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Immune Targets Druggability of *Mycoplasma synoviae* in Chicken

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ABSTRACT

Background: *Mycoplasma synoviale* (Ms), the smallest and simplest bacteria, is categorized as an atypical organism that lacks cell wall. It is a worldwide economically important pathogen of poultry, causing respiratory affections, synovitis, abnormalities in eggshell and drop in production in chickens and turkeys. The present study was conducted to evaluate the druggability targets of *Mycoplasma synoviae* on cytokines and antioxidant changes in commercial broilers.

Methods: Two hundred and eighty, day-old, unsexed broiler chicks of strain *Vencobb* were randomly allotted to 7 treatment groups with each treatment having 4 replicates and each replicate consisting of 10 birds. Cytokines and antioxidant parameters were determined.

Result: Cytokines, TBARS, SOD, catalase and GSH levels showed a significant (P<0.05) difference between various treatment groups. Therefore, it is necessary to havea regular survey on poultry pathogenic mycoplasmas and to monitor antimicrobial susceptibility patterns in order to ensure that effective chemotherapy is being used to treat mycoplasmal infections.

Key words: Antioxidants, Chicken, Cytokines, Mycoplasma, TBARS.

INTRODUCTION

Mycoplasmosis is one of the major problems among avian diseases in emerging poultry industry of India and the world. *Mycoplasma synoviae* (Ms), the smallest and simplest bacteria, is categorized as an atypical organism that lacks cell wall. It is a worldwide economically important pathogen of poultry, causing respiratory affections, synovitis, abnormalities in eggshell and drop in production in chickens and turkeys (Kleven, 2004). *Mycoplasma synoviae* both as synovial infection and as a respiratory infection causes economic losses to the poultry industry. Cytokines, essential proteins of immunity, have been recognized as endogenous signaling molecules that mediate the cellular defense system against inflammatory response. Oxidative stress (OS) is regarded as a factor responsible for the deleterious effects on poultry health and welfare. *In vitro* tests have shown that Ms isolates are sensitive to tilmicosin and tylosin; enrofloxacin, sarfloxacin and oxytetracycline (Jordan and Horrocks, 1996). The strains of *M. synoviae* are becoming more resistant to antibiotics than other avian mycoplasmas, which indicates that it is more difficult to treat infected flocks successfully. Inactivated Ms vaccines have been used however, they are expensive due to the large amount of antigenic material needed to trigger a sufficient immune response (Wang *et al.,* 2001). Hence, the present study was conducted to evaluate the druggability targets of *Mycoplasma synoviae* on cytokines and antioxidant changes in commercial broilers.

MATERIALS AND METHODS

Two hundred and eighty, day-old, unsexed broiler chicks of strain *Vencobb* were purchased from a local hatchery for this study and were randomly allotted to 7 treatment groups, as tabulated in Table 1, with each treatment having 4 replicates and each replicate having 10 birds. The brooder house as well as other equipment were thoroughly disinfected before the arrival of the chicks and maintained as per the Cobb Broiler Management Guide. Additional source of heat was provided during the brooding period. Birds of all the groups were vaccinated with Marek's disease vaccine (Day 1), New castle disease (ND) vaccine on 7th and 21st and 31st day and infectious bursal disease (IBD) vaccine on 14th day. Duringthe first three weeks of trial, the chicks were fed *adlibitum* with standard starter mash and there after with

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finisher mash. Water at ambient temperature and diets (starter and finisher phases) was supplied *adlibitum* throughout the period. All the birds from group T0 to T7 were inoculated with 10^4 ccu/ml of Ms Culture to each bird on day 16.

Assessment of plasma cytokines

Blood samples (2 ml) were collected aseptically from the brachial vein, centrifugated for 10 minutes at 4000 rpm and the serum samples were then kept at -70°C in a deep freezer. Tumor Necrosis Factor-Alpha, Interleukin - 1β, Interleukin-6 and Prostaglandin E2 were estimated using ELISA kit was procured from Mybiosource, USA.

Estimation of tissue antioxidant profiles (liver)

Thiobarbituric acid reacting substances (TBARS)

Estimation of TBARS were done according to the standard procedure. The concentration of test samples was obtained using molar extinction coefficient of MDA. The units of activity were expressed as nmoles of MDA/mg of tissue.

Superoxide dismutase (SOD)

Values of superoxide dismutase were estimated according to standard procedure (Madesh and Balasubramanian, 1998). Theenzyme activity was expressed as Units/mg protein.

Estimation of catalase

Catalase were estimated according to standard procedure (Asru, 1972). The enzyme activity was expressed as µg of H_2O_2 decomposed/mg protein/min.

Reduced glutathione (GSH)

Reduced glutathione were estimated according to standard procedure (Moron *et al.,* 1979). The values were expressed as nmoles of GSH/mg of protein.

Statistical analysis

Data were subjected to statistical analysis by applying Two-way ANOVA using statistical package for social sciences (SPSS) version 16.0. Differences between means were tested using Duncan's multiple comparison test and significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

The concentration of TNF-α (pg/ml) of the negative control group was significantly ($p<0.05$) higher (15.735 and 13.570 during 3rd and 5th week) than those of positive control group (9.766 and 7.054 during 3rd and 5th week) (Table 2). The groups treated with Taimulin and Nizatidine showed ($p<0.05$) significant increase in IL-6 concentration as compared to negative control group at the end of 5th wk. Allthe groups showed significantly ($p<0.05$) higher IL-6 concentration compared to group T2 at the end of 5th wk (Table 2). The IL-6 values of groups Enrofloxacin and Tilmicosin were comparable to that of group T7 at the end of 5th wk. All the treated groups revealed significant ($p<0.05$) decrease in IL-1 and PGE-2 (Table 2) concentration at the end of 5th wk as compared to 3rd wk. The interaction of *Mycoplasma* species with the host immune system is complex. The pathogenicity of some species involves resistance to host clearance mechanisms. A polysaccharide capsule occurs in some *Mycoplasma* species. The capsule decreases production of tumour necrosis factor (TNF) and interleukin (1L)-1 and decreases utilization of glucose by alveolar macrophages (a measure of activation), when compared with non-encapsulated organisms. The capsule also resists phagocytosis in the absence of specific anti-capsular antibodies (Almeida, 1992). Some host species seem more susceptible than others to certain *Mycoplasma* species, possibly because of differences in immune function.

Table 1: *In vivo* Experimental protocol for pharmacological evaluation of drugs against *Mycoplasma synoviae.*

Group	Birds/group	Group treatment
T0: Negative control	40	Control group T0 was fed standard basal ration without any antibiotic/herbal medicine added to it (inoculated with Ms Culture on Day 16)
T1: Treatment	40	Tylosin dose @MIC value from Day 1-3; Day 16-18; Day 26-28 and Day 34-36 inoculated with Ms Culture on Day 16
T2: Treatment	40	Enrofloxacin dose @MIC values; from Day 1-3; Day 16-18; Day 26-28 and Day 34-36 (inoculated with Ms Culture on Day 16)
T3: Treatment	40	Taimulin dose @MIC value; from Day 1-3; Day 16-18; Day 26-28 and Day 34-36 (inoculated with Ms Culture on Day 16)
T4: Treatment	40	Erythromycin dose @MIC value; from Day 1-3; Day 16-18; Day 26-28 and Day 34-364 (inoculated with Ms Culture on Day 16)
T5: Treatment	40	Tilmicosin dose @MIC value; from Day 1-3; Day 16-18; Day 26-28 and Day 34-36 (inoculated with Ms Culture on Day 16)
T6: Treatment	40	Nizatidine@MIC value; from Day 1-3; Day 16-18; Day 26-28 and Day 34-36 (inoculated with Ms Culture on Day 16)
T7: Positive control	40	Positve control group was fed standard basal ration without any antibiotic/herbal medicine added to it (Not inoculated with Ms Culture on Day 16)

Table 2: Effect of drug treatment on cytokine profiles in broiler chickens 3rd and 5th week.

	T0	T1	T2	T3	T4	T5	T6	T7
					TNF-α levels			
Day 21 (3rd week)	15.735^{a}	17.615^{a}	20.987^{b}	22.905^{b}	18.816^{b}	12.186^{a}	16.396^{b}	9.766^{a}
Day 42 (5th week)	13.570^{b}	16.875^{b}	25.189^{c}	22.514^{c}	17.800^{b}	9.474^{a}	15.38^{b}	7.054^{a}
					IL-1 levels			
Day 21 (3rd week)	0.142^{a}	0.150^{a}	0.152^{ab}	0.155^{b}	0.147^{a}	0.143^{a}	0.127^{a}	0.123^{a}
Day 42 (5th week)	0.150^{a}	0.188^{b}	0.190^{b}	0.158^{a}	0.151^{a}	0.152^{a}	0.131^{a}	0.132^{a}
					IL-6 levels			
Day 21 (3rd week)	0.473^{a}	3.756^{c}	3.779^{c}	4.763^{c}	2.547^{c}	2.442^{c}	2.227^{c}	2.122^{c}
Day 42 (5th week)	3.619^{a}	6.754^{c}	8.136^{c}	9.997^{c}	7.511^{c}	6.255^{bc}	7.191^{c}	5.935^{bc}
					PGE 2 levels			
Day 21 (3rd week)	0.115^{a}	0.128^{a}	0.212^{c}	0.165^{b}	0.134^{ab}	0.121^{a}	0.102^{ab}	0.106^{a}
Day 42 (5th week)	0.113^{a}	0.131^{a}	0.137^{b}	0.133^{ab}	0.122^{a}	0.119^{a}	0.116^{a}	0.119^{a}
	T0	T1	T2	T3	T4	T5	T6	T7
Day 42 (5th week)	3.619^{a}	6.754^{c}	8.136^{c}	9.997^{c}	7.511^{c}	6.255^{bc}	7.191^{c}	5.935^{bc}

Mean±SE values with different superscript differ significantly ($p<0.05$).

Several species of *Mycoplasma* have activity against host immunoglobulins. Immunoglobulin Fc receptors, which might hinder phagocytosis even in the presence of antibodies, have been identified. Three surface proteins of *M. salivarium* are associated with the binding of this organism to the Fc fragment of human immunoglobulin. Two Fc receptors for chicken immunoglobulin have been described (Lauerman and Reynolds-Vaughn, 1991) and are reported to occur in atleast nine strains of *M. synoviae* (Madesh and Balasubramanian,1998).

Some properties of pathogenic *Mycoplasma* species seem to be related to the induction of host immune-mediated self injury *M. Capricolum* membranes have been found to stimulate production of oxygen radicals by murine peritoneal macrophages (Avron and Ruth, 1995). Some mammalian *Mycoplasma* species stimulate the production of TNF and natural cytotoxic (NC) activity (Lin *et al.,* 1988), which may mediate host destruction of infected cells. Stimulation of TNF-α was found to be greater with virulent strains than with less virulent strains of *M. pulmonis* in mice.

Innate/intrinsic host defenses are an imperative component of early and natural defenses against pathogenic microorganisms infection (Hietbrink *et al.,* 2006). During infection, MS interacts with host epithelial cells and generates an inflammatory response, resulting in increased levels of cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-2 (IL-2) (Berzat and Hall, 2010). The increased levels of inflammatory mediators appear to play a protective role or to initiate an irreversible immune response leading to cell death (Hoffmann, 2003). TNF-like activity has been found in the chicken but has not been well characterized (Klasing, 1991; Klasing and Peng, 1990 and Kleven, 1997). Other forms of immune-mediated host injury involve stimulation of ineffective or autoimmune host responses. Arthriditis produces a soluble T cell mitogen (MAM) which binds directly to rat, mouse and human major histocompatibility complex class II molecules without the need for processing by antigen-presenting cells (Posnett, 1993). The MAM-class II complex is recognised by 5-50% of T cells, in contrast to the few T cells that recognise a specific antigen-class II complex (Posnett, 1993). MAM appears to stimulate Th2 activity rather than Thl, resulting in decreased IL-2 and increased IL-4 and IL-6 secretion. Polyclonal activation of B cells, probably as a result of IL-4 and IL-6 stimulation, potentially leads to an autoimmune state. The detection of increased concentrations of rheumatoid factor in the serum of chickens infected with the WVU 1853 strain of *M. synoviae* was suggested as a possible indication of autoimmune induction (Sells, 1976 and Walker *et al.*, 1978). Autoimmunity through immune complex formation has also been suggested in *M. synoviae*infection, as immunoglobulin G was identified in numerous affected organs without any evidence of *M. synoviae* organisms (Kawakubo *et al.,* 1980). This possibility has, however, been disputed (Sells, 1976). Cross-reaction of antibodies against *Mycoplasma* attachment proteins could therefore lead to autoimmune tissue damage and decreased immune function. Currently, TNF-α, IL-1β and IL-6 inflammatory factors are usually responsible for the systemic effects of inflammation. Many studies have shown that TNF-α expression is associated with several diseases, including CRD, rheumatoid arthritis, pneumonia and various forms of inflammation in birds and mammals (Liang *et al.,* 2017). Moreover, IL-1β and IL-6 participate in the occurrence and development of the disease by inducing extensive chemokine expression and upregulating adhesion molecules in human endothelial cells (Tian *et al.,* 2016). These results suggest that proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, play a key role in the development or progression of such inflammatory diseases by promoting inflammation and tissue injury.

Table 3: Druggability on hepatic antioxidant profiles in broiler chickens (5th week).

	MDA (nmol/mg protein)	SOD (activity/mg protein)	CAT (activity/mg protein)	GSH (µ mol/mg protein)
T0	0.31±0.10[a]	0.16±0.21[a]	0.16±0.13[a]	0.22±0.07[a]
T1	0.26±0.14[a]	0.27±0.16[b]	0.37±0.16[b]	0.33±0.09[b]
T2	0.29±0.17[a]	0.37±0.22[b]	0.54±0.15[b]	0.46±0.11[b]
T3	0.24±0.21[a]	0.53±0.13[b]	0.51±0.19[b]	0.47±0.14[b]
T4	0.25±0.18[a]	0.34±0.17[b]	0.33±0.23[b]	0.36±0.17[b]
T5	0.29±0.11[a]	0.14±0.16[a]	0.24±0.12[a]	0.33±0.11[a]
T6	0.16±0.17[b]	0.38±0.13[b]	0.29±0.17[b]	0.42±0.13[b]
T7	0.12±0.12[b]	0.48±0.21[b]	0.45±0.14[b]	0.71±0.14[b]

Mean±SE values with different superscripts differ significantly (P<0.05).

The concentration of TBARS (nmoles MDA/mg protein) in liver at 5th week revealed a significant (P<0.05) rise in group negative control group (0.31±0.10) as compared to group positive control group. Enrofloxacin treated group showed a significant (p<0.05) increase in TBARS concentration as compared to positive control group. The TBARS levels in the Nizatidine treated group were significantly (p<0.05) lower as compared to other treatment groups at the end of 5th week (Table 3).

SOD, is the main driving force in cell/body adaptation to various commercially relevant stress conditions. In the present study, the concentration of SOD (U/mg protein) in liver at 5th week revealed a significant (P<0.05) decrease in group negative control group (0.16±0.21) as compared to group positive control group (0.48±0.21). The Tilmicosin group (T7) showed a significant (p<0.05) decrease in SOD concentration as compared to other treatment groups at 5th week. The SOD levels in the Tiamulin (T3) group were significantly (p<0.05) higher as compared to all other groups at the end of 5th week (Table 3).

The concentration of catalase (U/mg) in Liver at 5th week revealed a significant (P<0.05) decrease in group T0 (0.16±0.21) as compared to group T7 (0.48±0.21). The group T5 showed a significant (p<0.05) decrease in SOD concentration as compared to other treatment groups at 5th week. The SOD levels in the Tiamulin group T3 were significantly (p<0.05) higher as compared to all other groups at the end of 5th week (Table 3).

The concentration of GSH (µ moles/mg protein) in liver at 5th week revealed a significant (P<0.05) decrease in group T0 (0.16±0.21) as compared to group T7 (0.48±0.21). The group T5 showed a significant (p<0.05) decrease in GSH concentration as compared to other treatment groups at 5th week. The GSH levels in the Tiamulin group T3 were significantly (p<0.05) higher as compared to all other groups at the end of 5th week (Table 3).

In the present study, an increase in plasma MDA levels of the experimental groupswas noted in comparison with the control group. This increase was found to be statistically significant in all groups on the 3rd week of the study. On the other hand, statistically significant difference was observed in the antioxidant levels of the experimental groups compared to the control group. The presence of statistically significant differences between MDA levels of some groups and the CAT activity of none of the groups in the experimental period despite antibiotic administration points out the involvement of cellular antioxidant defence systems and decrease in the severity of lipid peroxidation (Halliwell and Gutteridge, 1990). Observations of an increase in MDA levels and decrease in antioxidant enzyme activity compared to the control group, in all periods, suggests that the oxidative impulse and free radical to form at levels that are tolerated by the body. On the other hand, since the differences observed at all dose quantities were not directly proportional to the increase in doses of the free radicals, which formed as a result of the administered doses were considered to be compensated by the antioxidant mechanism. Yazar *et al.* (2002) administered 25 mg/kg/b.w. of tilmicosin as a single dose to mice and found a decrease in cardiac SOD and GSH-Px activity. Mezes *et al.* (1992) administered 50 mg/kg/b.w.of tiamulin or 140 mg/kg/b.w. of salinomycin to 28-day-old broiler chickens, reared on feed containing 60 ppm of salinomycin and determined MDA and glutathione levels, as well as GSH-Px and CAT activities in the liver. They reported hepatic MDA and CAT activity to increase and glutathione levels and GSH-Px activity to decrease in the both groups treated with salinomycin and tiamulin. Despite the presence of many studies on the capacity of antibiotics to cause lipid peroxidation in other animal species, the number of studies carriedout in poultry species is quite low. Since so far no study was carried out on oxidative stress caused by *Mycoplasma synoviae* and antibiotics in poultry species, it is difficult to compare the results of this study with any similar investigations in poultry. In this respect, this study bears significance with regard to being a reference for future studies in poultry.

CONCLUSION

Cytokines, TBARS, SOD, catalase and GSH levels showed a significant (P<0.05) difference between various treatment groups. Therefore, it is necessary to havea regular survey on poultry pathogenic mycoplasmas and to monitor antimicrobial susceptibility patterns in order to ensure that effective chemotherapy is being employed to treat mycoplasmal infections.

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