THE EFFECT OF TILMICOSIN AND APRAMYCIN ALONE OR TOGETHER IN TREATMENT OF EXPERIMENTALLY INFECTED CHICKENS WITH *MYCOPLASMA GALLISEPTICUM* ONLY OR WITH *ESCHERICHIA COLI*

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ABSTRACT

Mycoplasmosis remains one of the most expensive and common diseases facing poultry industry. The presence of Mycoplasma gallisepticum (MG) predisposes birds to other infections as Escherichia coli which together causes mortality, and sub-optimal performance. Therefore, this study was carried out to evaluate the efficacy of tilmicosin, apramycin and both drugs together on experimentally infected chicken with local field isolates of MG only or with E. coli as CRD and CCRD treatments. The present investigation clarified that infected groups treated with both tilmicosin and apramycin showed additive or synergistic interaction between them, which in turn confirmed the safety use of this combination therapy. The use of the recommended therapeutic dose of tilmicosin (1ml/L) or apramycin (0.5gm/L) alone or in combination for 5 successive days after appearance of the clinical signs are of considerable value in the treatment of MG infection in broiler chickens as antimycoplasmal drugs through displayed valuable improvement in clinical symptoms, survival rate, lesion scoring and termination of infection by the mycoplasmacidal effect of both drugs. The results also indicated that combined tilmicosin-apramycin therapy as antimicrobial drugs gives

relatively stable results in the treatment of CCRD in chickens with a superior activity and efficacy than apramycin or tilmicosin alone followed by apramycin in reducing the severity of lesion, mortality rate, E. coli count and improve the general activity of chickens, so it can be used as alternative strategy that may contribute to the treatment programs of CCRD. Co-infected group treated with tilmicosin showed less improvement than other MG and E. coli infected treated groups this returned to its restricted effect on E. coli infection.

INTRODUCTION

Owing to the substantial losses caused in both performance and production, MG has been described as the most economically important of the 4 pathogenic Mycoplasma species affecting poultry *(Evans et al., 2005)*. In Egypt the prevalence of the MG isolation was 58.3% in broiler samples. Early intervention may help decrease the associated morbidity and mortality of chickens affected by chronic respiratory disease *(Osman et al., 2009)*. CRD caused by MG is one of the most important veterinary diseases, which cause mortality, reducing of weight gain and increasing of FCR. There are several drugs which are used for its prevention and control *(Zakeri and Kashefi, 2011)*.

Chemotherapeutic approaches are often necessary and consider the most practical way to minimize MG transmission in the case of outbreaks *(Wu et al., 2005)* preventing clinical signs, lesions as well as economic losses *(Kleven, 2008)*. Uses of recent antimycoplasmal drugs in the therapy is still recommended in the eradication programs. It remains the most economic method and powerful tool in veterinary medicine in controlling of mycoplasma infection than application of vaccine *(Jordan et al., 1999)*.

MG predisposes the birds to *E. coli* yielding significant losses in performance and associated economics to all sectors of the poultry industry *(Khairy, 2011)*. Colibacillosis continues to be one of most important respiratory and systemic diseases infecting all ages of the poultry and resulting in the heavy morbidity and mortality, loss of body weight, decreased feed conversion efficiency and condemnations *(Satyajit et al., 2013)*. Control of *E. coli* is an important in poultry farm which depend mainly on the use of certain antimicrobials to avoid hazard effect of these infections *(Hafez, 2008)*. If antibiotic use is restricted in poultry production, it would be anticipated that colibacillosis would become an even greater problem *(Huff et al., 2002)*. Farms with a history of respiratory disease had an increased risk of *E. coli* as a secondary infection of the respiratory tract and may spread from there to the peritoneal cavity *(Barnes, 2008)*.

The way of use of antibiotics in veterinary medicine is very important for the poultry health security *(Butcher, 2006)*. Combination therapy can be used to achieve a broad antimicrobial spectrum of empirical therapy, treat a polymicrobial or severe infection and reduce the likelihood of antimicrobial resistance *(Neu, 1994)*. It has become common for veterinarians to use cost/effective medications to treat severe respiratory and enteric disease challenges. By improving the products used and understanding of their pharmacokinetics (PK), pharmacodynamics (PD) and relating them to the antimicrobial susceptibility of the pathogenic bacteria, their effective use will be enhanced, their therapeutic benefits maximized and, at the same time, resistance development minimized. The use of PK and PD is generally very helpful in determining the choice of antimicrobial and dose that should be used; but other factors often influence this, with cost being the major one *(Burch, 2003)*.

The macrolide including tilmicosin is among the antibiotic families most widely used in the veterinary field for treatment of mycoplasmosis in poultry in many countries *(Gerchman et al., 2011)*. Tilmicosin is a broad-spectrum bacteriostatic macrolide antibiotic synthesized from tylosin for veterinary use only. It has an antibacterial spectrum that is predominantly effective against *Mycoplasma* spp, so it is recommended for the treatment of respiratory diseases in poultry *(Prescott, 2000)*.

The aminocyclitole antibiotics incuding apramycin are used in the treatment of acute and chronic infections of the respiratory organs and gastrointestinal tract, caused by Gram negative bacteria *(Ferenc et al., 1987)*. The spectrum of activity, bioavailability and low risk of toxicity to gain more therapeutic efficiency and fewer side effects, are regarded among these newly developed aminocyclitol antibiotics as apramycin that seem promising in veterinary use *(Ahmed, 1990)*.

The aim of the present study was conducted to study the effect of tilmicosin and apramycin interaction and evaluate their effectiveness as antimycoplasmal and antimicrobial drugs against experimentally infected chickens with MG only or with *E. coli* in order to achieve the optimal control to CRD and CCRD using some *in-vivo* studies; such as, improving clinical signs, lesion scoring, mortality rate, assessment of the severity of post-mortem lesions and bacterial re-isolation.

MATERIALS AND METHODS

Drugs:

Tilmicosin posphate (Timosin®) is semisynthetic antimycoplasmal drug belongs to macrolide antibiotics for poultry manufactured by: Arabco Med for Ultra vet and distributed by I vet. Each 1 ml of Timosin contains 278.21 mg of tilmicosin phosphate, (eq. to tilmicosin base: 250

mg). It is used in the recommended therapeutic dose from the producer company at a level of 1ml/liter given for 5 successive days.

Apramycin sulfate (Apracin®) is broad-spectrum aminocyclitol antibiotic water-soluble powder for poultry developed and produced by Unipharma. Each 150 gm packet contains 78 gm (eq. 50 mema units of apramycin) used in the recommended therapeutic dose from the producer company at a level of 0.5gm/liter given for 5 successive days.

Experimental chickens:

One hundred and thirty five, one-day-old unsexed avian 48-broiler chicks (a division of Cobb) were obtained from commercial breeder farm, El Kenana Company for Grand Parents, Tanta, Egypt. They were divided randomly into 9 equal groups each contains 15 chicks, housed in a well-isolated floor pens and kept under complete hygienic conditions. Water and food were provided *ad libitum* during the experimental period (42 days) using standard broiler ration free from any medications obtained from FATY HENS company. Vaccination was carried out against Newcastle and Gumboro diseases according to ***Giambrone and Ronald, (1986)***. Birds were evidenced to be free from MG and *E. coli* infections according to ***Belah et al., (2012)***.

Bacterial strain:

***M. gallisepticum* strain:** Pathogenic field MG strain, morphologically, biochemically, serologically and molecullary characterized, was used for this study.

E. coli* strain:** Pathogenic *E. coli* strain serotype O78 of avian origin was isolated from a field case of coliseptisaemia in a local chicken and serotyped using the somatic antigen according to ***Ørskov and Ørskov, (1984). The identification was done by studying their

morphological and staining characteristics according to ***Quinn et al., (1994)***, cultural, sugar fermentation and standard biochemical reactions according to ***Jalil and Das, (2001)***. Both strains were obtained originally from the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

Grouping and experimental design:

Table (1): The experimental design.

Group	No of chicks	Age of infection		Medication			Experimental Period (day)
		MG	*E.coli*	The used drugs	Dose	Time of medication	
1	15	--	--	--	--	--	42
2	15	8 day	--	--	--	--	42
3	15	8 day	--	Tilmicosin	1ml/L	From 28th to 32nd day	42
4	15	8 day	--	Apramycin	0.5gm/L	From 28th to 32nd day	42
5	15	8 day	--	Tilmicosin + Apramycin	1ml/L + 0.5gm/L	From 28th to 32nd day	42
6	15	8 day	23 day	--	--	--	42
7	15	8 day	23 day	Tilmicosin	1ml/L	From 28th to 32nd day	42
8	15	8 day	23 day	Apramycin	0.5gm/L	From 28th to 32nd day	42
9	15	8 day	23 day	Tilmicosin + Apramycin	1ml/L + 0.5gm/L	From 28th to 32nd day	42

The groups were treated with the drugs in drinking water after the clinical signs of both infections had appeared (20th day post of MG infection and 5th day post of *E. coli* infection) for 5 successive days from 28th day to 32nd day of age.

Preparation of bacterial inoculation and experimental infections:

M. gallisepticum* strain:** It was grown in Frey's Modified Mycoplasma Growth Broth culture according to ***EL-Shabiny, (1984) at 37 °C for 48 hr. Broiler chickens of all groups except the negative control group were infected with 0.1 ml aliquots of PPLO (Pleuro Pneumonia Like Organism) broth culture equal (100µl) containing 1×10^6 CFU/ml was given by automatic micropipette/chick intra-tracheal to induce infection according to ***Belah et al., (2012)*** at 8th day of age.

E. coli **strain:** It was suspended in peptone water and enumerated by McFarland nephelometer standards obtained from Difco (USA) according to ***Finegold et al., (1998)***. The experimental infection occurs to last 4 groups via inoculation of 0.2 ml containing (1x10^8 CFU/ml) intatracheally according to ***Khairy et al., (2012)***, at the 23rd day of age.

Serological tests:

The commercially stained MG standard antigen (Nobilis) produced by Intervet international, (B.V. Boxmeer, Holland) was used to check for the presence of MG antibodies using serum plate agglutination (SPA) test according to ***OIE Terrestrial Manual, (2008)***. The agglutination has been standardized against a standard serum.

The *in vivo* evaluation of the efficacy of the tested drugs:

a) Clinical signs: All chickens were left under observation during the experimental period to record the clinical symptoms. The infected birds were examined for the clinical signs of both diseases according to ***Khairy et al., (2012)***.

b) Lesion scoring: Typical air-sac lesions of mycoplasma infection were recorded and scored from (0 to 4) to assess the severity of lesions in air sacs of dead and sacrificed chickens in all groups according to ***Stipkovits, (1997)***.

c) Mortality rate: Mortality for each group was recorded daily until the end of the work (42 day of age) and calculated as a percentage. The exact cause of mortality was confirmed by postmortem examination, which was carried out on the day of death.

Postmortem examination of dead or slaughtered birds: On 27th, 33rd and 42nd day of age. Three, 5 and 3 birds from each group were sacrificed, after the onset of clinical signs, after treatment and at the end of the study respectively Birds were opened and examined macroscopically for any lesions especially for CRD and/or collibacillosis *Khairy et al., (2012)*.

d- Bacterial re-isolation: Samples were collected from the sacrificed birds, on 27th day of age after appearance of clinical symptoms to ensure the occurrence of the intended infections and on 33rd day of age, after the treatment period to evaluate the efficacy of the tested drugs *(Salama, 1994)*. Planting was insured in the respective culture to MG and *E. coli*.

1- Isolation of *M. gallisepticum* from respiratory organs: MG in trachea, lungs and air sacs was isolated on PPLO medium according to *OIE Terrestrial Manual, (2008)*. The plates were considered negative if no growth appeared after 21 days of incubation. Characteristic colonies appear as tiny, smooth, circular translucent masses and sometimes have a "fried egg" appearance with a dense raised central area *(Ley and Yoder, 1997)*. Planting took place in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

2- *E. coli* strain:

For bacterial re-isolation: Specimens from liver, spleen, kidneys and a loop full of heart blood were put in to peptone water tube and incubated at 44^0c /24 hr, then streaked into Tryptone Bile X-Glucuronide medium (TBX) agar as a selective chromogenic medium for detection and isolation of *E. coli* bacteria and incubated at 44^0c /24 hr. Typical colonies appear blue/green coloured colonies *(Torlak et al., 2008)*.

For bacterial count: Aforementioned specimens were inoculated into nutrient broth tube and incubated at 37^0c/24 hr, 10 fold serial dilutions were prepared from an overnight broth culture of *E. coli,* then two plates was cultured from each dilution. 0.1 ml from each tube was spread on Eosin methylene blue (EMB) agar plate as a selective differential medium for *E.coli* isolation, identification and count. Then they were incubated at 37^0c/24 hr. Counting, typical colonies, which appear green metallic sheen colonies with dark purple center, by plate count method, was carried out according to **Chowdhuri *et al*., (2011)**. *E. coli* count was calculated by getting the mean of each two plates and expressed as CFUs/plate.

Statistical analysis:

Data are represented as mean ± SE (standard error). One-way analysis of variance (ANOVA) was used to compare the means of values of all groups at a significance level of $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$. Statistical analysis was performed using the method of *Petrie and Watson, (1999)* and computerized using *SPSS 11 (2001)*.

RESULTS

Antimycoplasmal and antimicrobial drugs' efficacy:

a- Clinical signs:

MG infected non treated group revealed clinical signs from the 7th day post-infection (PI) and typical signs of mycoplasmosis began on the 2nd week PI (3 weeks of age), respiratory symptoms including firstly sneezing later on coughing, mucoid nasal discharge, air gasping, difficult breathing, ocular signs as eyelid edema, conjunctivitis or ocular secretion with bubbles and tracheal rales were observed in most of birds; these signs ended by poor growth and reduced feed consumption. All these

symptoms were not recorded in groups, which received tilmicosin and/or apramycin and birds showed normal behavior. Treatment with both drugs leads to rapid and great improvement in general body conditions with complete ending to any respiratory symptoms. Curing began after 3 days from the onset of the treatment with no noticeable differences when compared to negative control group all over the experiment.

The non-medicated super infected chickens with *E. coli* induced rapid decline in birds' condition (severe depression, dehydration, tendency to huddle together, reluctance to move, anorexia), respiratory signs and foul smelling watery brownish greenish diarrhea were the main clinical signs in most of birds from the 3rd day post *E. coli* inoculation. Dyspnea, rattling noises were more aggravated than only MG infection. Slow growth and decreased feed intake were apparent. Variable degrees of illness were recorded until the end of the experiment. Coinfected group treated with tilmicosin improved only the respiratory signs but still presence of watery diarrhea and bad body condition. The medicated groups by apramycin or both drugs together decreased the respiratory distress and other clinical signs till ending it and restore bird's activity.

b- Lesion score:

The number of typical affected birds showing air-sac lesions and the mean air-sac lesion scores in different groups were recorded and scored in (table 2 and 3).

The air sacs of the 2 positive control groups either infected with MG only or with *E. coli* showed the highest lesion scores, which ranged from (3 to 4). Treated groups with tilmicosin and/or apramycin showed scores from (o to 2). The lesion scores were significantly decreased (p $\leq$ 0.001) by all treatments with no significant difference of air sacculitis in

different treated groups examined till complete curing at the end of the study except coinfected tilmicosin treated group, which showed a relatively high lesion scores mainly due to *E. coli* infection.

Air sac lesion scores of the dead birds scored 2, 3, 4 and 4 at PM examination for 1st case of mortalities in (G2), 2 cases in (G6) and last case of mortalities in (G7) respectively.

Table (2): The number of chickens showing air sacculitis lesion in all groups.

G 9	G 8	G 7	G 6	G 5	G 4	G 3	G 2	G 1	Groups / No. of slaughtered birds	Days of age
Numbers of chickens showing air sacculitis lesions										
3	2	2	2	3	2	2	2	0	**(3) Post infection**	27th day
100	67	67	67	100	67	67	67	0	%	
1	2	3	5	1	2	2	5	0	**(5) Post treatment**	33rd day
20	40	60	100	20	40	40	100	0	%	
0	0	2	3	0	0	0	3	0	**(3) At the end of the study**	42nd day
0	0	67	100	0	0	0	100	0	%	

Table (3): Mean air sac lesion scores in all groups.

ANOVA		Mean air sac lesion scoring									Items	
P-value	**F**	**G9**	**G8**	**G7**	**G6**	**G5**	**G4**	**G3**	**G2**	**G1**		
***	19.167	3.67 [bc]	3.67 [bc]	3.33 [bc]	4.00 [c]	2.67 [bc]	2.33 [b]	3.00 [bc]	2.67 [bc]	0.00 [a]	**Mean**	27th day
		± 0.33	± 0.33	± 0.33	± 0.00	± 0.33	± 0.33	± 0.00	± 0.33	± 0.00	**± SE**	
***	16.446	0.20 [a]	0.60 [a]	1.40 [ab]	3.80 [c]	0.20 [a]	0.60 [a]	0.40 [a]	2.60 [bc]	0.00 [a]	**Mean**	33rd day
		± 0.20	± 0.40	± 0.60	± 0.20	± 0.20	± 0.40	± 0.25	± 0.25	± 0.00	**± SE**	
***	21.083	0.00 [a]	0.00 [a]	0.67 [a]	2.33 [b]	0.00 [a]	0.00 [a]	0.00 [a]	1.67 [b]	0.00 [a]	**Mean**	42nd day
		± 0.00	± 0.00	± 0.33	± 0.33	± 0.00	± 0.00	± 0.00	± 0.33	± 0.00	**± SE**	

Different alphabetic super scripts indicate statistically significant differences in each experiment.

The same alphabetic super scripts indicate statistically no significant difference. *** $P \leq 0.001$

G1=Non-Non   **G2**=inf MG-Non   **G3**= MG+T   **G4**= MG+A   **G5**= MG+T+A.
G6= MG+*E. coli*   **G7**= MG+*E. coli* +T   **G8**= MG+*E. coli* +A   **G9**= MG+*E. coli* +T+A.

c- Mortality rate:

The MG infected non treated group elicited (6.7 %) mortality rate on (15th) days of age suffered from severe tracheitis and airsacculitis. While all MG infected treated groups did not record any mortalities. Super infected chickens with *E. coli* untreated group showed the highest mortality rate (13.3 %) on (33rd and 34th) days of age suffered mainly from coligranuloma, colisepticemia severe enteritis and airsacculitis, whereas infected groups treated with tilmicosin or apramycin or both drugs together showed lowered mortality rates to 6.7%, 0% and 0% respectively(on 39th day of age). Four chickens died from artificial infections.

Post mortem lesions of sacrificed chickens:

Most MG infected chickens showed airsacculitis, tracheitis and variable lung affections. All infected chickens medicated with the used drugs or their combinations, showed a great decrease in percentage of the gross pathological lesions. However both drugs together give the best result.

Most MG and *E. coli* infected chickens showed tracheitis with hemorrhagic exudates, severe airsacculitis, adhesive pericarditis, fibrinoperulent perihepatitis, enteritis with colisepticemia appeared as sever congestion and subcapsular hemorrhage in most of the parenchymatous organs specially the liver. Lung affections included congestion, hemorrhage, indurations or pneumonic batches. All infected chickens that receive treatments showed favorable degrees of improvement but treatment with apramycin or both drugs was more effective in controlling these lesions.

d- Bacteriological findings (infection assessment):

Results of reisolation were calculated as percent (table 4) and *E. coli* counts from internal organs after treatment were shown in (table 5).

Concerning to *M. gallisepticum* re-isolation:

Non infected birds showed no bacterial re-isolation in all tested periods, however, higher frequencies of re-isolation of the pathogen were recorded in infected groups post infection (PI). Medicated groups with tilmicosin and/or apramycin were bacteriologically negative to MG, so they completely reduced the frequency of re-isolation to be 0%.

Concerning to *E. coli* re-isolation:

Positive cultural colonies found only in *E. coli* challenged groups from (G6 to G9) and this demonstrated that the intratracheal respiratory infection became systemic infection. Treatment of super inoculated groups with tilmicosin did not affect the *E. coli* reisolation frequency, while apramycin treated groups either alone or combined with tilmicosin show reduction in number of the positive cases.

E. coli count:

The coinfected non treated group showed the highest count reaches to (5.000 ± 0.02) x 10^3. All coinfected groups treated with tilmicosin, apramycin or both drugs together displayed a significant decrease in total *E. coli* count when compared to the positive control group with apparent differences between infected treated groups. The lowest count was recorded in birds given both therapies as (2.233 ± 0.02) x 10^3, followed by apramycin (2.333 ± 0.01) x 10^3 and finally tilmicosin treated group (4.225 ± 0.02) x 10^3 at $(P \leq 0.001)$.

Table (4): The rate of re-isolation of both microorganisms with its incidence.

G 9	G 8	G 7	G 6	G 5	G 4	G 3	G 2	G 1	Age	Group		
3	2	2	2	3	2	2	2	0	**MG**			
100	67	67	67	100	67	67	67	0	**%**	**27th day (PI)**		
3	3	3	3	0	0	0	0	0	*E. coli*	**n= 3**		
100	100	100	100	0	0	0	0	0	**%**			
0	0	0	5	0	0	0	5	0	**MG**			
0	0	0	100	0	0	0	100	0	**%**	**33rd day (PT)**		
3	3	5	5	0	0	0	0	0	*E. coli*	**n= 5**		
60	60	80	100	0	0	0	0	0	**%**			

Header: **Numbers of chickens yielding positive culture**

n= number of slaughte\red birds

Table (5): *E. coli* count $(x10^3)$/gm organs.

ANOVA		E. coli count $(x\ 10^3)$ CFU/gm				Items	
P-value	**F**	**G9**	**G8**	**G7**	**G6**		
*****	10546.242	2.233 [a]	2.333 [b]	4.225 [c]	5.000 [d]	**Mean**	**33rd day**
		± 0.02	± 0.01	± 0.02	± 0.02	**± SE**	

Different super scripts indicate statistically significant difference.

The same super scripts indicate statistically no significant difference. *** $P \leq 0.001$

G1=Non-Non   **G2**=inf MG-Non   **G3**= MG+T   **G4**= MG+A   **G5**= MG+T+A.
G6= MG+*E. coli*   **G7**= MG+*E. coli* +T   **G8**= MG+*E. coli* +A   **G9**= MG+*E. coli* +T+A.

DISCUSSION

1-Clinical manifestations:

MG infected untreated group showed a various clinical symptoms as sneezing, mild nasal and lacrimal discharge, mild to moderate loss of appetite, slow growth, mouth breathing in most of cases, similar clinical signs have been reported by *Abd El-Daeem, (2008) and Stipkovit, et al., (2012)*.

Treated groups with tilmicosin or apramycin showed gradual decrease in clinical signs till disappeared after the treatment course. Similarly, *Guo et al., (2004)* assessed that apramycin has a good result in treatment of MG infection. *Majima, (2004)* reviewed the clinical benefits of macrolides in treatment of chronic sinusitis, which include decreased nasal secretions and postnasal drip, with improvement in nasal obstruction, affecting mucus production, and the transportability of airway secretions, so they have been used successfully to treat diffuse panbronchiolitis and a progressive inflammatory lung disease. Our data clearly reinforced those obtained previously by *Amer et al., (2009 b) and Abd El-Ghany, (2009)* who noticed the positive effect of tilmicosin in reduceng the clinical signs of MG. Signs subsided by introducing the medication in both drugs treated group and chickens were more apparently healthy with normal activity during the experiment. This may be attributed to their efficacy and additional or synergistic antimycoplasmal effect on MG microorganisms.

Coinfected non medicated chickens showed both mycoplasmosis and colibacillosis appeared as severe depression, inability to stand, more inappetance, loss of weight, complicated respiratory manifestations and diarrhea. Rapid appearance of these signs within few days post

inoculation may be attributed to the previously damaged respiratory system by MG infection which allows *E. coli* to enter the systemic circulation directly through the air sacs. Severe brown runny diarrhea appeared at the 3rd day post *E. coli* inoculation and reached its peak at the 5th day PI, which was self limiting at the 35th day of age. ***Monckton and Hasse, (1988)*** suggested that interaction between bacterial cells and mucosa may be mediated by adhesions, permitting close contact between bacteria and enterocytes which may help to reduce the wash down of the organism and promote colonization. Also ***Hafez, (2008) and Younes, (2012)*** recorded that experimentally *E. coli* infected broilers at 23rd day of age suffered from diarrhea as well as respiratory symptoms including nasal discharge, gasping, rales and cough. Additionally ***Ameh et al., (2011)*** reported that all the challenged chicks with a field strain of O78 developed foul smell yellowish brown watery diarrhea which ranged from 1-4 days. Some chicks showed anorexia, ruffled feathers and matted vent with 8 chicks died of the diarrhoea while the diarrhoea was self limiting in the rest of the chicks.

In coinfected group treated with tilmicosin, chickens were apparently normal from complicated respiratory signs but throughout the experiment there was a mild to moderated depression and persistent diarrhea for a period. Results may be explained by its only antimycoplasmal effect. It does not affect the *E. coli* organisms; this confirmed by ***Huang et al., (2009)*** who found that nearly 98.20% of *E. coli* isolates were resistant to tilmicosin.

Treatment of coinfected chickens with apramycin alone or combined with tilmicosin resulted in disappearance of the clinical symptoms and improved the health status of the infected chickens. ***Burch, (2003)*** reported that poorly absorbed products from the gut, such

as apramycin are very useful for treating infections there, as *E. coli* and salmonella. Nearly similar results were obtained by ***EL-Sheikh, (2004)*** who proved the efficacy of apramycin in the treatment of poultry colisepticemia. This may be attributed to the effective antimicrobial effect of apramycin toward both infected microorganisms, without any negative effect after its use with tilmicosin.

2- Lesion scoring:

MG infected untreated group revealed mucous exudates in nasal passages, trachea and air sacs. Some lungs showed congestion and others light colored foci. Most affected air sacs scored 2 or 3 degree. Mycoplasmosis is primarily a disease of respiratory system, initially of air sacs and later intends to trachea and upper respiratory passages. ***Much et al., (2002)*** found that as the infection starts with colonization of the respiratory tract, tracheitis and airsacculitis are the predominant symptoms of a localized infection in chickens. Similar lesions were mentioned by ***Abd El-Daeem, (2008) and Stipkovit, et al., (2012).***

MG chickens treated with tilmicosin and/or apramycin showed various air sac lesions from apparent normal to slight turbidity without marked difference between medicated groups ; they scored 1 or 2 in some slaughtered cases till reach to (0) score at the end of the study. Similarly ***Charleston et al., (1998)*** proved that 5-day; "in water" tilmicosin medication at dose levels of 50-300 mg/liter had a great efficacy for the treatment of experimental MG infection and showed a significant reduction in the incidence and severity of air sacculitis and peritonitis lesions caused by MG. This may be attributed to the pharmacokinetic characters of tilmicosin as concluded by ***Modric et al., (1999)***that tilmicosin has good tissue penetration and infection/ inflammation further improves its tissue penetration. Results indicated

that tilmicosin played a role in limitation of gross lesions *(Guarini et al., 1999)* and in controlling infection *(Saggiorato et al., 2000). Papich and Riviere, (2001)* attributed the high success rate of tilmicosin treatment to the prolong presence of its therapeutic concentrations intracellularly and in respiratory organs especially the lung tissues; the habitat of the MG. Our data clearly reinforced those obtained previously by *Amer et al., (2009 b)* who found that tilmicosin is still effective in controlling mycoplasmosis in broilers, decreasing the mean gross air sacs and microscopic tracheal lesions scores of treated group to be significantly less than those of un-treated infected group.

Similar observations were recorded by *Ryden and Moore, (1977)* who concluded that apramycin contributes to the successful treatment of poultry pneumonia and respiratory diseases. These findings coordinated also with *Guo et al., (2004)*. By the end of the experiment no differences existed between the incidences of air sacculitis in all MG infected treated groups. Thus far, our work proved that all the used treatments caused complete elimination of mycoplasmas from respiratory organs as supported by the MG reisolation in addition to prevent the appearance of visible complicated lesions or decrease their severity. So no special advantage was obtained from giving both drugs together; however, their combination improved their effectiveness in decreasing the severity of the early air sacculitis lesion scores detected directly after treatment course to (0) in most of sacrificed chickens.

Disease severity is judged by criteria including air sac lesions, bacterial count and mortality rate. Gross pathological lesions displayed that there were more serious involvements of the trachea, lung, air sacs, heart, liver and intestine in coinfected group without medication. The

liver was enlarged with focal congestion and covered by white cloudy covering as fibrinous perihepatitis. Lungs revealed dark red congested appearance or hemorrhagic in other cases. The heart was enlarged, inflamed and covered with white cloudy covering in most of the cases. Trachea revealed tenacious exudate with linear hemorrhages. Macroscopic lesions of air sacculitis appeared more severe, air sacs were opaque in most of cases and some cases containing caseous material in addition to great increase in the thickness of many thoracic or abdominal air sacs that appear as flesh so all these cases taken 3 to 4 lesion scores. Splenomegaly was recorded in few birds; moreover, *E. coli* infection produced enteritis and peritonitis. Similar gross lesions were reported by ***Peighambari et al., (2000)*** who studied the susceptibility of enhancement as a result of secondary invasion of the respiratory tract by *E. coli* bacteria after preceeding infection with MG. They found an initial respiratory infection (airsacculitis) followed by septicaemia, inflammation and enlargement of several organs, such as liver and spleen, peritonitis, perihepatitis and pericaiditis as a generalized infection. Also ***Ameh et al., (2011)*** mentioned that postmortem findings of *E. coli* infection were enteritis, few cases of ascities, fibrinous airsacculites, mucoid exudates in the trachea, enlarged and dark congested liver. The detected gross lesions in both infected groups to the end of the experiment explained by the long period needed by damaged lungs and air sacs to heal and this opinion supported by ***Khairy et al., (2012)*** who studied the capability of MG and/or *E. coli* to cross the mucosal barrier of the respiratory tract, by their virulence factors then enter the blood stream to disseminate throughout the body causing lesions and damage in various internal organs of experimentally infected birds via intra-tracheal route.

Lesions detected in coinfected tilmicosin treated group were similar to complicated lesions recorded in coinfected untreated group but with less severe incidence of tracheaitis and airsacculitis. These results may be attributed to the great pathogenic effect of *E. coli* infection not affected by tilmicosin. Only MG lesion in respiratory system air ways respond to tilmicosin treatment. ***Charleston et al., (1998)*** proved that even in the presence of an exacerbating virus; tilmicosin treatment appears to be effective in reducing mean lesion score. The severity of air sac lesions can reduce mortality in severely diseased birds with MG.

Coinfected chickens treated with apramycin resulted in reduced PM lesions. Similar results were obtained by ***Umesha et al., (2010)*** who assessed that the severity of *E. coli* gross lesions were mild to nil with apramycin medication. Different grades of lesion scores could reflect the individual response to infection and/or treatment and explained by the various levels of birds' immune defense against the infection.

Coinfected group treated with both drugs showed the best improvement in all gross lesions and this confirmed by bacterial reisolation and the absence of mortalities, our data coordinated with ***Kleven, (1998)*** who clarified that control of the mycoplasma infection is simplified when concurrent infections are minimized. ***Toutain et al., (2002)*** attributed the persistence of lesions for several weeks even though the infection has been resolved to the inability of birds to produce the enzymes necessary to liquefy the fibrinous lesion and commenced once treatment to stop total resolution of lesions. Also ***Drlica, (2003)*** stated that early treatment, before major damage has been caused, is vital to the successful treatment of infections to achieve the bacteriological cure.

3- Mortality rate and postmortem lesions:

In all cases of mortalities both inoculated microorganisms were the main cause of death, this supported by the postmortem lesions. We noticed that the 1st case of mortalities recorded in MG infected untreated group was associated with the appearance of clinical signs (1 week PI) which may be as a result of complete failure of respiratory system to work effectively or accommodate with the inoculated dose. This was confirmed by severely detected tracheitis and airsacculitis without any other obvious causes of death. This partially differed from *Yoder, (1978)* who reported that mycoplasma leads to mortality in later course of disease due to decreased birds' viability. No mortality was recorded between MG infected treated groups. These results agree with *Abd El-Ghany, (2009) and Zakeri and Kashefi, (2011)* who recorded that tilmicosin significantly reduced MG mortality and gross lesions. Our results support the still desired antimycoplasmal therapeutic effect of tilmicosin and apramycin, attributing their effectiveness to their mode of action.

Untreated coinfected group induced a high mortality rate 13.3% and started after the *E. coli* super infection. Gross lesions in these dead cases were severe congestion in lungs and liver, septicaemia, completely thickened air sac, fibrinous exudates on liver capsule and heart's pericardium, we attributed these severe lesions mainly to the inoculated *E. coli*. The mortality rate recorded in this study coordinated with that obtained by *Khan et al., (2006)* who found 2 mortality cases in combined MG and *E. coli* infection. *Hafez, (2008) and Younes, (2012)* also recorded 3 cases, as a mortality rate, in *E. coli* infected broiler chickens which constitute 12% and 15% mortality respectively to the authors. The main detected gross lesions were enteritis, airsacculitis, pericarditis and

perhepatitis. ***Pattison et al., (2008)*** emphasized that presence of *E. coli* with MG plays an important role in increasing mortalities which resulted from invasion of massive number of infective and pathogenic microorganisms and this was confirmed by the high *E. coli* count reisolated from different internal organs of this group.

Mortality rate reached 6.7% and 0% in coinfected groups treated with tilmicosin and apramycin respectively. This result indicated the effective use of apramycin in treatment of CCRD, while the only effect of tilmicosin on MG cannot help chickens to overcome the virulence pathogenic effect of the *E. coli* and this was clearly seen from the PM picture of the dead chickens. Also ***Cracknell et al., (1986)*** proved the efficacy of apramycin as a treatment for *E. coli* infection in broilers at 27-39 days of age at 3 levels of medication: 125, 250 and 500 mg activity/liter, administered for 5 successive days in reduction of mortality rate in diseased birds. Furthermore, ***EL-Sheikh, (2004)*** supported the use of apramycin sulphate in the drinking water as a treatment of *E. coli* in broilers. Infected group treated with both drugs did not record any mortality and this attributed to the antimycoplasmal and antimicrobial effect of both used drugs in increasing the stability of birds to face the pathogenic effects of both infections.

4- Bacterial reisolation and *E. coli* count:

Samples collected from tilmicosin and/or apramycin treated groups after 5 days of treatment were negative to MG, this confirmed that the used drugs are highly effective antimycoplasmal drugs causing complete MG cure or elimination and remain a mainstay in treating of MG infection. ***Moore et al., (1996) and Diarra et al., (1999)*** attributed initially the treatment success of tilmicosin to its pharmacodynamic concentration in appropriate tissues and low inhibitory concentrations.

Our results agree with that cited by **Jordan and Horrocks, (1996), Abd El-Ghany, (2009) and Amer et al., (2012)** who observed that infected chicks with a virulent strain of MG and treated with tilmicosin, showed negative serological results with a significant decrease in reisolation percentage till reached (0%) with no recovery of the organism either during life or after death. As well, **Charleston et al., (1998)** stated that increasing the dose of tilmicosin to the extent of 200-300 mg/l, no bird was serologically positive on day 21. In **Beba et al., (1998)** investigation, macrolides enhanced cell mediated immunity as evidenced by increased lymphocyte stimulation index, phagocytosis, killing percent of polymorphnuclear (PMN) cells and impressively concentrated in both macrophages and PMN cells and activate them. Needless to say, the enhancement of cell-mediated immune response is important for resolution of many infectious diseases. This opinion supported by **Scorneaux and Shryock, (1998)** that tilmicosin has prolonged retention in phagocytic cells, which then migrate to sites of infection, the tilmicosin phagocyte association constitutes a valid antibiotic delivery system as a "sustained-release" in the presence of bacteria again producing locally high concentrations of active drug. This may explain in part why tilmicosin is effective *in vivo* against avian pathogens such as MG. Also **Prescott et al., (2000)** proved the ability of tilmicosin to concentrate intracellular in phagocytic cells 20 times higher than those in extracellular fluids a property that would possibly augment the expected lethal effect of tilmicosin against intracellular mycoplasma. In keeping with this line many recent authors favor it's use in pulmonary infections as **Fedrizzi et al., (2008)**. They mentioned that tilmicosin, has a great clinical efficacy in respiratory diseases, easily attains high intrapulmonary concentrations as alveolar macrophages can concentrate the drug. It has a fast absorption rate, a rapid distribution to target tissues with a slow

release. The authors confirmed that oral tilmicosin could attain concentrations exceeding the target pathogen's MIC in lungs and alveolar macrophages, much faster and longer than in blood.

MG reisolation proved that apramycin is of high efficacy when used in its recommended therapeutic dose (0.5 gm/L) as CRD treatment. The ability of apramycin to reach to some preferable adjacent serous coats to the localized MG infection and accumulated in high concentrations in the mucosal membranes of the respiratory tracts explained its efficacy in the treatment of pulmonary diseases in poultry, in addition to its bactericidal effect. This proposition was supported by *Pechere and Dugal, (1979)* who found that aminoglycosides attain therapeutic concentration in the pleural and even peritoneal fluid, especially if inflammation is present. *Ziv et al., (1995)* reported that apramycin is a potent inhibitor of protein synthesis in bacteria at low concentrations by inhibiting the translocation step of protein synthesis and induces translation errors.

The use of both drugs together also recorded negative MG colonies in the examined samples; this is a great support to their additive and not antagonistic interaction as none of the used drugs declined the efficacy of the other one. The antimicrobial activity of tilmicosin and apramycin on some important gram-negative respiratory tract pathogens were studied by *Diarra et al., (1999)* and found that the presence of these drugs in the medium decreased the growth rate of these bacterial strains using drug concentrations as low as 1/4 MIC. The effective therapeutic dose of both drugs recorded by *Carpenter et al (2001)* to be in a dose of 0.5 gm powder/liter drinking water for poultry mycoplasmosis and colibacillosis for apramycin. Tilmicosin, in a dose of 200-300 mg/l for 5 days was used for MG elimination and in a dose of 250-500 mg/l as mycoplasmacidal to MG and MS.

Our data was not fit with those previously recorded by **Kempf, (1991)** who reported that the treatment of MG infection with antimycoplasmal drugs may also prevent a significant immune response and make it difficult to detect the microorganism giving a false impression that the flock is free from infection. We confirmed our results by the completely absence of MG colonies on PPLO media till 3 weeks of incubation, which not depend on presence or absence of antibodies, in addition to the general improvements in various health sings and absence of any gross lesions at the end of the study in all treated groups except coinfected tilmicosin treated group. That was mainly due to unsusceptible *E. coli* infection to tilmicosin. So these drugs have a real antibacterial effect on MG infection not only mask their presence or detection. Our data was also not fit with previous works showed that the use of antibiotics does not result in complete elimination of mycoplasmas from host tissues as **Salama, (1994)** who reported that there is no used chemotherapy, which has the ability to eradicate the mycoplasma microorganisms from tissues of infected birds. Such contradiction may be attributed to the use of different drugs in our study other than that used by the author.

The highest *E. coli* count was recorded in untreated *E. coli* super infected group. This assured dissemination of the inoculated strain throughout the respiratory system to the internal organs, this means that disease occurrence is associated with specific virulence factors which grant the bacteria the ability to proliferate in the poultry host and cause disease **(Van der Westhuizen and Bragg, 2012)**.

Tilmicosin treated group had the largest count within treated groups and this result is scientifically logic due to the resistant ability of *E. coli* toward tilmicosin as antibiotic treatment. Macrolides are known to be minimally effective against gram-negative bacteria in the intestine

because of their outer membrane permeability barrier, which cannot be easily penetrated due to the molecular size of these antibiotics in relation to the bacterial porin channels *(Norcia et al., 1999)*. Nevertheless, some enterobacteria strains have an incomplete lipopolysaccharide structure, permitting increased penetration of the macrolides and these strains could have been decreased by tilmicosin, this can explain the remarkable decrease observed. In keeping with this line *Williams et al., (2005) and Bosi et al., (2011)* recorded that tilmicosin had antibiotic-mediated clearance of overt pathogens present at a subclinical level in the gastrointestinal tract and had a potent negative effect on enterobacteria. There is also evidence that it can directly interact with the immune response after induction by lipopolysaccharides; however, in that instance, macrophages and monocytes responded by decreasing various inflammatory cytokines *(Cao et al., 2006)*. These immunomodulatory properties can support the antibacterial action of the drug on *E. coli*.

In this study we recorded a less degree of *E. coli* count in apramycin treated coinfected group. This was supported by many researchs that handled the efficacy of apramycin against gram negative bacteria specially the *E. coli*. *Sander, (1980)* assessed that enteritis and other pathological changes may allow significantly greater absorption to take place to aminoglycosides antibiotics, however they are poorly absorbed (usually 10%) from intact gastrointestinal tract. Additionally *Leitner et al., (2001)* examined the effect of apramycin sulphate on the decreasing colonization of pathogenic *E. coli* to an undetectable level in the intestines of chicks if used in (0.5g/L) for 24 or 48 h before *E. coli* inoculation, which could lead to a reduction of colibacillosis in poultry. The presence of *E. coli* infections post treatment may had been explained by *Van den Bogaard et al., (2001)* who found that the prevalence of resistance in faecal *E. coli* nearly to all antibiotics was significantly higher in broilers with relatively high antibiotic use. In keeping with this

line *Toutain et al., (2002)* mentioned that when infection reached the affected organ caused inflammation and production of exudate and other debris, which presumably inhibit the antibiotic penetration and complete destruction of the organism. Finally according to **FAO and WHO, (2012)** apramycin remains today our most important weapon against gram –ve pathogens. This antibiotic has been successfully used in avian medicine against both MG and *E. coli* infections.

Coinfected group given both treatments greatly reduced the frequency of reisolation and give the lowest *E. coli* count. This result may attribute mainly to the great antimicrobial effect of apramycin on *E. coli* and secondary to the supportive effect of tilmicosin in fighting the previous mycoplasma infection as well as the anti-inflammatory and immunomodulatory actions of this antibiotic contribute to its efficacy. All these causes provide a good body condition against bacterial infection which help by different ways in increasing and supporting body's immune system and its defense mechanism. In keeping with the same line *Fischer et al., (2011)* demonstrated that macrolide antibiotics have potent immunomodulatory activity. Their spectrum of action extends to regulation of leukocyte function and production of inflammatory mediators, control of mucus hypersecretion, resolution of inflammation, and modulation of host defense mechanisms. Another explanation may be due to the different modes of actions of both drugs on bacteria supporting each other, tilmicosin interferes bacterial protein synthesis by binding reversibly to subunit 50S of the bacterial ribosome *Yerramilli et al., (2011)* while apramycin blocks translocation in translation elongation by binding to particular proteins of 30s subunit resulting in miscoding of the genetic code of the mRNA template *Tsai et al., (2013)*. Consequently, incorrect amino acids are incorporated into the growing peptide chains and faulty bacterial proteins are produced.

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تأثير كل من التلميكوزين والأبراميسين منفردين أو مجتمعين في علاج الدجاج المعدي معملياً الميكوبلازما جاليسبتكم منفرة أو مع الإيشريشيا كولاي

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تعتبر الميكوبلازما جاليسبتكم التى تسبب مرض الجهاز التنفسي المزمن من أهم البكتريا التى تهاجم صناعه الدواجن فى مصر وتعمل الإيكولاى بالتآزر معها فى إحداث المرض المزمن المعقد الذي يسبب زيادة نسبه النفوق ونقص معدلات النمو في دجاج التسمين. استهدف هذا البحث دراسة فاعلية التيلميكوزين بجرعة (ملى/لتر) والأبراميسين بجرعة (0.5جرام/لتر) ودراسة مدى التداخل بينهم في علاج الدجاج المعدي معمليا بالميكوبلازما جاليسيبتيكم منفردة أو مجتمعه مع الإيكولاى. تمت العدوى بالميكروبين عن طريق الحقن داخل القصبة الهوائية بجرعة 0.1 ملى للميكوبلازما عند عمر 8 أيام و 0.2 ملى للإيكولاى عند عمر 23 يوم . تم مراقبة وتسجيل الأعراض ومعدلات النفوق يومياً وتشريحهم لمعرفه الصفة التشريحية للمرض وإعادة عزل البكتريا المحقونة من عينات (الرئة والقصبة الهوائية والأكياس الهوائية) للميكوبلازما والأعضاء الداخلية للإيكولاى بعد العدوى للتأكد من الإصابة وبعد العلاج لتقييم فاعليه الأدوية المستخدمة بعد ظهور الأعراض لمدة خمسه أيام متتالية من اليوم 28 الى اليوم 32 من العمر. وأظهرت نتائج العلاج بأحد العقارين أو كليهما للمجموعات المصابة كفاءة في علاج الميكوبلازما نظراً للتحسن الذي نتج عن استخدامهما المتمثل فى اختفاء الأعراض المرضية، تحسن حاله الأكياس الهوائية، الصورة التشريحية، تقليل النافق، وكانت نتائج العزل سلبيه لكل المجاميع المعالجة مقارنه بالدجاج المصاب وغير معالج ولذلك يمكن استخدام كلاً من العقارين منفردين أو مجتمعين بالجرعات العلاجية بأمان كمضادات للميكوبلازما حيث تبين أن العلاقة الدوائية بينهما هي علاقة تآزريه وأن العلاج بالدوائين معا لم يؤثر على نشاطهما العلاجي ولم يظهر أي تأثير سلبى على الحالة العامة للطيور. كما أن استخدام الأبراميسين في المجموعة المعديه بالميكوبلازما والإيكولاى له تأثير علاجي قوى كمضاد للإيكولاى وأكثرها فاعلية بدرجة أفضل من التيلميكوزين منفرداً في خفض عدد مستعمرات الإيكولاى نظراً لضعف تأثير التيلميكوزين على الإيكولاى. وأن إعطاء الدوائين معاً أعطى نتائج جيده حيث أدّوا إلى تحسن الحالة العامة للطيور وانخفاض معنوي كبير في عدد مستعمرات الإيكولاى لذا يوصى باستخدام التيلميكوزين مع الابراميسين للسيطرة على المرض التنفسي المزمن المعقد بميكروب الإيكولاى.