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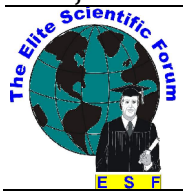

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Pathogenicity and Effect of Exogenous Antibodies on the Viability of *Mycoplasma gallisepticum* in Chicken Embryos

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ABSTRACT
Isolation of *Mycoplasma gallisepticum* (MG) was attempted from different organs including trachea, air sacs and lungs (n=27 for each organ) of commercial layer chickens. Out of all the organs examined, 18.51% trachea (n=5/27) and 7.41% air sacs (n=2/27) were found positive for MG isolates; whereas, no isolation was made from lungs. The isolates were identified by microbiological and biochemical tests i.e. change in the color (red to orange) and turbidity of broth in addition to the typical colony characteristics (fried egg shaped colonies) of MG on PPLO (pleuropneumonia like organism) agar. Isolates were purified and sub-cultured in PPLO broth and pathogenicity of MG was observed in chicken embryos on the basis of mortality pattern. Gross pathological lesions in inoculated chicken embryos included oedema, stunted growth and hemorrhagic embryos. In second trial, antigen-antibody complex was inoculated in 7-days old (n=10) embryonated eggs to observe the effect of the exogenous antibodies on the viability of MG in chicken embryos. Results showed that simultaneous administration of anti-MG antibodies along with MG antigen failed to neutralize the antigen inside the embryonated chicken eggs.

INTRODUCTION

Poultry industry is an excellent source of low cost animal proteins and has a major role in national economy of Pakistan by generating revenue. In Pakistan, this industry is flourishing very rapidly and has become the second biggest industry after textile but still facing various problems including disease threats. Among these diseases, avian mycoplasmosis is one of the most important maladies hampering the growth of poultry industry in the country. Among twenty different species of Mycoplasma, isolated from poultry birds, *Mycoplasma gallisepticum* (MG) is the most important avian pathogen responsible for chronic respiratory disease (Buim et al., 2009). It causes significant economic losses in the form of mortality, carcass condemnation, decreased egg production, poor hatchability and feed conversion ratio, in addition to the medication cost, spent on the treatment of diseased birds. Moreover, it has been reported that MG infected hens lay at least 16 eggs lesser than the non-infected hens (Munir, 2011). Similarly in infected breeders, this reduction has been estimated up to 21 less eggs per bird (Mohammad et al., 1987). Furthermore, their progeny shows poor FCR, carcass quality and economic losses due to prevention and treatment costs. MG infection causes sneezing, conjunctivitis, air sacculitis, and decreased egg production in affected birds (Ley, 2008). In Pakistan, the disease is quite prevalent and is causing heavy economic losses.

This study was planned to observe the pathogenicity of field isolates of MG and an attempt was made to neutralize the MG in embryonated chicken eggs by simultaneous inoculation of exogenous MG antibodies along with the antigen.

MATERIALS AND METHODS

Sampling

A total of 81 samples of trachea, air sacs and lungs (n=27 for each organ) were collected from recently dead or slaughtered birds from 19 MG positive commercial layer flocks. The samples were placed in sterilized Petri plates and brought to Poultry Disease

Diagnostic laboratory, Poultry Production, Faisalabad, Pakistan for further processing.

Isolation of *Mycoplasma gallisepticum*

For isolation of MG, all the samples were processed on Modified Frey's medium (Frey et al., 1968) with minor modification. The media base was supplemented with 15% horse serum, 1% glucose and 10% yeast extract. To prevent contamination, 1% thallium acetate and 0.5% penicillin were also added to the media. For isolation, about 0.5 g of each tissue (trachea, air sac and lungs) were cut into small pieces and ground in a tissue grinder containing 5 ml of mycoplasma medium. A total of 0.2 ml suspension was inoculated in Pleuro-pneumonia like organism (PPLO) broth. Cultures were examined daily for evidence of growth for 7 to 10 days. Positive growth on broth (with turbidity and color change in medium) was transferred to (PPLO) medium and plates were incubated at 37 °C temperature and 70% relative humidity. The plates were examined daily for the evidence of typical fried egg shaped colonies up to 14 days after inoculation (Yoder, 1980). The isolates of MG were identified morphologically and confirmed by biochemical tests (fermentation of glucose and tetrazolium).

Experimental Design

The whole study was split into two experiments. The first experiment was designed to observe the pathogenicity of field isolates of MG and in the second study, the effect of exogenous antibodies upon MG in embryonated eggs was measured.

Experiment No. 1

Day old fertile chicken eggs (n=30) collected from MG free flocks were cleaned with 70 % ethyl alcohol and fumigated with 37% formalin and Potassium permanganate with a ratio of 2:1 before they were inoculated with MG. Twenty eggs were inoculated with field isolate (0.2 ml of broth culture/egg) via yolk sac route. The other ten eggs were kept as control .The site of inoculation of all the eggs were sealed with sterile wax and incubated at 37 °C for 18 days. The candling of each egg was performed on each of day 6^{th}, 9^{th}, 12^{th}, 15^{th} and 18^{th} post MG inoculation. Infertile eggs from both the groups (test and control) were discarded at first candling. The eggs showing embryonic mortality were removed at each candling and were stored at 4°C till further processing.

Experiment No. 2

Hyper immune sera from two naturally infected birds with MG were collected. The sera were pooled and inactivated by heating at 56 °C for 30 minutes in water bath. The positive serum was titrated by using MG antigen (Intervet International®). An antigen-antibody complex was prepared by mixing equal volume of isolates propagated in PPLO broth and the specific hyper immune serum (HA titer =4). Thirty 7-days old chicken embryonated eggs were obtained and divided into three equal groups. In first group, each embryonated egg was inoculated with above complex (0.2 ml per egg via yolk sac route).The embryonated eggs of second group were inoculated with the same quantity of the pure broth culture (positive control) via the same route. In the third group, the embryonated eggs were kept as negative control and PPLO broth (Uninoculated) was inoculated (0.2 ml) via yolk sac route. The embryonated eggs of all the three groups were re-incubated at 37 °C in the incubator for further 12 days. The candling was performed every day post-inoculation till 18^{th} day. The embryos showing mortality were transferred to 4 °C till further processing. At 18^{th} day, the remaining eggs were also transferred to refrigerator.

The yolk material and yolk sacs from all the chilled embryos of both the trials were inoculated into PPLO broth and PPLO agar for re-isolation of MG. The numbers of positive cultures were noted. Embryonic development, embryonic death and recovery percentage were also noted.

RESULTS AND DISCUSSION

Mycoplasma species are very fastidious organisms and require special growth media supplemented with activated amino acids, co-enzymes and vitamins (Razin, 1984). MG is a nutritionally deficient organism that needs a complex medium enriched with 10 to 15 per cent heat inactivated swine, avian or horse serum as a source of cholesterol (Rottem et al., 1978). It has been reported that broth containing 0.5-1% (W/V) glucose and 10-15 % (V/V) horse serum produced the greatest dry weight of Mycoplasma. In this study, the isolation of MG was attempted from various suspected organs including trachea, air sacs and lungs. Trachea and air sacs are routinely used to isolate MG as these are reported to be the predilection sites for its multiplication and have been widely used for isolation purposes (Hitchner et al., 1980; Giap, 2008). In the present study, PPLO broth and agar were prepared according to the method of Frey et al. (1968) with a minor modification i.e. the replacement of swine serum with that of horse. The colonies developed on PPLO agar were very small, smooth, circular and flat with dense elevation in the centre showing fried egg shaped appearance and similar findings have been reported previously by some other researchers (Gharaibeh and al Roussan, 2008 and Heleili et al., 2011; Ahmad et al., 2011) who also used Frey's medium for the isolation of MG.

Giemsa staining of smears prepared from the broth revealed that the organisms were highly pleomorphic and usually coccoid in shape. Such morphological characters of MG have also been reported earlier (Khalil, 1984; Tariq el al., 1987).

Out of 81 samples, MG was isolated only from 7(8.64%) samples. Results are in line with those of Hanif and Najeeb, (2007) and Helail, (1998) who reported that MG was isolated at rate of 9.29% and 11.89%, respectively from different organs. Maximum isolation 5(18.5%) was made from trachea followed by 2(7.41%) from air sacs; whereas no isolation was made from lungs (Table 1).

Table 1: Rate of isolation of *Mycoplasma gallisepticum* from field specimens on the basis of colony morphology

Organs	No. of samples examined	No. of MG isolates	Percentage
Trachea	27	5	18.5
Air sacs	27	2	7.41
Lungs	27	-	-
Total	81	7	8.64

MG isolation rate from trachea was 18.5% in the present study. Our results were lower than Osman et al., 2009 (25.5%) Gharaibeh and al Roussan, 2008 (31.6%) Feberwee et al., 2005 (33%), Ahmad, 1989 (44.3%), Pakpinyo and Sasipreeyajan, 2007 (44.66%) and Heleili et al., 2011 (62.96%). Moreover, from air sacs the isolation rate was 7.41%; whereas, Helail (1998), Ahmad (1998) and Heleili et al. (2011) showed 36.29%, 47.9% and 90 % isolation rates, respectively and this revealed that the isolation rate in the current study was lower than other studies.

Further, MG is somewhat difficult to be isolated and low percentage of isolation in the present study might be due to premedication of birds with antimycoplasmal drugs. MG is also difficult to isolate from commercial layer because of overgrowth with nonpathogenic mycoplasma that grow very faster than pathogenic ones. Other workers have also reported the low percentage of MG isolation (Khalil, 1984).

Pathogenicity of *Mycoplasma gallisepticum*

Inoculation of chicken embryo provided a model for characterization of MG by observing virulence and pathogenicity. Certain highly pathogenic strains have clear relationship between the titer of MG inoculated, the embryo mortality and time of death. Less pathogenic strains usually caused embryo mortality during the later stages of incubation (Levisohn, 1985; Giap, 2008). In the present study, 20 fertile eggs were inoculated with field isolates of MG cultured on PPLO broth and incubated at 37 °C. During candling at 6th day of post-inoculation (PI), the infertile eggs (4 from experimental and two from control) were discarded. All the 16 inoculated embryos died within 18 days of inoculation, 11(68.75 %) embryos died within first 9 days. Among the control group, only one embryo died at 6th day (Table 2).

Dead and live embryos were chilled at the end of experiment (18th days PI). The pathological lesions recorded in dead embryos were edematous, hemorrhagic and stunted growth of chicken embryos. MG was re-isolated from yolk sac of all the chilled dead or live embryos. No isolate was recorded from control group. Dead embryos from the control did not show specific lesions of MG. The cause of death of one embryo in control group might be non-specific. The results are in agreement with the findings of Renchu et al. (1997) and Akhtar et al. (1991). Renchu et al. (1997) reported that 33 SPF chick embryos inoculated with 4 MG strains showed up to 80% embryo mortality within the first 8 days; whereas, Akhtar et al. (1991) reported that four strains MI-203, MI-225, MI-211, MI-2000) with few exceptions caused 100% embryo mortality in late phase of incubation (5-10 days PI). MI-211 caused higher embryo mortality in the early period of incubation (3-5 days post inoculation). Edema, hemorrhages and stunted growth of chicken embryos resulting in dwarfism has also been reported by Levisohn (1985), Akhtar et al. (1991) and Renchu et al. (1997). The study of Pakpinyo et al. (2010) revealed that 16 embryos died in early phase (3-7 days PI) and 30 embryos died in later stage of incubation when 108 embryos were inoculated with three MG strains (F, 6/85 and Thai strains) with different dose levels. They also described that F strain caused the highest 23 (50 %) embryos mortality followed 14(30.43 %) by 6/85 strain and 9(19.56 %) Thai strain. Giap (2008) also reported that MGS6, 144 and 1-18 strains caused early embryonic death as compared to ts-11, H21 8T, H2 1 IIT, H24 5C and H26 9C strains that caused embryo mortality during later stages of incubation.

Effect of exogenous antibodies on *Mycoplasma gallisepticum*

In the second trial, antigen-antibody complex was inoculated in 10 embryos (7-days old) to see the effect of exogenous antibodies upon MG in the eggs during incubation. Only one embryo died at 3rd day post inoculation in the experimental group. In the positive control, all the embryos died within 5 days PI; whereas, all the embryos in the negative control remained alive. This indicated that the antibodies prevented the embryonic deaths in eggs inoculated with Ag-Ab complex but the embryonated eggs still remained carriers. Although the high doses of antibodies might have some effects to kill carrier organisms. This result was in line with Levisohn (1985) and Whithear (1996) who reported that embryo mortality due to virulent MG was completely blocked in eggs containing maternal antibodies against MG although the Mycoplasma could be re-isolated from the yolk sac membrane of the live embryonated eggs after 17 days of incubation. Attempts to mimic the effect of maternal antibodies by

Table 2: Mortality pattern of embryos inoculated with *Mycoplasma gallisepticum* field Isolates

Group	No. of eggs inoculated	Infertile* eggs	Embryonated eggs	Mortality at various days of incubation					
				6th	9th	12th	15th	18th	Total
MG inoculated fertile eggs	20	4	16	6	5	2	2	1	16/16
Control	10	2	8	1**	-	-	-	-	1/8

* Observed by candling at days 6th post incubation; **The death of embryo might be non-specific

Table 3: Effect of Exogenous Antibodies on the mortality in Chicken Embryos infected with *Mycoplasma gallisepticum*

Group	Embryos inoculated	Mortality (days post inoculation)												
		1	2	3	4	5	6	7	8	9	10	11	12	Total
Ag-Ab complex	10	-	-	1	-	-	-	-	-	-	-	-	-	1/10
Positive Control	10	-	-	3	5	2	-	-	-	-	-	-	-	10/10
Negative Control	10	-	-	-	-	-	-	-	-	-	-	-	-	0/10

inoculating exogenous MG antiserum were not successful.

It was concluded that isolated cultures were proved to be highly pathogenic as they showed high early embryonic mortality. Further investigations on the sub-typing of Mycoplasme species are highly desired. Moreover, antibodies against MG and other pathogens present in embryos might interfere in the pathogenesis of MG but attempt to eradicate the carrier stage during incubation did not prove to be fruitful.

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