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 yielded 118 positive samples with a total incidence rate 59%. The highest recovery rate was from respiratory organs (72.7%) followed by swabs from nasal cleft (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). Unlike other bacteria, it lacks an rigid cell wall but bounded by a plasma membrane, what makes it very sensitive to adverse environmental conditions (Raviv and Kleven, 2009). Avian mycoplasmosis constitutes 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 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 worldwide (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 contamination 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 treatment 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 sensivity 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 0.1 ml of the viable Mycoplasma culture was inoculated into 0.9 ml 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 0.1 ml of the viable Mycoplasma culture was inoculated into 0.9 ml 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 Committee 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 temperature 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

<table>
<thead>
<tr>
<th>Sample types</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory organs</td>
<td>110</td>
</tr>
<tr>
<td>Swabs</td>
<td>75</td>
</tr>
<tr>
<td>Fluid of swollen joints</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
</tr>
</tbody>
</table>

Results
1- Primary isolation of Mycoplasma from collected samples

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

<table>
<thead>
<tr>
<th>Site of isolation</th>
<th>No. examined</th>
<th>Isolation</th>
<th>Precentage of positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory organs</td>
<td>110</td>
<td>80  30</td>
<td>72.7%</td>
</tr>
<tr>
<td>Swabs</td>
<td>75</td>
<td>35  40</td>
<td>46.7%</td>
</tr>
<tr>
<td>Swollen joints</td>
<td>15</td>
<td>3   12</td>
<td>20%</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>118 82</td>
<td>59%</td>
</tr>
</tbody>
</table>
characteristic morphological appearance of mycoplasma colonies on PPLO agar medium (fried egg appearance).

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

<table>
<thead>
<tr>
<th>Site of isolation</th>
<th>No. of positive samples</th>
<th>Digitonin positive</th>
<th>Digitonin negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Respiratory organs</td>
<td>80</td>
<td>65</td>
<td>81.2</td>
</tr>
<tr>
<td>Swabs</td>
<td>35</td>
<td>28</td>
<td>80</td>
</tr>
<tr>
<td>Swollen joints</td>
<td>3</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>96</td>
<td>81.3</td>
</tr>
</tbody>
</table>
3- Biochemical characterization of isolated Mycoplasma:

Table (4) Biochemical identification of isolates:

<table>
<thead>
<tr>
<th>Bio group</th>
<th>No. of isolates (118)</th>
<th>Incidence</th>
<th>Glucose Arginin</th>
<th>Films &amp; spot Formation</th>
<th>Suspected type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>58</td>
<td>49%</td>
<td>+ve -ve</td>
<td>-ve</td>
<td>M. gallispticum</td>
</tr>
<tr>
<td>Group II</td>
<td>3</td>
<td>2.5%</td>
<td>+ve -ve</td>
<td>Late +ve</td>
<td>M. synoviae</td>
</tr>
<tr>
<td>Group III</td>
<td>18</td>
<td>15.3%</td>
<td>-ve +ve</td>
<td>+ve</td>
<td>M. gallinerum</td>
</tr>
<tr>
<td>Group IV</td>
<td>7</td>
<td>5.9%</td>
<td>-ve +ve</td>
<td>-ve</td>
<td>M. arginine</td>
</tr>
<tr>
<td>Group V</td>
<td>32</td>
<td>27%</td>
<td>-</td>
<td>-</td>
<td>Un typed Mycoplasma</td>
</tr>
</tbody>
</table>

Fig. (2) Digitonin test for obtained isolates

Fig. (4) Biochemical identification of isolates:
4- Serological identification of *Mycoplasma* isolates:

Growth inhibition test (GIT):

**Table (5) Serological identification of *Mycoplasma* isolates by GIT**

<table>
<thead>
<tr>
<th>Biotype</th>
<th>No. of positive isolates</th>
<th>Identified Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>40/58</td>
<td>MG</td>
</tr>
<tr>
<td>Group II</td>
<td>3/3</td>
<td>MS</td>
</tr>
</tbody>
</table>

*fig. (5) Serological identification of *Mycoplasma* isolates by GIT*
Table (6): Showing results of minimal inhibitory concentration test of mycoplasma isolated from different sites:

<table>
<thead>
<tr>
<th>MIC</th>
<th>1st End point</th>
<th>2nd End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Ciprofloxacin</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2- Doxycyclin</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3- Erythromycin</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>4- Lincospectin</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>5- Streptomycin</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6- Tilmicosin</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>7- Tylosin</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

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 ELghany(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. gallinorum 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 Ciprofloxacins, 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 enrofloxacin 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 by Doxycyclin and Tylosin while Ciprofloxacin and Lincospectin were less effective.

References


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الملخص العربي
تعتبر الميكوبلازما من ميكروبات واسعة الانتشار في مزارع الدواجن المختلفة مسببة خسائر اقتصادية كبيرة للمربيين وأصحاب المزارع وتتميز بانتقالها من الأم المصابة إلى الكتاكيت (انتقال رأسي). وهى أيضا تهيج الطيور للاصابة الميكروبى الأخرى حيث أن العدوى تؤدي لقصر الانتاج.

وفي خلال هذا العمل:
1- تم جمع عدد 200 عينة من الدجاج الذي يظهر عليه الاصابه باعراض تنفسية من عدد من المحافظات (الاسمايلية والشرقية والقاهره) تشمل مسحات حلقيه واجزاء من الاعضاء التنفسية والمفاصل المتورمة كالالتالي:
   • الاعضاء التنفسية 110 عينة والمفاصل الحلقيه 75 عينة والمفاصل المتورمة 15 عينة.
2- تم العزل الأول للميكوبلازما من العينات المختلفه بعدها عدد 118 عينة ايجابية وذلك بمعدل اصابة 59%.
3- تم اختبار الديجيتونين للتعرف على المعزولات وتعتبر الميكوبلازما حساسة للديجيتونين حيث تظهر منطقة تثبيط ملحوظ تحيط قرص الديجيتونين والذي أعطى 96 عينة من إجمالي 118 عينة ايجابية معزوله ميكوبلازما والباقي سلبي لهذا الاختبار.
4- اجراء التصنيف البيوكيميائي للمعزولات التي تم الحصول عليها وكانت النتيجه كما التالي:
   • مجموعة I: ميكوبلازما جاليسبتكم 58 معزوله 49% إيجابية.
   • مجموعة II: ميكوبلازما سينوفي 3 معزوله 100% إيجابية.
   • مجموعة III: ميكوبلازما جاليبرم 18 معزوله 15,3% إيجابية.
   • مجموعة IV: ميكوبلازما ارجينيني 7 معزوله 5,9% إيجابية.
   • مجموعة V: ميكوبلازما غير مصنفة 22 معزوله 27% إيجابية.
5- تم فحص عينات مضاد السيرم للمجموعة I والمجموعة II وذلك باختبار تثبيط النمو ولكن لا تم اجراء الاختبار للمجموعة III والمجموعة IV لعدم وجود مضادات السيرم لهم ووجد ان 40 عينة من 58 عينة ايجابية للميكوبلازما جاليسبتكم وان 3 عيان ايجابية للميكوبلازما سينوفي.
6- تم اجراء اختبار مانع النمو (GIT) باستخدام مضاد سيرم مرجعي لتأكيد التعرف على المعزولات.
7- تم اجراء اختبار أقل تركيز فعال (MIC) لبعض المضادات البكتيرية المختلفة ضد عوارض مماثله.