CHICK EMBRYO MORTALITY STUDIES USING DIFFERENT STRAINS OF *MYCOPLASMA GALLISEPTICUM*

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SUMMARY: Four M. gallisepticum strains isolated from cases of chronic respiratory diseases (CRD) were studied and compared with MG-S6 and MG-F strains for their pathogenic potential for embryonated chicken eggs. The embryo mortality was found to be dose dependent. However, all four strains with few exceptions, caused 100% embryo mortality, showing that apart from the virulence factor the bulk multiplication may also result in embryo death. Amongst the four strains used MI-211 was found to be the most pathogenic one. The present investigation indicates that the pathogenicity of MI-211 is comparable with that of MG-S6 which is reported to be highly pathogenic.

Key Words: Mycoplasma gallisepticum, pathogenicity, chick embryo mortality.

INTRODUCTION

Avian mycoplasma serotypes are found to differ in their potential for producing chick embryo mortality, with most of the strains of *M. gallisepticum* being pathogenic for chicken embryos (20). The cultural history of a strain of *M. gallisepticum* also correlates with the embryo mortality (15). Pathogenic *M. gallisepticum* strains cause a high embryo mortality, but it may be possible that the *in ovo* virulence is enhanced by egg adaptation (7). Some non-pathogenic or less pathogenic strains may also cause embryo mortality, but this may be attributable to the massive multiplication rather than the actual virulence of a particular strain and this requires further study (7). In addition there may be marked variations in results between repetitive trials using the same technique (10,16,17) as well as between different techniques of pathogenicity study (6,7,9,13,18,19).

The present study was conducted with a view to test the ability of four locally isolated *M. gallisepticum* strains, along with two other strains (MG-S6 and MG-F), to cause embryo mortality, so that their pathogenic potential and any variation among them in this particular respect could be determined. Such a study might also be useful to classify these local isolates of *M. gallisepticum* for future studies.

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MATERIALS AND METHODS

Eggs

Fresh fertile chicken eggs (from a mycoplasma free flock) were obtained from a local hatchery (PIA Shaver).

Pretreatment of eggs

The eggs were washed with soap and water and then with 70% alcohol for surface disinfection. After drying at 37°C for 6 hours the eggs were immersed in 0.1% W/V chilled erythromycin solution at 4°C for 30 minutes in order to remove residual bacteria (1). These eggs were then incubated at 37°C for 7 days which was considered enough to eliminate the residual effect of erythromycin which may interfere with the subsequent experiments. On the eight day, before inoculation, every egg was candled.

Mycoplasma strains

For locally isolated strains of MG (MI-200, MI-203, MI-211 and MI-225) were randomly selected to study their pathogenic potential. MG-S6 and MG-F were used for comparison as the former one is reported to be highly pathogenic (5, 14) and the later one as a less pathogenic species which is also used as a vaccine strain (2, 4, 11).

Isolation and identification of mycoplasma strains

All the local strains used in the present study were originally isolated from the respiratory tract of chickens clinically suspected of suffering from mycoplasmosis using Jordan and Amin mycoplasma isolation medium (5). The locally isolated

mycoplasma strains were identified first by morphological, cultural and biochemical means followed by confirmation by different serological means.

Storage of mycoplasma strains

These strains were kept lyophilized for it is known that mycoplasma lose their pathogenicity rapidly during sub-culturing in the laboratory (8).

Determination of mycoplasma titre

Before inoculation they were grown in Jordan and Amin broth (5) for 24 hours and the total number of colony forming units per ml (CFU/ml) of every strain was determined by the Miles and Miisra technique (12).

Embryo inoculation

Eggs were divided into groups so that there were 24 eggs for each inoculum for each mycoplasma strain as shown in Table 1.

Table 1: Inoculation protocol for embryonated chicken eggs.

Strains	Size of inoculum (CFU/ml)			
	1	2	3	4
MG-S6	$4.5x10^8$	$4.5x10^7$	$4.5x10^6$	$4.5x10^5$
MG-F	$8.1x10^8$	$8.1x10^7$	$8.1x10^6$	$8.1x10^5$
MI-200	$3.4x10^9$	$3.4x10^8$	$3.4x10^7$	$3.4x10^6$
MI-203	$1.5x10^9$	$1.5x10^8$	$1.5x10^7$	$1.5x10^6$
MI-211	$4.4x10^8$	$4.4x10^7$	$4.4x10^6$	$4.4x10^5$
MI-225	$6.7x10^8$	$6.7x10^7$	$6.7x10^6$	$6.7x10^5$

One group of 24 embryonated chicken eggs was inoculated with plain broth. Another group of 24 embryonated eggs served as uninoculated control.

Every inoculation was done with 0.2 ml culture into the yolk sac with a 1 inch long 26 gauge needle. After inoculation the punctured shells (over the air sac) were sealed with sterile wax. All the eggs were then incubated at 37°C for 12 days with daily candling. Embryos dying within 48 hours after inoculation were discarded. Dead embryos were examined for lesions and yolk was cultured for isolation of mycoplasma using Jordan and Amin medium (5).

RESULTS AND DISCUSSION

As shown in Table 2, MI-211 caused higher embryo mortality in the early period of incubation (3-5 days post inoculation, PI). This is comparable with the embryo mortality pattern shown by MG-S6, which is considered to be comparatively more pathogenic among various strains of *M. gallisepticum* (5,14). On the other hand, the remaining three strains (MI-200, MI-203, and MI-225) were found to be nearly similar in their embryo mortality pattern as all of them caused embryo mortality in the late phase of incubation (5-10 days PI). This pattern is comparable with that of MG-F which is reported to be a relatively less pathogenic strain of *M. gallisepticum* (2, 4,11,14).

Table 2: Time of chick embryo mortality after inoculateon with different MG strains at different inoculation levels.

MG-Strains	Inoculum (CFU/ml)	Early mortality (3-5 days PI)		Late mortality (5-10 days PI)	
		No	%	No	%
MG-S6	$4.5x10^8$	24	100	24	100
	$4.5x10^7$	15	62.5	24	100
	$4.5x10^6$	9	37.5	24	100
	$4.5x10^5$	6	25	24	100
MG-F	$8.1x10^8$	12	50	24	100
	$8.1x10^7$	7	29	16	66.6
	$8.1x10^8$	4	16.6	16	66.6
	$8.1x10^6$	0	0	15	62.5
MI-200	$3.4x10^9$	1	50	24	100
	$3.4x10^8$	7	29	24	100
	$3.4x10^7$	6	25	24	100
	$3.4x10^6$	3	16.6	20	83.3
MI-203	$1.5x10^9$	8	33.3	24	100
	$1.5x10^8$	4	16.6	24	100
	$1.5x10^7$	6	25	24	100
	$1.5x10^6$	4	16.6	19	79.1
MI-211	$4.4x10^8$	24	100	24	100
	$4.4x10^7$	15	62.5	24	100
	$4.4x10^6$	12	50	24	100
	$4.4x10^5$	9	37.5	24	100
MI-225	$6.7x10^8$	10	41.7	24	100
	$6.7x10^7$	8	33.3	24	100
	$6.7x10^6$	6	25	24	100
	$6.7x10^5$	2	8.3	20	83.3

PI=Post inoculation
Mortality is the mean of five separate experiments
Number of birds per dose used were 24

On the basis of the embryo mortality data an attempt is made to calculate LD_{50} of every test strain (Table 3). The LD_{50} values reported in Table 3 are calculated on different days as the incidence of mortality below and above 50% occurred in different strains on different days.

The values reported in Table 3 clearly indicate the relative pathogenic potential of each strain of *M. gallisepticum*. The pathogenicity of *M. gallisepticum* MI-211 seems to be comparable with that of the standard strain MG-S6 which is reported to be the most pathogenic by most workers (5,14). The high degree of pathogenicity of MI-211 also coincides with its history that is the strain was isolated from a severe case of CRD. The other 3 test strains of *M. gallisepticum* showed a lower degree of virulence in chick embryos and the results are comparable with that of MG-F strain. The LD_{50} of MG-S6 and MI-211 was calculated on the 5th day while those of the other strains it was calculated on the 7th day.

Table 3: The LD_{50} of *M. gallisepticum* strains.

Strains	LD_{50}	Day LD_{50} calculated
MG-S6	$7.0x10^6$	5 th day
MG-F	$9.29x10^8$	7 th day
MI-200	$3.7x10^6$	7 th day
MI-203	$1.3x10^8$	7 th day
MI-211	$3.05x10^6$	5 th day
MI-225	$3.3x10^8$	7 th day

* Finney DJ (3) Probit Analysis. Second edition, London, Cambridge University Press. MG-S6 is reportedly highly pathogenic strain of *M. gallisepticum*. MG-F is reportedly less pathogenic and used a vaccine strain. MI-200, MI-203, MI-211 and MI-225 are four strains of *M. gallisepticum* isolated locally.

The data in the present investigation show a relationship between the number of cells inoculated and the mortality of chick embryos observed. In general embryo mortality at lower inoculation levels occurred at a later period after inoculation. It was possible to isolate mycoplasma from all the dead embryos. Reduced embryo growth with hemorrhage and oedema were the main gross pathological changes. The severity of these changes were found to be dose dependent.

The distinct feature of the study is the embryo mortality pattern. Lower doses of the more pathogenic strains caused more early mortality of embryos, while higher doses of less pathogenic strains caused embryo mortality in later periods. These results substantiate earlier studies (7).

As already reported even a less pathogenic strain of *M. gallisepticum* would show almost 100% mortality of chick embryos due to massive multiplication of cells in the inoculated animal specially where the observation period is long (7). In the present investigation it is 10 days. The recording of early embryo mortality within the first 3-5 days seems to give a meaningful conclusion with respect to the relative pathogenicity of these strains. The recording of early mortality has also been emphasized by Levisohn *et al.* (7).

A cumulative approach of early mortality observations within 3-5 days PI (Table 2) would indicate that above 50% chick embryos were killed by MG-S6 or MI-211. This approach of indicating relative pathogenicity of these strains correlates well with the LD_{50} determination (Table 3). On the contrary if the same cumulative approach is applied on the mortality data after 10 days PI the results would become much less meaningful (i.e. 62-100% mortality by all strains after 5-10 days PI) and then it would be difficult to draw a worthwhile conclusion. Based on the present study it is suggested that some more work should be done and that recording of embryo mortality data within 3-5 days PI may be more appropriate for determining relative pathogenicity of MG in embryonated eggs and compared with late mortality data.

In the investigation the mortality pattern of chick embryos by MG-F was similar to that reported by earlier workers (8). However, the same strain has also been described to cause a high degree of mortality in chick embryos on long term exposure (7). In addition a review of the literature would indicate that mortality pattern of chick embryos by less pathogenic or moderately pathogenic strains of MG is largely influenced by the immunological status of the growing chick embryos (7) as well as the history of exposure of the flocks, from where the fertile eggs are obtained, to other pathogens (13). However, in view of paucity of data related to the interaction of maternal antibodies and other pathogens with MG pathogenicity determination, further studies are required.

The validity of results of the *in ovo* pathogenicity evaluation of MG has been questioned (7). However, *in ovo* method has its own importance as it would reflect upon reduced hatchability. The lack of information on the relationship between *in vivo, in ovo* and *in vitro* method of pathogenicity determination suggest some more comprehensive investigation on this aspect should be conducted.

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