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Treasing Study on Tylvamyco® as a Novel Immunomodulatory Medication for Broiler Chickens

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Abstract

Present study aims at the evaluation of the efficacy of Tylvamyco® as a new macrolides generation in control of avian mycoplasmosis in broilers chickens with special attention to its immunomodulatory effects. A total of 500 a-day-old broiler Ross 308 chicks were equally subdivided into two treatments of 250 birds in each. The Tylvamyco® treated group, and the control non treated group were kept in a separate house. Blood samples and tracheal tissues collected at one day old and also each week till the end of the trials for isolation *M. gallisepticum* and also measuring the immune status of the experimental chicks. *M. gallisepticum* occurrence rate in broilers chickens was 12% which confirmed by PCR. The minimal inhibitory concentration values Tylvamyco® against recovered 12 *M. gallisepticum* isolates standard strain showed that the Tylvamyco® has MIC_{90} value of 0.008. In the Tylvamyco® treated group the immune status profiles record that there are marked increase in the immunological parameters by age as; HI test results for *Mycoplasma*, NDV, AI, INF-γ conc., IL-6 conc., phagocytic cell count, nitric oxide conc. and lysozyme conc. at 1, 15 and 30 day old, respectively. The molecular analysis of *CXCL8 gene* as an indicator for inflammation reduction potency in In the Tylvamyco® treated group by using real-time PCR showed that the cycle threshold of *CXCL8 gene* reduced by age from 13.6 to 10.7 at 15 and 30 day old with fold change 0.57 and 1.4, respectively. Performance parameter in Tylvamyco® treated group was 3.22kg/bird with mean weight gain 2.33kg/bird and FCR 1.4. The mortality rate was 5%. We concluded that Tylvamyco® acts as a potent immunomodulatory medicine in broilers.

Key words: Tylvamyco, Mycoplasmas, Immunomodulation, Broiler chickens.

INTRODUCTION

Avian mycoplasmosis causes considerable economical losses to the poultry industry, especially in chickens all over the world. In broilers, *M. gallisepticum* cause a reduction in weight gain, a decrease in feed conversion efficiency, an increased mortality rate, and increased condemnations at slaughter (Ley, 2008). Even with the monitoring and control programs in place, many chicken flocks become infected vertically and horizontally (Bradbury, 2001). The three main approaches for the control of the disease are eradication followed by prevention, vaccination or medication. While eradication and vaccination provide long-term solution for the control of mycoplasmosis, medication can be a prompt and effective tool to reduce the economic losses by mitigating egg transmission and clinical signs (Kleven, 2008). However, antibiotic susceptibility profile should first be determined to maximize treatment efficacy (Landman *et al*., 2008). Immunity is split into innate and adaptive systems. Although it was once thought that the inflammation was not very highly regulated, further research into the key players and processes that make up the phenomenon have revealed that there is complex web of processes that coordinate this innate immune response. As the first line of the innate immune response, including inflammation is necessary to remove an invading pathogen post-infection (Jain and Pasare, 2017). Macrophages are tissue resident phagocytes; similar to neutrophils, they are derived from myeloid precursors and play a key role in antimicrobial activity. They also have a more diverse repertoire of functions including tissue surveillance, remodelling and antigen presentation, which helps link innate and adaptive immune responses

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(Schneberer *et al*., 2012). Pro-inflammatory mediators of inflammation are crucial to the onset and perpetuation of inflammation (Levy and Serhan, 2014). The cytokines are mainly produced by activated macrophages and epithelial cells in which it is constitutively produced. Interleukin-6, which is secreted by T cells and macrophages, can have dual function in the body. Macrolides constitute a class of drugs implicated in immune modulation (Kanoh and Rubin, 2010). Macrolides can be naturally occurring compounds like erythromycin and tylosin or semisynthetic ones like azithromycin, tilmicosin and tulathromycin (Villarino *et al*., 2013). Macrolides are biostatic antibiotics; they bind to the 50S ribosomal subunits of Gram-positive and a limited number of Gram-negative bacteria to inhibit protein synthesis (Kannan *et al*., 2014). Besides, they preferentially accumulate in tissues and phagocytes as opposed to circulation. Notably, some macrolides have been shown to reach intracellular concentrations up to 500 times greater than systemic levels (Kanoh and Rubin, 2010). This localization gives these drugs superior pharmacodynamics as these compounds can be transported to the site of inflammation (Bosnar *et al*., 2005). Interestingly, macrolides have also been shown to have antimicrobial action below the threshold required for bacteriostatic activity (Steel *et al*., 2012). Tylvamyco® contains 625mg tyvalosin/g, which is a 2nd generation macrolide antibiotic developed by addition of an isovaleryl group to the tylosin molecule to potentiate its ability to penetrate the lipid membranes of the host and *Mycoplasma* bacterial cells till binding bacterial ribosomes, therefore, prevents bacteria from protein synthetization and give the compound both a bacteriostatic and bactericidal effect with high absorption and intracellular concentration in respiratory tissues and circulating heterophils which are drawn to sites of infection. Tylvalosin developed by Japanese researchers using a patented process, is highly effective against macrolide-resistant *Mycoplasma spp.* The minimum inhibitory concentrations (MIC) were determined from the lowest concentration of the antibiotics where no pH and color change of the broth was detected, meaning that the growth of the bacteria was completely inhibited in the broth. Initial MIC values were determined when the growth controls showed color change. Final MIC values were determined when no further growth was detected, generally after two weeks of incubation. MIC50 and MIC90 values were defined as the lowest concentrations that inhibited the growth of 50 or 90% of the strains (Hannan, 2000), so this study aims at evaluation the Tylvamyco® as a potent agent in control of broilers chickens mycoplasmosis with its antimicrobial and immunopotentiation criteria.

MATERIALS AND METHODS

Birds and sampling

Five-hundred-day-old broiler commercial Ross chicks were reared until 5 weeks of age. All birds were raised in environmentally controlled rooms in the Animal House Facility at Cairo University. All vaccination program including IBV, NDV and IBDV were given, and they divided equally in two groups, first group: Tylvamyco® treated group, in recommended dose at 25mg/kg in the first 4 days for 12 hours daily and repeated at 19, 20, 21, 22 day old. Second group: control non treated group. Blood samples from each bird species were collected and tested by HI. At this time, tracheal swabs from chickens were collected and tested by culture and PCR to detect occurrence of *M. gallisepticum*. Chickens were weighed to ensure that the groups had similar average body weights. The birds were then observed daily for clinical signs.

Isolation of *M. gallisepticum* and confirmation by PCR

Dry tracheal swabs were dipped several times in Frey's media and then discarded. Inoculated media were incubated at 37°C for at least 10 days or until a color change was evident (Kleven, 2008). PCR was used to test for *M. gallisepticum* growth in the Frey's broth. DNA was extracted as previously described (Liu *et al*., 2001) and PCR was performed using *M. gallisepticum*-specific primers as previously described (Nascimento *et al*., 1991).

Antibiotic susceptibility profiles of *M. gallisepticum* recovered isolates

Microbroth dilution examinations were performed on 10^4-10^5 CCU/ml of the strains. In brief, the tests were performed in 96-well microtiter plates containing modified Frey's broth medium, using growth controls (broth medium without antibiotic), sterility controls (broth medium without antibiotic and *Mycoplasma* inoculum), pH controls (broth medium adjusted to pH 6.8) and all strains were tested in duplicates (Hannan, 2000).

Immunological studies

HI titer

Blood samples were collected and tested by HI test as described previously by (Kleven, 2008). Briefly, *Mycoplasma* HI test was conducted in a microtiter plate using 4 HA units of antigen per test, A HI titer of 1:40 or greater was considered positive. While Inactivated H5N1 antigen (A/chicken/Egypt/18-H/2009) was used for detection of AIV-H5 antibodies and Lasota strains (8HA units) for detection of NDV antibodies (OIE, 2014).

INF-γ assay

Chicken INF-γ ELISA kits (Novatein Bio, Massachusetts, USA) were used according (Raheel and Orabi, 2019) as the interferon concentrations were calculated from the standard curve by means of a software product.

IL-6 assay

Chickens Sandwich-ELISA where the Micro-ELISA plate provided has pre-coated with antibody specific to chicken IL-6. The concentration of IL-6 in the sample was calculated by comparing the OD of the sample to the standard curve according to (Raheel and Orabi, 2019).

Phagocytosis assay

CytoSelect™ 96-Well Phagocytosis Assay (Red Blood Cell Substrate) Catalog Number: CBA-220 according to (Yu *et al*., 2015) on blood samples collected at 7, 15, 28 and 35 days of age from the two groups.

Nitric oxide and Lysozyme

Lysoplates were prepared by using uniform suspension of *M. lysodeikticus* for measuring lysozyme conc. in serum samples, while 100 µl serum were used to measure NO conc. by using Griess reagent according to (Raheel and Orabi, 2019).

Real Time PCR for determination of down-regulation and fold change of CXCL8 and GAPDH genes

Changes in gene expression of CXCL8 in monocyte-derived macrophages were measured using qPCR techniques by using the following primers:CXCL8 forward: TAG GAC CAG AGC CAG GAA GA , reverse: GCT GCA GAA AGC AGG AAA AC at 95ºC for 5min, 40 cycles of 95ºC for 10s, 60ºC for 30s and 72ºC for 1min. RT-PCR results were analyzed using comparative threshold cycle (CT) (Ruth, 2017).

Performance parameters

Feed intake, feed efficiency and weight gain were recorded during the period of experiment till 5th week of age (Orabi and Raheel, 2019).

Statistical analysis

The results were presented as mean±SE. All given parameters were compared between studied groups using the one-way ANOVA with fixed effects of the factors using (Start Soft Inc). Differences were considered significant at P≤0.05.

RESULTS

Occurrence of *M. gallisepticum* in broiler chickens and its MIC profiles

In the present treasing *M. gallisepticum* occurrence rate in broilers chickens was 12% which confirmed by PCR in Fig 1. The mycoplasmacidal activity of Tylvamyco® is both concentration and time dependent. The MIC values Tylvamyco® against recovered 12 *M.gallisepticum isolates* standard strain showed that the Tylvamyco® had the lowest MICs with an MIC90 value of 0.008µg/ml.

Tylvamyco® immunomodulation and performance effects on broiler chickens

In the Tylvamyco® treated group the immune status profiles record that there are marked increase in the immunological parameters by age as; HI test results for *Mycoplasma* was 1.8±1.32, 2.9±1.53 and 3.8±1.46 at 1, 15, 30 day old, while for NDV was 1.7±1.61, 4.2±1.66 and 4.8±1.68, although for AI was 1.5±1.35, 2.3±1.43 and 4.5±1.56 at 1, 15, 30 day old. Consequently the INF-γ conc. (45±0.453, 195±0.532 and 450±0.365pg/ml), IL-6 conc. (12±0.432, 86±0.573 and 250±0.896pg/ml), phagocytic cell count (10^2, 10^4 and 10^5), nitric oxide conc. (2.3, 14.6 and 28.4µmol/ml) and Lysozyme conc. (1.6, 5.9 and 6.7µmol/ml) results recorded in table 2 at 1, 15, 30 day old, respectively. The molecular analysis of *CXCL8 gene* as an indicator for inflammation reduction potency in In the Tylvamyco® treated group by using real-time PCR showed that the cycle threshold of *CXCL8 gene* reduced by age from 13.6 to 10.7 at 15 day old and 30 day old with fold change 0.57 and 1.4, respectively.

Table 1: Tylvamyco® MIC against local recovered *M. gallisepticum* isolates from broiler chickens

Isolate #	MIC	MMC	MIC/ MMC
1	0.008	0.023	1
2	0.007	0.016	1
3	0.0077	0.028	1
4	0.006	0.045	1
5	0.054	0.065	1
6	0. 065	0.036	2
7	0.0046	0.023	1
8	0. 065	0.036	2
9	0. 065	0.036	2
10	0.0077	0.028	1
11	0.006	0.045	1
12	0. 065	0.036	2
ATCC 19610	0.0095	0.056	1

MIC; minimum inhibitory concentration, MMC; minimum mycoplasmacidal concentration.

Performance parameter in this group were represented in feed consumption rate which recorded as 3.22kg/bird, with mean weight gain 2.33kg/bird and feed conversion rate 1.4. The mortality rate was 5% with slight air saculitis, as post-mortem records. In the other hand the untreated control group *Mycoplasma* HI results were 1.8±1.32, 2.2±1.22 and 2.6±1.55, while for NDV were 1.7±1. 61, 2.6±1.33 and 2.9±1.44, although for AI 1.5±1.22, 1.8±1.66 and 2.5±1.77 at 1, 15 and 30 day old respectively. The results recorded in Table 2: INF-γ conc. (45±0.453, 86±0.532 and 130±0.422pg/ml), IL-6 conc. (12±0.432, 18±0.564 and 45±0.365pg/ml), phagocytic cell count (10^2, 10^3 and 10^3), Nitric oxide conc. (2.3, 5.4 and 8.6µmol/ml) and lysozyme conc. (1.6, 2.5 and 3.4µmol/ml. *CXCL8 gene* cycle threshold of *CXCL8 gene* increased by age from 16.3 to 19.5 at 15 day old and 30 day old with fold change 0.92 and 2.3, respectively. Performance parameter in this group were represented in feed consumption rate which recorded as 2.6 kg/bird with mean weight gain 1.4 kg/bird and feed conversion rate 1.85 with significant gasping, rales, nasal discharge and lacrimation. The mortality rate was 40% with air-sacculitis, pericarditis, perihepatitis and pneumonia as post-mortem records and this reflect on the medical and immunological benefits of the used medicine.

DISCUSSION

Mycoplasma gallisepticum is the causative agent of chronic respiratory disease, a prevalent disease of poultry, which is responsible for significant economic losses in farms (Tavio *et al*., 2014). The primary signs of *M. gallisepticum* infections include nasal discharge, kerato-conjunctivitis, air-sacculitis, and depression. *Mycoplasma gallisepticum* should be cultivated on specially formulated media, for the reason that *Mycoplasma* is dependent on outside sources of precursor molecules for macromolecular syntheses (Levisohn and Kleven, 2000). In the present investigation the occurrence rate *M. gallisepticum* occurrence in broilers chickens rate was 12% in day old chicks, which represent high percentage of vertical transmission from the breeder farms with special attentions to bad hygienic condition of the chicks industry. Our performance parameter in untreated control group were represented in feed consumption rate which recorded as 2.6kg/bird, with mean weight gain 1.4kg/bird and feed

Table 2: Tylvamyco® immunological and performance criteria in broiler chickens

Parameters/Age	Tylvamyco® treated gp			Control gp		
	1 day	15 day	30 day	1 day	15 day	30 day
Mycoplasma HI	1.8±1.32	1.5±1.53	1.3±1.46	1.8±1.32	2.7±1.22	3.4±1.55
NDV HI	1.7±1. 61	4.2±1.66	4.8±1.68	1.7±1. 61	2.6±1.33	2.9±1.44
AI HI	1.5±1.35	2.3±1.43	4.5±1.56	1.5±1.22	1.8±1.66	2.5±1.77
INF-γ conc. (pg/ml)	45±0.453	195±0.532	450±0.365	45±0.453	86±0.532	130±0.422
IL-6 conc. (pg/ml)	12±0.432	86±0.573	250±0.896	12±0.432	18±0.564	45±0.365
Phagocytic cell count	10^2	10^4	10^5	10^2	10^3	10^3
Nitric oxide conc.(µmol/ml)	2.3	14.6	28.4	2.3	5.4	8.6
Lysozyme conc. (µmol/ml)	1.6	5.9	6.7	1.6	2.5	3.4
Cycle Threshold of CXCL8 gene	--	13.6	10.7	--	16.3	19.5
Fold change of CXCL8 gene	--	0.57	1.4	--	0.92	2.3
Feed consumption		3.22 kg/bird			2.6 kg/bird	
Mean weight gain		2.33 kg/bird			1.4 kg/bird	
Feed conversion rate		1.4			1.85	

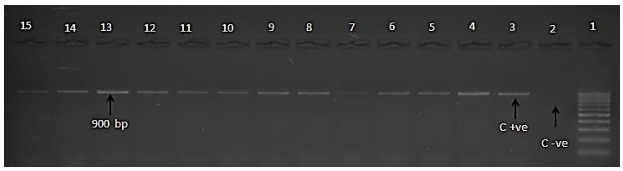

Fig. 1: Gel electrophoresis showed positive amplification at 900 bp. for *M.gallisepticum* by using the specific primers. (Lane 1: 100 bp DNA ladder; Lane 2: control negative; Lane 3: control positive ATCC 19610; Lane 4 till 15: recovered isolates).

conversion rate 1.85, while in the Tylvamyco® treated group treated group the feed consumption was 3.22kg/bird, with mean weight gain 2.33kg/bird and feed conversion rate 1.4. Tylvalosin is a new broad spectrum, third generation veterinary macrolide. Derived from tylosin, it shares its 16-membered ring (Zhao *et al*., 2014). Tylvalosin is currently used to treat bacterial infections in livestock such as *Clostridium* and *Mycoplasma* infections in poultry and swine (Tavio *et al*., 2014). The presence of the *isovaleryl* group increases its lipophilicity, allowing it to rapidly penetrate the lipid membrane of host and bacterial cells and enabling highly effective binding to bacterial ribosomes, so tylvalosin binds to bacterial ribosomes and prevents protein synthesis, this can lead to inhibition of bacterial growth or death of the bacteria, also tylvalosin's first metabolite, known as 3-AT, also has antimicrobial activity macrolide (Märit *et al*., 2012). It attaches to a different site on the ribosome, enhancing the effectiveness and clinical efficacy of tylvalosin. This dual action may be responsible for its favorable resistance profile (Tavio *et al* 2014). The minimum mycoplasmacidal (MMC) for Tylvamyco® is similar to the minimum inhibitory concentration (MIC). The MMC/MIC ratio is often considered highly important in determining cidal effect, and the fact that this is low indicates that Tylvamyco® has cidal activity in vivo, so the present study recorded that the MIC values Tylvamyco® against recovered 12 *M. gallisepticum isolates* standard strain showed that the Tylvamyco® had the lowest MICs with an MIC90 value of 0.008 µg/ml. Studies conducted in Japan and the EU showed that strains of *Mycoplasma gallisepticum* resistant to tylosin remained sensitive to tylvalosin. Additional MIC studies with *M. synoviae* comparing tylvalosin to other macrolides antibiotics showed that tylvalosin had the lowest MIC value (Guedes *et al*., 2009). Like its macrolide counterparts, it has been shown have to have anti-inflammatory properties (Zhao *et al*., 2014). Studies show that mice had markedly reduced levels of LPS-induced pro-inflammatory cytokines such as IL-1β, IL-6, CXCL8, and TNF-α as well as the lipid mediator PGE2 (Tavio *et al* 2014). The molecular analysis of *CXCL8 gene* as an indicator for inflammation reduction potency in In the Tylvamyco® treated group by using real-time PCR showed that the cycle threshold of *CXCL8 gene* reduced by age from 13.6 to 10.7 at 15 day old with fold change 0.57 and 1.4, respectively, while cycle threshold of *CXCL8 gene* increased by age from 16.3 to 19.5 at 15 day old and 30 day old with fold change 0.92 and 2.3 respectively in untreated control group. Tyvalosin exerts both a direct and indirect effect on the immune system driving the change from monocytes to macrophages, activating macrophages and concentrating within lysosomes within macrophages. The combination of tylvalosin together with the potent lysosomal enzymes assists the innate immune system to combat pathogens (Pallares *et al*., 2015). In the current study the results of immunological parameters in Tylvamyco® treated group as INF-γ conc. (45±0.453, 195±0.532 and 450±0.365pg/ml), IL-6 conc. (12±0.432, 86±0.573 and 250±0.896pg/ml), Phagocytic cell count (10^2, 10^4 and 10^5), Nitric oxide conc. (2.3, 14.6 and 28.4µmol/ml) and lysozyme conc. (1.6, 5.9 and 6.7µmol/ml) were recorded in Table 2 showed that these parameter concentration increased gradually by age at 1, 15, 30 day old, respectively, in the other hand there are marked decrease in these parameter in the control group at the same age of chickens which give a potent evidence for the regulatory and immunomodulatory effects of Tylvamyco®. Macrolides such as tylvalosin can reduce the inflammation caused by pathogens further reducing the severity of lesions. Clinical efficacy studies were undertaken on commercial poultry units to evaluate tylvalosin for control of *M. gallisepticum* (Qui and Zhong, 2017). Tissue macrophages are derived from blood monocytes and can exist in either a resting or an activated state. Once in an activated state, the macrophage is more metabolically active, has more lysosomes and has greater phagocytic activity and thus has a greater ability to destroy invading pathogens (Tavio *et al*., 2014). Although the present study give attention for humoral immune response parameter in broilers chickens which represented in HI test results for *Mycoplasma* that was 1.8±1.32, 2.9±1.53 and 3.8±1.46 at 1, 15, 30 day old, while for NDV was 1.7±1.61, 4.2±1.66 and 4.8±1.68, although for AI was 1.5±1.35, 2.3±1.43 and 4.5±1.56 at 1, 15, 30 day old Tylvamyco® treated group, while the results for control untreated group were *Mycoplasma* HI results were

1.8±1.32, 2.2±1.22 and 2.6±1.55, while for NDV were 1.7±1. 61, 2.6±1.33 and 2.9±1.44, although for AI 1.5±1.22, 1.8±1.66 and 2.5±1.77 at 1, 15 and 30 day old, respectively. Therapeutics and treatments for self-amplifying inflammatory diseases like bacterial pneumonia must then deliver a 'one-two punch' in their action. Indeed, these therapeutics must tackle both the microbe and the resulting acute inflammation. Over the past decade, researchers both in human and veterinary medicine have been screening for therapeutics combining both properties (Derek *et al*., 2004). Macrolide antibiotics are a group that have been of interest in the past due to their ability to induce neutrophil apoptosis, down regulate inflammatory cell recruitment and enhance efferocytosis (Cramer *et al*., 2017). Though their immune modulating and antimicrobial effects have been thoroughly investigated, the underlying mechanisms of action have yet to be fully characterized (Bosnar *et al*., 2005).

Conclusion

Tylvamyco® may be considered as superior macrolides that has dual effect in broiler chickens as it is advisable in control of mycoplasmosis with immunomodulation and anti-inflammation criteria at 25mg/kg dosage with no side effect on the bird but also improve body weight gain and feed conversion rate.

Author's Contribution

BMM carried out consultation and reviewing results while IR was involved in immunological studies. HE reviewed the experiment and AO collected data, issues and wrote the manuscript.

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