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ISOLATION AND CHARACTERIZATION OF VARIANT STRAINS OF MYCOPLASMA GALLISEPTICUM (MG) FROM BROILER BREEDER CHICKEN AND RATS

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SUMMARY

The serological profile of *Mycoplasma gallisepticum* (MG) in a broiler breeder flock showed negative result until 30 weeks of age, where it became MG positive (100%), when tested at both of 40 and 55 weeks of age. *Mycoplasma* could be detected from tracheal swabs in this broiler breeder flock at age 49 weeks by culture and polymerase chain reaction (PCR) .Isolation and characterization of the recovered isolates using culture, biochemical, serological and PCR testes proved to be MG. Dot ELISA was applied in the current study and it was potential enough in rapid confirmation of *Mycoplasma* isolates. Histopathological examination of the trachea and lungs of infected broiler breeder flock, revealed the destructive effect of MG. In a trial to investigate shedding of MG in four hatched chicks from the infected broiler breeder flock, the result revealed that the examined hatches were negative by PCR, culture and serology. The observed number of rats in the MG infected broiler breeder farm directs the attention for the role of rats in transmitting MG. Trials for isolation and characterization of *Mycoplasma* from such rats were conducted and the isolate was proven to be MG strain. A pathogenicity study was carried out on the two MG isolates (broiler breeder and rat strains) in SPF chicks. The results showed that, the two isolates were different in their effect reflecting the variable behavior of MG in different species or highly raise the presence of a mutant or variant strain of MG, with variable degree of pathogencity in chickens. The study reports, the isolation and characterization of MG from rats and chicken with different pathogencity. Also, the presence of MG in rat

taken from chicken house may explain the persistence of MG in the chicken houses and the role of rats in transmission and virulence of MG field strains.

INTRODUCTION

Mycoplasma gallisepticum (MG) is considered one of important respiratory pathogens of broilers, and reproductive pathogens of commercial layers (Brown et al .,1995). Also, MG infects turkeys, other gallinaceous birds, duck and geese (Yoder, 1991) . MG causes conjunctivitis in house finches (Ley et al., 1996).The capability of MG to enter the non phagocytic host cells provides this pathogen an opportunity for resisting host defenses and selective antibiotic therapy, establishing chronic infection and passing through the respiratory mucosal barrier to cause systemic infection (Winner at al., 2000). House finches infected with MG may be capable of transmitting the infection horizontally to other avian species which are susceptible to infection and disease (ley et al., 1997). MG has a terminal structure that mediates adherence to its target tissues. Variation in the major surface antigens of MG is considered the cause of its remarkable ability to persist and evade the immune response. MG is considered a successful pathogen because they can enter the host, multiply, evade the defense mechanism, cause damage and escape to infect new hosts (Bradubury, 2005) .Like other mycoplasmas, MG has a short survival time in the environment but they could be able to be transmitted vertically or horizontally successfully to new hosts. Horizontal transmission is mainly caused by intensive husbandry and stress factors (Bradubry, 2005).The modern molecular methods of strain typing help in understanding the pathways of transmission and the possible role of game and wild birds as intermediate vectors between poultry flocks (Bradubry, 2005).

The use of controlled environmental conditions, acclimatization, a high plan of nutrition and low stocking density are all important factors in the outcome of MG infections in domestic poultry and other species (Kollias et al., 2003). It is necessary to have an effective and reliable tool for confirming *Mycoplasma* infection in order to control and eradicate infection in flocks (Abdel-Moumen and Roy, 1995). In unvaccinated flocks , serum plate agglutination (SPA) is a commonly used screening test ,and is often backed up by Enzyme linked immunosorbent assay (ELISA) . Mycoplasma culture is often used to confirm the diagnosis in flocks with suspicious serological reaction (Salisch et al., 1998).

The aims of the present study were:

1- Isolation and characterization of MG from infected chickens housed in controlled environmental condition.
2- Investigation of the possible MG infection of rats caught from the same house.
3- Study the pathogencity of the isolated MG strains in SPF chicks.

MATERIALS AND METHODS

1- Study the serological profile of MG infection in broiler breeder chicken flock using ELISA.

From MG unvaccinated broiler breeder flock, 72serum samples were collected aseptically from the wing vein of the birds. To detect the presence of antibodies for MG ELISA kits (Synbiotic com.)were applied as recommended by manufactures. Using ELISA reader at wave length of 405, under optimal conditions the valid optical density values ranges were 0.06 -0.12 for MG negative control, 0.35-0.75 for MG positive control serum, and the calculation of the reading for the tested serum was applied as recommended.

2- Detection of *Mycoplasma gallisepticum* by polymerase chain reaction (PCR) in comparison to culture and serological methods from broiler breeder chicken flock.

Detection of MG by :

a- PCR:

A total of 25 tracheal swabs obtained from 49 weeks age broiler breeder flock were subjected to DNA extraction according to Fan et al. (1995).The PCR assay was applied on the extracted DNA according to Lauerman (1998). Analysis of the PCR products was done by using gel electrophoresis 2% with ethidium bromide staining and visualized by UV transilluminator. Mycoplasma gallisepticum, F strain (Shiering Plough) was used as control positive for PCR test. As expected , PCR product with 186 bp is considered positive for the presence of MG.

b-Culture conditions:

Tracheal samples were cultured on PPLO (pleuropnemmonia like organisms) broth and agar for *Mycoplasma* isolation according to Razin (1978) and propagated as described by Razin and Tully (1983) . Purification was carried out as mentioned by Tully (1983b). Genus determination was carried out according to Erno and Stipkovits (1973).Maintenance of MG was followed as previously described by Leach 1983). Biochemical characterization was conducted as described by Erno and Stipkovits (1973).Immunological identification was conducted using growth inhibition test (Clyde, 1983) and Dot ELISA (Lobo et al., 1996).

c- Serological tests:

Serum plate agglutination test (SPA) was done according to Kleven and Yoder (1989)using MG stained antigen (Intervet) on 54 serum samples obtained from 49 weeks age broiler breeder flock

ELISA kits were supplied by Synbiotic, Europe and were conducted according to the manufacture recommendation. Reference antisera (Synbiotic Com.) for MG and MS were used for immunologic identification of the isolates .

3- Detection of MG in hatched chicks originated from the infected broiler breeder flock.

Detection of MG in four successive hatche

chicks, originated from the same infected broiler breeder flock was done by using culture, PCR and serological tests as mentioned above.

4- Histopathological studies on lung and trachea of MG infected broiler breeder flock:

At post mortem , trachea and lung were removed from birds for histopathological examination as described by Bancroft et al. (1996).

5- Isolation of *Mycoplasma* from tracheal swab of rats caught from broiler breeder house and characterization of the isolate:

Rats caught from the broiler breeder house were examined for the presence of Mycoplasma in their tracheal swabs using PCR technique and isolation .

6- Experimental study to compare the pathoginicity of the two Mycoplasma isolates (Broiler breeder strain and Rat strain) in SPF chicks.

Fifty four, one day old SPF (specific pathogen free) chicks were divided into 3 groups each of 18:Group (1) was inoculated intranasally (I/N)with 0.2ml MG broiler breeder isolate $8X10^6$ (CFU/ml) broth culture.

Group (2) was inoculated (I/N)with 0.2ml of MG rat isolate $8X10^6$ (CFU/ml) broth culture.

Group (3) was considered as control non infected group.

The period of the experiment was15 days. Trachea and lung were taken from each bird on the1st, 2nd, 4th, 7th, 10th 15th post infection, for histopathological examination, as described by Bancroft et al. (1996) .Degree of histopathology was applied according to Ouda et al. (2004).

Serological examination on the 15th day post infection, was done using SPA and ELISA testes as mentioned above.

RESULTS AND DISCUSSION

Table (1) Serological profile of MG infection in broiler-breeder flock using (ELISA)

Age / Result	35 weeks	40 weeks	50 weeks	55 weeks
Total No. of samples.	18	18	18	18
No. of + ve	16	18	14	18
%of positive	88.9	100	77.8	100
Mean	2508	4895	2232	2677.5
C.V	42.5	22.9	50	34

C. V= coefficient variantion

Table (2): Results of detection of MG in infected broiler breeder flock using different techniques.

	PCR & Culture	Serology (ELISA)
Type of sample	Tracheal swab	Serum
No. of sample	25	54
Age	49 weeks at isolation	49 weeks
% of positive	100	88.9 (48/54)

Table (3): Results of MG detection in hatches of the MG infected broiler-breeder flock

Hatch No.	Age of B.B. flock	No. of sample	Culture	PCR	SPA	ELISA
H1	40 W	20	- ve	- ve	- ve	- ve
H2	45 W	20	- ve	- ve	- ve	- ve
H3	50 W	20	- ve	- ve	- ve	- ve
H4	55 W	20	- ve	- ve	- ve	- ve

- ve :negative results

Table (4): Identification of *Mycoplasma* strains isolated from chickens and rats caught from the chicken house

Tests	Results for both isolates
Culture on Mycoplasma medium (PPLO)	+
Digitonin sensitivity	+
Glucose fermentation	+
Arginine deamination test	-
Growth inhibition test (GI)	+ MG
Dot ELISA	+ MG
PCR	+ MG

+ Ve culture : Fried egg appearance
+ Ve digitonin : inhibitory zone indicating Mycoplasma
+ Ve Glucose fermentation test: change in co lour from red to orange or yellow co lour
- Ve Arginine deamination test: no change in co lour
+ Ve Growth Inhibition test : inhibitory zone around the disc of Mycoplasma gallispticum antisera
+ Ve Dot ELISA : means it is Mycoplasma gallispticum strain
+ Ve PCR : appearance of MG band

Table(5): Results of Histopathology examination of MG infected broiler breeder flock

Result of Histopathological examination	
Lung	Trachea
Oedema noticed in the inter lobular C.T stroma with sever dilatation of blood vessel & capillaries. Degree (I).	Epithelial lining of the mucosal layer with the basement membrane was completely destructed & desquamated, while the underlining C.T , Laminapropria and muscularis mucosa showed massive no. of mononuclear leucocytes inflammatory cells infiltration in diffuse manner with dilatation of blood capillaries. Degree (III).

Table (6): Results of pathogenisity test of chicken and rat MG isolated strains in SPF chicks expressed by the degree of histopathology

Organ	Days post infection	Group infected with Boiler breeder isolate	Group infected with Mice isolate
Trachea	1st day	-	I
	2nd day	0	II
	4th day	I	II
	7th day	I	III
	10th day	II	0
	15th day	III	0
Lung	1st day	-	I
	2nd day	I	I
	4th day	II	II
	7th day	III	III
	10th day	III	III
	15th day	III	III

**** Grades of histopathological changes :**

Trachea: O = No alterations

I = Dilated and hyperemic blood vessels and capillaries of the mucosa, sub mucosa , cartilaginous layer with thickening and metaplasia in the mucosal layer and associated with mononuclear leucocytes inflammatory cells.

II = Goblet cell formation all over the lining covering epithelium with hyperplasia diffuse goblet cells formation all over mucosal lining epithelium and muscular layer unfiltered by mononuclear leucocytes cells , extravasated RBCS.

III = Goblet cell formation with focal desquamation in the lining mucosal epithelium with mononuclear leucocytes inflammatory cells in the muscular layer.

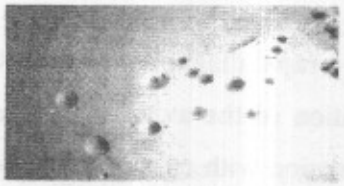
Lung : O = No alterations.

I = Severe dilatation of blood vessels, hyperplasia in epithelial lining of bronchial connective tissue dispersed with edema.

II = Dilated stromal blood vessels with thickening and hypertrophy in the vascular wall , perivascular edema and bronchial showed Goblet cell , hyperplasia of lining epithelial with mononuclear inflammatory cells and edema in lamina propria while other bronchials showed extra vasated blood cells with goblet cells.

III = Focal mononuclear leucocytes and dilated blood capillaries with desquamation of mucosal lining, mononuclear leucocytes aggregated in lamina propria of bronchioles with hyperplasia.

****The serology of the serum of SPF chicks at 15 days post inoculation was negative by SPA and ELISA.**



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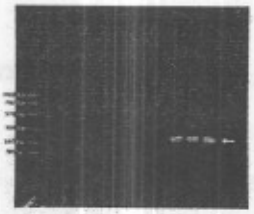

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Photo 1 : The identified colonies of MG on PPLO agar showing fried egg colonies
Photo 2 :The PCR product of amplified fragment 16srRNA of isolated MG at186 bp.
Lane,(M): Represent1000 bp DNA molecular marker (50,150,300,500,750,1000bp).
Lane,(H) : Negative control.
Lane,(I) :MG reference F strain.
Lane,(J) : Isolated Broiler breeder MG strain.
Lane,(K) : Isolated Rat MG strain .
Photo 3 : Trachea grade I Photo 4: Trachea grade II Photo 5: Trachea grade III
Photo 6 : Lung grade I Photo 7: Lung grade II Photo 8: Lung grade III

In spite of their simple appearance and their minimal genome, mycoplasmas put their genetic material to maximum use and they managed to be highly successful and sophisticated pathogens (Bradbury, 2005). A high positive percentage of MG infection by serology was recorded in the present investigation from age 35 weeks (88.9%).The MG sera positive cases reached to 100% at both of 40 and 55weeks of age, as shown in Table (1). Mycoplasms have the ability to resist the host immune defenses and selective antibiotic therapy, establishing chronic infection (Winner et al. 2000) and adsorb host protein on their surface so they can not be recognized for while as an antigen (Stipcovits, 2001). Therefore the immune response to mycoplasmas is delaye .A trial for detection of MG in tracheal sample was investigated by cultural method and PC .Mycoplasma was isolated from such broil breeder flock at the age of 49 weeks , (Table, 2

The isolate was characterized using cultural, biochemical, serological and PCR testes and proved to be MG strain (Table ,4and Photo, 1 and 2). Previously, Tully, (1983a) and Hirsh and Zee (1999), showed the sensitivity of MG to a paper disc saturated with 1.5% digitonin while Erno and Stipkovits (1973) maintained that MG has the ability to ferment glucose. Growth inhibition of isolated Mycoplasma colonies by the incorporation with specific MG hyper immune sera was carried out. Quinn et al. (2002) recommended the identification of Mycoplasma by the growth inhibition test. Similarly, we employed those tests to prove that the isolated Mycoplasma strains were MG. Also ,Dot ELISA was applied in the current study and proved its potentiality in rapid confirmation of *Mycoplasma* isolates. Although , Mycoplasma gallisepticum is difficult to be isolated by conventional methods yet , MG isolation may be obtained within 4-7days and up to 30 days may be required to conclude a negative results (Salisch et al., 1998). Trails for MG isolation in the present study were successful and proved the conditions used. Mycoplasma isolates required at least 3 to 7days before colonies appear and cultivation techniques are laborious, slow and expensive and require sterile conditions and personal skills (Hirsh and Zee, 1999). Histopathological examination of the trachea and lungs of infected broiler breeder flock revealed the destructive effect of MG. Table (5) illustrate these findings which in accordance with those observed by Tajima et al. (1982) and Stipkovits (2001). Application of a rapid diagnostic tool for detection and identification of the avian Mycoplasma by PCR in combination with cultural and serological tests is the final judgment of true infection by MG. The PCR technique employed in the present study has proven its specificity and sensitivity in amplification of low amount of nucleic acid to a level that can be easily detected. Nascimento et al. (1993) applied PCR technique to detect MG. PCR detects DNA of viable and non viable Mycoplasma, however only viable mycoplasmas should be considered as a potential source of infection (Marios et al., 2002). Electrophoresis of the PCR products of amplified 16SrRNA gene of MG from the tested sample revealed products with 186 bp in size, photo (2). Hartup and kollias (1999) proved that in the absence of positive culture, a positive PCR test only supports the presence of a non viable organism not active infection. MG infection can spread vertically, (transovarian transmission) from hens to progeny (Ley et al ., 1997, and Barbour et al ., 1998 and Bradbury, 2005). In a trial to investigate shedding of MG in four hatched chicks from the infected broiler breeder flock, the results revealed that the examined hatches were negative by PCR, culture and serology. Treatment with antibiotic, in parent flocks and the presence of immunosuppressive agents, results in very low level of antibodies in progeny (Stipkovits, 1994). The inability of cultural and PCR tests to detect Mycoplasma gallisepticum DNA in these hatches (Table 3) might be due to the presence of atypical MG

strain of low pathogencity. The incidence of transmission of MG to progeny may be in the low range between 0.5 to 5% at the late phase of infection (Stipkovits, 2001). Due to improving diagnostic techniques, the theory of host specificity may need to be reviewed. MG has been isolated from natural infection in a variety of bird species including ducks, geese, game birds, guinea fowl, pigeons and pheasants, (Bradbury,2005). The role played by wild birds or game birds in transmitting avian Mycoplasma had greater attention since MG caused conjunctivitis in Finches in the USA (Ley etal.,1996)and spread of infection into other north American birds (Luttrell et al., 2001 and Mikaelian et al., 2001). The observed number of rats in the MG infected broiler breeder farm directs our attention to the role of rats in transmitting MG. So trials for isolation and characterization of *Mycoplasma* from such rats were conducted and the isolate was proved to be a MG strain as shown in Table (4). Bradbury (2005) mentioned that the detection of the intermediate vectors between poultry flocks is now greatly aided by the availability of modern molecular methods of strain typing. A pathogenisty study was conducted using the two MG isolates (Broiler breeder and rat strains) in SPF chicks in order to illustrate the virulence of the two isolates on the trachea and the lung of infected SPF chicks. The study period was 15days and samples from trachea and lungs were taken for histopathological examination on the first, second, fourth, seventh, tenth and fifteenth days post inoculation. The results of this experiment proved that the MG isolated from rats was more virulent for the SPF chicks than those isolated from the broiler breeder in the trachea and the pathogenic effect was early and severe in the first week. The lesions were ameliorated by the tenth day, while the broiler breeder MG strain showed retarded or delayed pathogenic effect .In case of the lungs, the pathogenic effect of the two MG strains was the same in the inoculated SPF chicks. The pathogenicity of MG was mainly due to the terminal tip structure that mediates adherence to its target tissues (Tajima et al., 1979,1984) , over come the mucociliary clearances by the host and taking micro-nutrient in situ (Nunoy et al., 1987). Table (6), photos (3-8) illustrate the degrees of histopathology and the results of pathogenicity test.

In conclusion of the pathogincity test conducted in the study, the MG strains isolated from broiler breeders and rats were different in their pathogincity. This may reflect the variable behavior of MG in different species or due to the presence of a mutant or variant strain of MG with variable degree of pathogenicity in chickens. The filed observation that samples from seropositive flocks might ended by culture or PCR positive or negative may be explained by the presence of a variant strains of MG. Indeed, the delayed serology detection of MG in the flock under the study may be due to the low virulence circulating MG strains among the chickens. The current tested flock was serology negative until 30 weeks of a

(data not shown).These result are in agreement with Nolan et al. (2004) who mentioned that the proportion of infected finches dying as a result of infection with MG decreased and asymptomatic infection was more common among wild birds than the past .Also, they mentioned that MG infection of breeding adults occurred late in the breeding season, suggesting that higher rates of reproduction increase risk of infection with MG. During a survey of wild avian species have no clinical signs of mycoplasmosis, Farmer et al. (2005) observed that some species had positive agglutination reaction for antibodies to MG, and tested negative by polymerase chain reaction (PCR) while others were PCR-positive for MG but antibodies to MG were not detected, Also, the experimental infections of some species showed clinical disease after exposure to MG, while others were infected without clinical signs suggesting that, they might represent potential MG reservoir.

In conclusion, the study reports the isolation and characterization of MG from rats and chicken with different pathogenicity. Also, it reports the presence of MG in rats taken from chicken house may explain the persistence of MG in the chicken houses and the role of rats in transmission and virulence of MG field strains which reflects the importance for the strategy of a rodent control programme. Molecular typing of the isolated strains needs to be addressed.

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