In *Vitro* Efficacy Of Some Antimicrobials On The *E.Coli* And Mycoplasmal Isolates From Cases Of Chronic Respiratory Disease In Broilers In Egypt

Abd El Aziz E. E.*, Hassan A. M.* and Jihan M. Badr**
*Mycoplasma Department, **Poultry Diseases Department
Animal Health Research Institute, Dokki, Giza, Egypt.

ABSTRACT

A total of 100 chicken cases of different ages showed clinical signs of chronic respiratory disease (CRD) collected from 20 different farms from Dakahlia governorate were used in the study. Tracheal, nasal swabs, lung and air sacs samples were collected for *Mycoplasma gallisepticum* (MG) isolation and liver, lung, kidney, bone marrow and air sacs were collected for *E coli* isolation. Primary isolation and biochemical characterization lead to five *Mycoplasma gallisepticum* and one M.gallinarum isolates (named DS1, DS2, DS3, DAS4 (*M. gallinarum*), DAS5 and DT6SC) which were confirmed by PCR except DAS4. The previous isolates and one MG reference isolate were included in Minimum inhibitory concentrations (MIC) assay for seven antibiotics (Doxycycline, Spiramycin, Erythromycin, Tilmicosin, Tiamulin, Tylocin and Enrofloxacin).

Isolation of *E coli* from clinical cases revealed an incidence of isolation of 90% of the examined cases, a total of 18 strains were included in the antimicrobial sensitivity for *E coli* using 10 antimicrobial discs (Ampicillin, Cephridin, Chloramphenicol, Enrofloxacin, Ciprofloxacin, Colistine sulfate, Doxycycline, Amikacin, Gentamycin and Sulphamethoxazol/ Trimethoprim). MIC testing for MG revealed that Doxycycline,Tiamulin and Tylocin were highly active against all of the MG strains used. Enrofloxacin was highly active against most of the strains used except against DS2, and DAS4 and DAS5 were fairly active. Spiramycin, Erythromycin and Tilmicosin were highly active against all the strains except against DAS4 were not effective

Antibiotic sensitivity testing of the isolated *E coli* strains revealed multi-resistance against two or more antibiotics for each strain. The isolated strains were highly sensitive to Colistine sulphate (100%) followed by Cephridin (88.9%), Amikacin (83.3%) then Gentamycin (77.8%). The sensitivity to Enrofloxacin and Ciprofloxacin was 55.6% and 50%, respectively. The sulphamethoxazole / trimethoprim sensitivity was 55.6%. The sensitivity to Ampicillin were only (33.3%) and to Chloramphenicol (22.2%).

INTRODUCTION

Mycoplasma gallisepticum is considered a significant pathogen of chickens and turkeys. Economic losses due to Mycoplasma infection resulted from mortality, reduced weight gain, feed conversion rate, decreased egg production and increased condemnations ***(1)***. The severity of the disease is greatly affected by the degree of the secondary infection with viruses such as Newcastle disease (ND) and Infectious Bronchitis (IB) and/or bacteria such as Escherichia coli ***(2)***.

Recently there is an increased concern to CRD due to the increased frequency in broiler farms which greatly influence the production and economy of these farms, *M. gallisepticum* infection results in increased susceptibility of the birds to *E coli* and/or other causatives of CRD and therefore increase the incidence of CRD. Socialization, environmental stress, coliform contaminated dust and ammonia resulting in deciliation of the upper respiratory tract which allows inhaled *E coli* to infect the air sacs ***(3 and 4)***. Susceptibility and sensitivity of the infection are greatest in young birds including developing embryo ***(5 and 6)***.

Antibiotic treatment is one of the major routes for control of the bacterial infection of the CRD (*Mycoplasma gallisepticum* and *E*

coli). Due to the lack of standards for disc sensitivity test or for minimum inhibitory concentration (MIC), the efficacy of different antibiotics for controlling *M. gallisepticum* had always been a problem but using minimum inhibitory concentration assayed and compared to the C-max of the same antibiotic in the chickens offers a guideline and recommendations for MIC testing of these organisms *(7).*

For *E coli* the commercial antibiotic sensitivity disc test can offer a standard method for examination.

The aim of this work was to investigate the prevalence of *E coli* and *M. gallisepticum* associated with CRD cases in different broiler farms, and to study the most effective antibiotics that can be used in controlling CRD infection caused by *E coli* and M. gallisepticum infection in broiler farms.

MATERIAL AND METHODS

Samples

In this study 100 chicken of different ages and showed clinical signs of CRD were collected from 20 different farms in Dakahlia Governorate. These birds were brought to Animal Health Research Institute. Case history, and clinical signs were recorded for each case. The respiratory signs were in the form of cough, sneezing, rales, nasal discharge, conjunctivitis and sinusitis. The birds were sacrificed, PM lesions were recorded and samples, tracheal, nasal swabs, lung and air sacs were collected aseptically from different internal organs and examined for *Mycoplasma* and *E coli*

Isolation and identification of MG

Liquid and solid media were used for the isolation and propagation of *mycoplasma* species *(8).*

Genus determination and biochemical characterization were carried out *(9).*

Polymerase chain reaction (PCR)

1-Extraction of chromosomal DNA *(10)*

A five ml quantity of overnight culture from each *mycoplasma* species isolated was centrifuged in a micro- centrifuge at 13000 r.p.m. for 3 minutes. The cell pellets were washed twice in 100 µl of 150 mM phosphate-buffered saline (PBS, pH 7.2) and suspended in 25 µl PBS. The cell suspension was heated directly at 100 °C for 10 minutes in a heat block and collected on ice for 10 minutes. Finally, the cell suspension was centrifuged for 3 minuets, and chromosomal DNA was collected and stored at 4°C.

2-Primer selection *(11)*

Two oligonucleotide primers were selected for the detection of MG. The sequence of primer (1) was: 5\- TAA CTA TCG CAT GAG AAT AAC-3\. The sequence of primer (2) was: 5\- GTT ACT TAT TCA AAT GGT ACA G-3\. The primer was locally prepared using 392 DNA/RNA synthesizer (Applied Biosystems) in Mycoplasma Department, Animal Health Research Institute, Dokki, Giza, Egypt.

3-DNA amplification *(11)*

The reaction mixture (total volume 50 µl) was 5µl of 10 X reaction buffer (Pharmacia), 1µl of 10 mM of nucleotide mix.(Sigma), 2 µl primer (containing 400 ng of each left and right primer), 2 µl DNA template (containing 40 ng DNA), 0.5 µl (2 units of *Taq* DNA polymerase (Promega) and the mixture was completed to 50 µl with distilled water. PCR was performed on a PROGENE thermal controller (UK),the amplification was performed by heating the samples for 5 minutes at 97°C then, using thirty cycles of denaturation for 15 sec. at 94°C, annealing for 1 minute at 55°C and extension for 2 minute at 72°C with the exception that final extension step was held for 10 min. The analysis of PCR amplified products and the DNA ladder (Pharmacia, 100 bp) was done using ten µl of the amplified PCR product, mixed with 2 µl loading buffer and electrophoresed through 1% agarose gel and DNA was visualized by UV flourescence after ethidium bromide staining, and then photographed.

Isolation and identification of E. coli

Liver, lung, kidney, bone marrow and air sacs samples were inoculated into Selenite

broth and incubated at 37 °C for 18-24 hours, loopfull of each broth culture of different organs were streaked onto MacConkey agar and Eosine Methylene blue (EMB) agar plates and incubated at 37 °C for 18-24 hours. Suspected colonies from different media were screened morphologically and biochemically *(12 and 13)*.

Minimum inhibitory concentration for MG *(14)*

In vitro activities of some drugs against seven mycoplasma isolates were made by minimum inhibitory concentration assay using the antibiotics listed in Table 1, which were examined against five field isolates of *M. gallisepticum* (DS1, DS2, DS3, DAS5 and DT6SC), one *M. gallinarum* (DAS4) and one reference *M. gallisepticum* (Kindly supplied by Prof. Dr. S. I. Eissa, Animal Health research Institute, Dokki, Giza).

The culture seeds of every strain used were titrated to be $1x10^4$ colony forming units (CFU).

Table 1. List of antibiotics used *.

Drug	Producing Company
Doxycycline	GMP (certified Spain -EU)
Spiramycin	GMP (certified Spain -EU)
Erythromycin	Pantex- Holland
Tilmicosin	ELANCO (Geneva)
Tiamulin	Sandoz GmbH (Basle-Switzerland)
Tylocin	ELANCO (USA)
Enrofloxacin	INVESA-Span

* Concentration at first well was 20 µg/ml

Antimicrobial sensitivity test for E. coli strains

Disc diffusion method was done *(15)* to E. coli isolates on Mueller Hinton agar using antibiotic discs produced by Oxoid LTD, London- England **(Oxoid manual, 1982)** including Ampicillin (10µg), Amikacin (30 µg), Cephridin (30 µg), Ciprofloxacin (5µg), Chloramphenicol (30 µg), Colistine sulphate (25µg), Doxycycline (30 µg), Enrofloxacin (5µg), Gentamycin (10µg) and Sulpha methoxazole / Trimethoprim (25µg). The results were interpreted *(16)*.

RESULTS AND DISCUSSION

Laboratory examinations revealed that the main clinical signs of the collected cases were anorexia, inactivity accompanied with different degrees of respiratory disorders (nasal discharge, rales, cough, etc...) with or without diarrhea.

The PM lesions revealed that most of the examined cases showed air-sacculitis, pneumonia, fibrenious pericarditis, sever perihepatitis and enteritis. The infected air sacs were thickened and often had cassious exudates. Similar lesions were previously reported *(17)*. The severity of the resulting CRD is directly related to the number of involved organisms.

The primary isolation of *mycoplasma* from the collected samples yield 6 mycoplasmal isolates (digitonin positive), 3/6 isolates from nasal swabs (50%) of birds suffered from sinusitis, 2/6 isolates from air-sacs (33.3%) of CRD cases and one out of six isolates (16.6%) from trachea as in Table 2.

Polymerase chain reaction was performed for the suspected *M. gallisepticum* field isolates, as seen in Figure 1. All the tested isolates were positive and gave specific *M. gallisepticum* band at 330 bp *(11)*.

More than one type of colonies could be isolated from the same site based on differences in morphological and biochemical properties. Several investigators described the

isolation of more than one species from a particular site in other hosts including bovine ***(18)***, dogs ***(19)***, avian ***(20)*** and humans ***(21)***.

Biochemical and PCR identification of the obtained isolates recorded in Table 2 showed that 5 isolates (83.3) were MG and one isolate (16.6) revealed the character of *Mycoplasma gallinarum* (Glucose negative, Arginin positive and Film and Spot positive), this strain was isolated from air-sac of bird suffered from CRD, *Mycoplasma gallinarum* is a fast grower mycoplasma which may hide the slow growing pathogenic mycoplasma or may be the cause of air-sacculitis ***(22)***.

Most of the MG isolated strainswere from aged birds (not less than 45 days), the rate of isolation of pathogenic mycoplasma was very low (6%). The low recovery rate may be due to anti-mycoplasmal substances in the form of drugs in tissues, anti-sera or inhibitors released from tissues after death and this influence the recovery of mycoplasma ***(23)*** and the incidence of mycoplasma isolation is very low in comparison with serological results ***(24)***.

Isolation of *E coli* from clinical cases (Table 3) revealed an incidence of isolation of 90% of the examined cases, this high isolation rate may be contributed to the inhaled coliform-contaminated dust, exposure to house dust and ammonia, young age, in addition to mycoplasmal, viral infection (IBV or NDV), and cold stress which decreasing the resistance to *E coli* and period of increased susceptibility begins earlier and persists longer ***(6 , 25*** and ***26)***.

Application of different antimicrobials as shown in Table 4 and comparison of MICs to the C-max revealed that most of antibiotics used gave MICs less than C-max of the same antibiotic in chicken so we divided the activity of the antibiotics in relation to C-max to highly active (1/4of C- max or less), fairly active (1/4 –1/2), suspect (1/2-1) and inactive if it is more than C-max.

According to the previous categorization, the Doxycycline,Tiamulin and Tylocin were highly active against all of the MG strains used. Enrofloxacin was highly active against most of the strains used except against DS2, DS3 and DAS4 and DAS5 was fairly active. Spiramycin, Erythromycin and Tilmicosin were highly active against all the strains except against DAS4 (*M. gallinarum*) were not effective.

The previous study indicated that Tylocin was highly effective against MG strains, while Erythromycin was less effective and was ineffective against *Mycoplasma synoviae* ***(27 and 28)***.

This also was in agreement with previous investigation which indicated that Tylocin and Tiamulin showed lower MICs ***(29)***.

Our results were also in agreement with that of ***(7)*** as they recorded that Tiamulin, Doxycycline and Danofloxacin had MICs ranges well below the C-max.

On the other hand, ***(30)*** stated that Tilmicosin showed slightly lower MICs than Tylocin.

Antibiotic sensitivity testing of the isolated *E coli* strains showed Table 5 multi-resistance against two or more antibiotics for each strain. The isolated strains were highly sensitive to Colistine sulphate (100%), followed by Cephridin (88.9%), Amikacin (83.3%) then Gentamycin (77.8%).

There is an increment in the resistance to Fluoroquinolones (Ciprofloxacin and Enrofloxacin), the sensitivity was 55.6% and 50%, respectively. The sulphamethoxazole / trimethoprim sensitivity was 55.6%.

Resistance of some strains of *E coli* to Fluoroquinolones was previously detected ***(31 and 32)***.

Most of the examined strains showed resistance to ampicillin that the sensitive strains were only 33.3% and to Chloramphenicol was 22.2%. *E coli* isolated from chickens was resistant to Flurophenicol, an antibiotic related to Chloramphenicol ***(33)***.

Isolates of *E coli* are frequently resistant to one or more of drugs especially if they have widely used in the poultry industry over a long

period *(34)*. However, antibiograms is imperative to determine drug sensitivity to *E coli* strain involved in a disease outbreak so that ineffective drug can be avoided. Under-dosing promotes the development of resistance and using the antibiotics as feed additives increase resistance to different antibiotics.

Conclusion

For protection and treatment of CRD complex in chickens, it is better to use Doxycycline, Tiamulin or Tylocin to control MG as they are highly active against MG. For *E coli*, it is advisable to use antibiograms to determine the drug of choice.

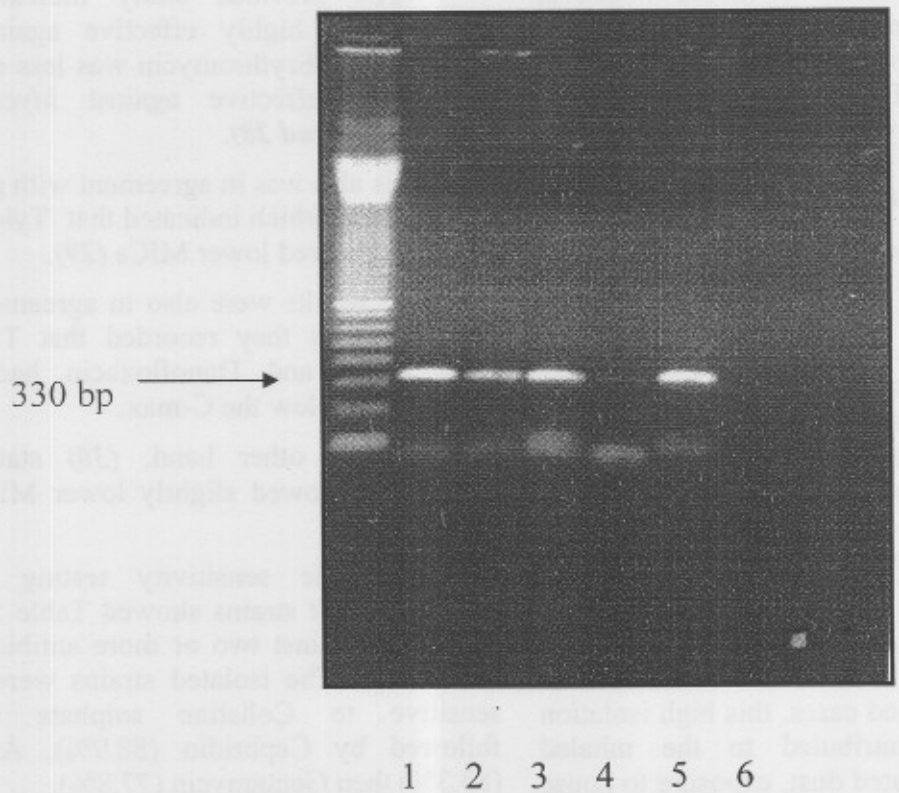

Figure 1. PCR results of field isolates *M. gallisepticum*.
(1) 100 bp ladder (Pharmacia)
(2) *Mycoplasma gallisepticum* (DS1)
(3) *Mycoplasma gallisepticum* (DS2)
(4) *Mycoplasma gallisepticum* (DAS3)
(5) *Mycoplasma gallisepticum* (DAS5)
(6) *Mycoplasma gallisepticum* (DT6SC)

Table 2. Mycoplasma isolation and identification (Biochemical and PCR) for the collected samples from different organs.

Site of isolation	Examined no.	No. of positive isolation	No. of Digitonin positive	Biochemical characterization			Susspected strain	PCR identification
				Glucose	Arginin	F & S*		
Nsal swab	100	3	3	+	–	–	*M. gallisepticum*	*M. gallisepticum*
Trache al swab	100	2	1**	+	–	–	*M. gallisepticum*	*M. gallisepticum*
Air-sac	100	2	1	+	–	–	*M. gallisepticum*	*M. gallisepticum*
			1	–	+	+	*M. gallinarum*	Not done

*= Film and Spot **= The other one was digitonin negative may be Acholeplasma

Table 3. Incidence of isolation of E.coli from CRD cases in broiler farms

No. of examined farms	No. of examined cases	E. coli positive cases	
		No.	percentage
20	100	90	90%

Table 4. Sensitivity test for *E coli* isolates from CRD cases in broiler farms

Antimicrobial discs	Disc conc. (µg)	Standard zone of inhibition (≥)	No. of examined strains	Sensitive strains	
				No.	Percent
Ampicillin (AMP)	10	19	18	6/18	33.3
Cephridin (CE)	30	18	18	16/18	88.9
Chloramphenicol (C)	30	28	18	4/18	22.2
Enrofloxacin (ENR)	5	30	18	9/18	50
Ciprofloxacin (CF)	5	11	18	10/18	55.6
Colistine sulfate (CT)	25	21	18	18/18	100
Doxycycline (Do)	30	18	18	8/18	44.4
Amikacin (AK)	30	19	18	15/18	83.3
Gentamycin (GM)	10	19	18	14/18	77.8
Sulphamethoxazole/ trimethoprim	25	24	18	10/18	55.6

Table 5. Minimum inhibitory concentrations (MIC) for different antibiotics used with different Mycoplasma strains isolated compared to the maximum plasma concentration (C-max).

Antibiotics	DS1*	DS2	**	DAS4***	DAS5	DT6SC****	S6 ref.	C max*****
Doxycycline	0.019	2.5	2.5	2.5	2.5	2.5	2.5	54.58
Spiramycin	0. 01	0.156	0.312	10	0.078	0.078	0.078	3.1
Erythromycin	0. 01	0.039	0.01	20	0.019	0.019	0.019	6.9
Tilmicocin	0. 01	0.078	0.039	20	0.312	0.156	0.156	2.46
Tiamulin	0. 039	0.156	0.312	0.01	0.625	0.312	0.312	3.56
Tylocin	0. 039	0.156	0.312	1.25	0.625	0.312	0.312	4.2
Enrofloxacin	0. 039	0.625	0.625	0.625	1.25	0.125	0.125	1.88

* = (D) means Dakahlia and (S) means sinus
** = (AS) means air sacs
*** = *M. gallinarum*
**** = (T) means trachea
*****= C-max of the previous antibiotics was put according to ***(7, 35 and 36).***

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الملخص العربى

دراسة معملية لتاثير بعض المضادات الميكروبية على الميكروب القولونى و معزولات الميكوبلازما من حالات مصابة بالمرض التنفسي المزمن فى بدارى التسمين فى مصر.

عيد السعيد عبد العزيز* عبد الواحد محمود حسن* جيهان مصطفى بدر**
*** قسم بحوث الميكوبلازما - ** قسم بحوث أمراض الدواجن-معهد بحوث صحة الحيوان – الدقي - الجيزة**

شملت الدراسة فحص عدد ١٠٠ حالة من الدجاج من مختلف الاعمار مثلت ٢٠ مزرعة تسمين تعانىجميعها من أعراض المرض التنفسى المزمن.تم تجميع مسحات رئوية،مسحات أنفية و عينات من الرئتين و الاكياس الهوائية لعزل الميكوبلازما ،بينما أخذت عينات من الكبد و الرئتين و الكلى و النخاع العظمى و الاكياس الهوئية لعزل الميكروب القولونى.بالعزل الاولى و الاختبارات البيوكيمائية أمكن عزل خمسة معزولات من الميكوبلازما جاليسبتكم سميت كالاتى DS1 , DS2 , DS3, DAS5 , DT6SC و معزولة واحدة ميكوبلازما جالينيرم (DAS4).

تم تاكيد السلالات المعزولة باسخدام تفاعل انزيم البلمرة المتسلسل (PCR) فيما عدا سلالة DAS4 و قد تم اختبار العترات السابق ذكرها بالاضافة الى عترة مرجعية للميكوبلازما لأقل تركيز مثبط MIC لسبعة مضادات حيوية (دوكسى سيكلين ،سبيراميسن، ارثرومايسن، تلميكوسين، تيامولين، التيلوسين،و الانروفلوكساسين).

تم عزل الميكروب القولونى من الحالات التى تم فحصها بنسة عزل ٩٠% و قد تم عمل اختبارات الحساسية لعدد ١٨ عترة معزولة باستخدام أقراص من المضادات الحيوية المختلفة (أمبيسلين، سيفرادين،كلورامفينيكول،انروفلوكساسين،سيبروفلوكساسين ،كولستين سلفات ،دوكسى سيكلين،أميكاسين،جينتاميسين ،و السلفاميثوكسازول-تراىميثوبريم).

أثبت اختبار أقل تركيز مثبط(MIC) للميكوبلازما أن الدوكسى سيكلين ،تيامولين،و التيلوسين كانو الاكثر كفاءه ضد كل عترات الميكوبلازما المعزولة وكان اللإنروفلوكساسين ذو كفاءة متوسطة ضد العترات DS2, DAS3 and DAS4 وDAS5 وذو كفاءة عالية ضد باقى العترات و أثبت السبيراميسين،و الارثرومايسين و التلميكوسين كفاءه عالية ضد كل العترات ماعدا DAS4 وجد أنه عديم الكفاءة ضدها.

أثبت اختبار الحساسية لمعزولات الميكروب القولونى تعدد المقاومة لتاثير اثنين أو أكثر من المضادات الحيوية لكل عترة. وقد وجد أن العترات المعزولة كانت شديدة الحساسية للكولستين سلفات (١٠٠%)، يليها السيفرادين (٨٨,٩ %) ،الاميكاسين(٨٣,٣ %)، و الجينتاميسين (٧٧,٨ %) بينما كانت درجة الحساسية للانروفلوكساسين و السيبروفلوكساسين (٥٥,٦ %و٥٠ %) على التوالى و كانت الحساسية للسلفاميثوكسازول-تراى ميثوبريم (٥٥,٦ %).

خلصت الدراسة إلي أن علاج المرض التنفسي المزمن في بداري التسمين يلزم استخدام أحد مضادات الميكوبلازما (دوكسي سيكلين أو تياميولين أو التيلوسين) وعمل أختبار الحساسية للميكروب القولوني بعد عزله لإختيار أفضل المضادات المؤثرة عليه.