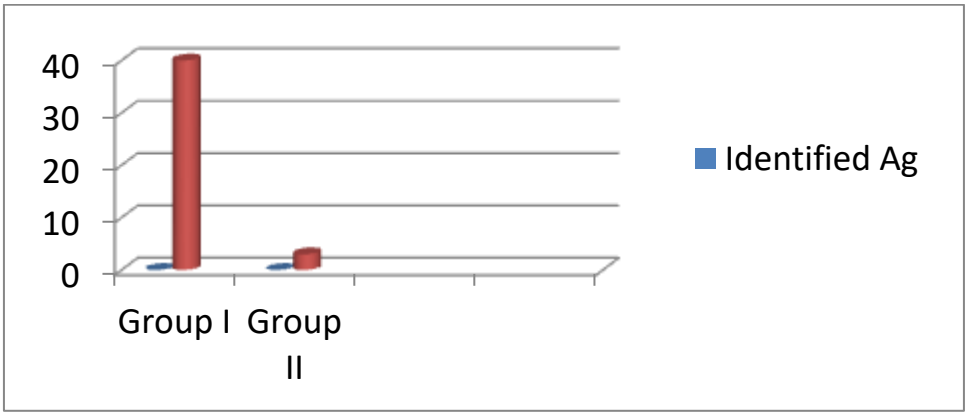

fig. (5) *Serological identification of Mycoplasma isolates by GIT*

Table (6): Showing results of minimal inhibitory concentration test of *mycoplasma* isolated from different sites:

MIC	1 st End point	2 nd End point
1- Ciprofloxacin	3	3
2- Doxycyclin	8	5
3- Erythromycin	7	6
4- Lincospectin	4	3
5- Streptomycin	4	4
6- Tilmicosin	7	6
7- Tylosin	6	5

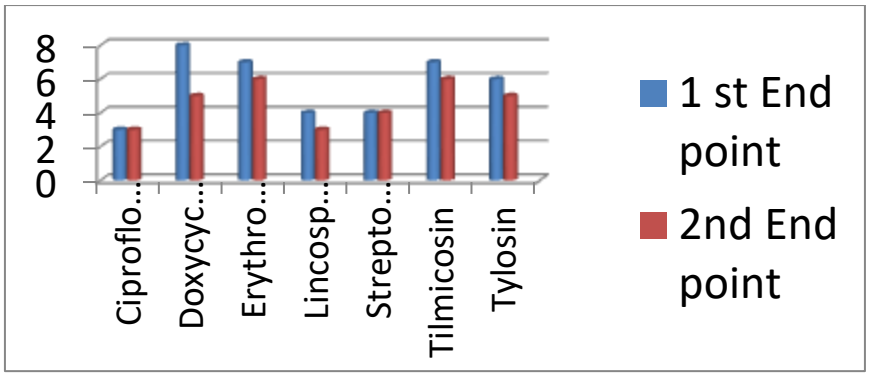

Fig. (6): *Results of minimal inhibitory concentration test of mycoplasma isolates*

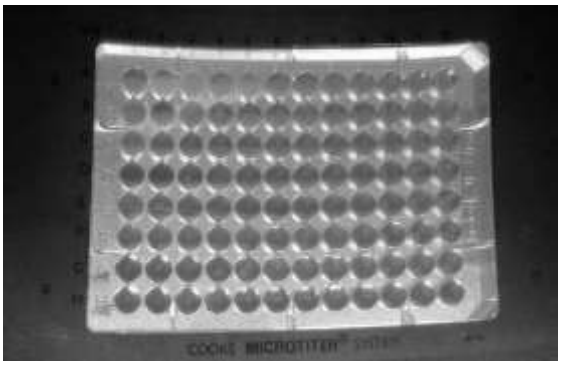

Photo (3) *Results of minimal inhibitory concentration test of* ***mycoplasma***

Discussion

Mycoplasma is a small free living highly fastidious and slow growing micro-organism, ***(Nicolas and Ayling, 2003).*** Avian Mycoplasmosis is considered as one of the major economic problems facing poultry industry all over the world because of its significant losses which are mainly due to

reduced egg production, poor feed conversion and carcass condemnation at processing ***(Yoder, 1984).***

The most economically significant mycoplasma pathogen of poultry is M. gallisepticum ***(Kleven S.H. & Levisohn S., 1996).*** Mycoplasma synoviae (MS) is recognized as pathogen in chickens and turkeys and is responsible for infectious synovitis ***(Kleven ., 1997)***. Infection with M. synoviae causes a respiratory disorder and infectious synovitis in chicken especially further highlight the economic significance of these bacteria in commercial poultry ***(Feberwee et al ., 2009).***

Culture is the gold standard for direct detection of the organism, but pathogenic avian Mycoplasmas are slow growing, relatively fastidious organisms, and might require up to 3 weeks for detectable growth . In some cases the isolation of avian Mycoplasmas is impaired by the culture over growth of saprophytic Mycoplasmas that inhabit the upper respiratory tract of avian species and contaminant bacteria and fungi that may not be inhibited by Mycoplasma- selective media ***(Kleven, 2003).***

In the present work ,Mycoplasma species grew well showed pure colonies like the characteristic fried egg appearance on Frey's agar medium by incubation at 37°C and 10% CO2 (tiny, smooth circular, translucent mass with a dense raised central area) as shown in photo (1) ***(Quinn et al., 2002).*** M. gallisepticum and M. synoviae replication requires a rather complex medium usually enriched with 10-15% heat inactivated horse serum.

In Table (2), The primary isolation of Mycoplasma spp. from the collected samples yielded 118 isolates out of 200 examined samples(59%). The highest recovery rate of Mycoplasma was from respiratory organs (72.7%) followed by swabs (46. 7%) and swollen joints (20%). These results agree with that recorded by ***Metwalli (1980)*** (50%), ***Mohamed (1997)*** (13.3%), ***Ulgen and Kahraman (1993)*** (15.3%), ***Saif-Edin (1997)*** (40%). Also ***Sharaf (2000)*** (22.85% of apparently normal 45 day old chickens and 57.14% of 45 day old diseased chickens) and ***Mohammed (2001)*** (21.2%) and ***Usama (2008)*** (89%).

It could be observed that mycoplasma organisms not only isolated from the respiratory organs, but also from the swollen joints as MS . from the above mentioned results, These results agree with those of ***Tebyanian et al.(2014)*** who isolated 17 M. synoviae species by microbiological method**.** M. synoviae culture and isolation are not easy and almost are not accurate in all the poultry laboratories.

Microbiological method is needed for some research projects and even for diagnosis. Many false negative PCR results might occur without

enrichment ***(Mardassi et al., 2005)***. Therefore, culturing should not be ignored but culturing can be costly and time-consuming, and can also be inconclusive because of low sensitivity ***(Ewing ML, et al., 1998)***.

The results of digitonin sensitivity test for differentiation between mycoplasma and acholeplasma species collected from different chicken flocks were reaveled in table (3). The positive ***mycoplasma*** species cultures showed inhibition zone around the digitonin impregnated discs. The total recovery rate was (81.3%) representing (81.2%) respiratory organs, (80%) swabs, and (100%) swollen joints were positive digitonine test. Nearly similar results were obtained by ***Salem et al. (1986), Saif-Edin (1997) and Mageed (2000)*** who concluded that the isolation rate of mycoplasma from different flocks in upper Egypt was ranged from 20-100% .In addition, ***Mansour (1995)*** and ***Serag (2005)*** isolated MG with percentages 58% from chicken's respiratory samples. In Table (4) , biochemical characterization was carried out to simplify identification. Four biochemical groups could be detected, group one was (49%) which is glucose positive, arginine negative and flim& spot formation negative. While group two (2.5%) which is glucose positive, arginine negative and late flim& spot formation, group three (15.3%) which is glucose negative, arginine positive and positive flim& spot formation and group four(5.9%) which is glucose negative, arginine positive and negative flim& spot formation . This result compared with that mentioned by ***Rania (2005)*** who classified the Mycoplasma organisms isolated from chickens into two biochemical groups. Furthermore, un-typed mycoplasma species were detected in 32 isolates(27%).Presence of un-typed mycoplasma species may refers to the synergistic situation between the field strains of MG and other types of class ***Mollicutes Wafaa Abd EL-ghany(2008)***.

In Table (5), the growth inhibition test showed positive parallel results with the biochemical test 40 isolates considered as MG and 3 isolates as MS. This result is in agreement with that reported by ***Mansour (1995)*** who isolated other types of mycoplasma and un-typed ones from the respiratory tract of broiler chickens from different Egyptian Governorates. GIT didn't applied to group III and group IV because of lack of specific antisera for M. gallinerum and M. arginini (they considered as commensal).So the test is great value in identification of Mycoplasma isolates as recorded by ***(Kleven , 1975).*** Avian Mycoplasmas have shown sensitivities to several antimicrobials. In this study a set of antibiotics including Ciprofloxacin, Erythromycin, Doxycycline, Lincomycin, Spectinomycin ,Tilmicosin and Tylosin were tested

against selected Mycoplasma isolates representing the different sites of isolation as shown in table(7). Erythromycin and Tilmicosin was the most effective tested antibiotic (6) followed by Doxycycline and Tylosin (5) while Streptomycin (4) and Ciprofloxacin and Lincospectin (3) less effective antibiotic against the tested isolates and these an agreement with ***Gautier-Bouchardon et al., (2002)*** and ***Gerchman et al., (2011)*** and ***Sabry (2004)*** who detected that Spectinomycin was the most effective tested antibiotic followed by lincomycin, doxycycline and tylosin while erythromycin and enrofloxcacin less effective against the tested isolates. ***Lin (2006)*** reported The highest in vitro sensitivity of MG isolates to ofloxacin, spiramycin and tylosin .

In conclusion, Mycoplasmas are worldwide pathogen in chickens and turkeys causing great economic losses. Isolation rate of mycoplasma in this present study was 59%. Application of digitonin test for the recovered isolates help in differentiation between Mycoplasma and Acholeplasma. Minimum inhibitory concentration (MIC) made for some representative isolates against some antimycoplasmal drugs. Erythromycin and Tilmicosin were of superior activity followed byDoxycyclin and Tylosin while Ciprofloxacin and Lincospectin were less effective.

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الملخص العربى

تعتبرالميكوبلازما من ميكروبات واسعه الانتشارفي مزارع الدواجن المختلفة مسببه خسائر اقتصاديه كبيره للمربين و اصحاب المزارع وتتميز بانها تنتقل من الام المصابه الي الكتاكيت (انتقال رأسي). وهى ايضا تهيئ الطيور للاصابه الميكروبات الأخرى حيث ان العدوى تؤدى لقة الانتاج.

وفي خلال هذا العمل:

1- تم جمع عدد 200 عينه من الدجاج الذي يظهر عليه الاصابه باعراض تنفسيه من عدد من المحافظات(الاسماعيليه والشرقيه والقاهره) تشمل مسحات حلقيه واجزاء من الاعضاء التنفسيه والمفاصل المتورمه كالتالي

- الاعضاء التنفسيه 110 عينه والمسحات الحلقيه 75 عينه والمفا صل المتورمه 15 عينه

2- تم العزل الاول للميكوبلازما من العينات المختلفه اعطي عدد 118 عينه ايجابيه وذلك بمعدل اصابه 59% كالتالي:

- الاعضاء التنفسيه 8 عينه 72.7% والمسحات الحلقيه 35 عينه 46.7%
- المفاصل المتورمه 3 عينه 20%

3- تم اختبار الديجوتنين للتعرف علي المعزولات وتعتبر الميكوبلازما حساسة للديجيتونين حيث تظهر منطقة تثبيط ملحوظ تحيط قرص الديجيتونين والذي اعطي 96 عينه من اجمالي 118 عينه ايجابيه معزوله ميكوبلازما والباقي سلبي لهذا الاختبار بمعدل 22 عينه وتعتبر اكولي بلازما.

4- اجراء التصنيف البيوكيميائي للمعزولات التي تم الحصول عليها وكانت النتيجه كالتالي:

- مجموعه**I** ميكوبلازما جاليسبتكم 58 معزوله 49%
- مجموعه**II** ميكوبلازما سينوفي 3 معزوله 2.5 %
- مجموعه**III** ميكوبلازما جالينيرم 18 معزوله 15,3%
- مجموعه**IV** ميكوبلازما ارجينيني 7 معزوله 5,9%
- مجموعه**V** ميكوبلازما غير مصنفه 32 معزوله 27%

5- تم فحص عينات مضاد السيرم للمجموعه **I** وللمجموعه **II** وذلك باختبار تثبيط النمو ولكن لا يمكن اجراء الاختبار للمجموعه **III** والمجموعه **IV** لعدم وجود مضاد السيرم لهم ووجد ان 40 عينه من 58 عينه ايجابيه للميكوبلازما جاليسبتكم و ان 3 عينات ايجابيه للميكوبلازما سينوفيي .

6- تم اجراء اختبار مانع النمو (GIT) باستخدام مضاد سيرم مرجعي لتأكيد التعرف علي المعزولات .

7-تم اجراء اختبار اقل تركيز فعال (MIC)لبعض المضادات البكتيريه المختلفة ضد عترات ممثله من الناتجه عزلها و تصنيفها لتحديد انسب هذة المضادات للعلاج ووجد ان اكثر هذة المضادات فاعليه هو الدوكسيسيكلين .