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ISOLATION, CHARACTERISATION AND PATHOGENICITY OF MYCOPLASMA GALLINARUM IN VILLAGE CHICKEN

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By

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To Abah and Emak; Noraini and Ahmad Nizam

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LIST OF ABBREVIATIONS

CRD	Chronic respiratory disease
RM	Ringgit Malaysia
SPF	Specific pathogen free
G & C	Guanine and cytosine
MG	*Mycoplasma gallisepticum*
MS	*Mycoplasma synoviae*
SEM	Scanning electron microscopy
LM	Light microscope
TEM	Transmission electron microscope
ELISA	Enzyme-linked immunosorbent assay
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
PCR	Polymerase chain reaction
CFU	Colony forming unit

Abstract of dissertation submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of the Doctor of Philosophy.

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Chairman: **Professor Abdul Latif Ibrahim, Ph.D.**

Faculty: **Veterinary Medicine and Animal Science**

This study was conducted to determine the presence of mycoplasma infections in village chickens in Malaysia. This study comprised the isolation and characterisation of *Mycoplasma gallinarum* and its pathogenicity in village chickens.

The occurrence of *M. gallinarum* in fresh eggs, infertile and/or early dead germs, dead-in-shell and/or pipped embryos was investigated. For mycoplasma isolation, samples were taken from the vitelline membrane, yolk, and yolk sac. *M. gallinarum* was not present in all the eggs and embryos sampled. Day-old-village chicken chicks and adult village chickens were swabbed at the choanal cleft region, an alternative site for mycoplasma isolation. *M. gallinarum* was not present in the day-old-chicks. However, of the 550 adult birds sampled, 294 were carriers of mycoplasma. The isolation rate varied from 26.3 to 73 per cent.

The mycoplasma species isolated from village chicken was identified as *M. gallinarum* based on biochemical and serological tests. The indirect immunoperoxidase test was easy to perform and results easy to read.

The morphological studies of the *M. gallinarum* isolates were studied by scanning electron and transmission electron microscopy and the result showed that morphologically *M. gallinarum* from village chicken was similar to that of earlier reports.

M. gallinarum remains viable in drinking water for up to a 48 hr period when the inoculum size is 10^8 colony forming units but not at 10^2 colony forming units.

The effect of *M. gallinarum* isolated from village chicken on embryonated village chicken eggs was investigated. Seven-, ten-, twelve- and eighteen-day-old embryos were inoculated with medium containing 10^6 colony forming units of *M. gallinarum* or uninfected medium and incubated at 37°C until hatched. No significant difference was observed between the infected embryos which failed to hatch and those which hatched or between them and the group inoculated with medium only. A much higher percentage of the eggs inoculated as 18-day-old embryos hatched than those inoculated at seven, ten and twelve days intervals.

In another pathogenicity study, mixed infection with *M. gallinarum* and Newcastle disease virus was examined. Day-old village chicken chicks were

vaccinated intranasally with F strain of Newcastle disease virus and inoculated intratracheally with *M. gallinarum* simultaneously and changes on the tracheal epithelium were observed by scanning electron microscopy. At day 3 post-vaccination/infection, major alterations of the epithelial surface were visible and the epithelial surface were apparently normal by day 7.

This study has successfully achieved its objectives by isolating, characterising and conducting pathogenicity studies of *M. gallinarum* in village chicken. Pathogenicity studies show that *M. gallinarum* is mildly virulent and the overall impact of *M. gallinarum* infection in village chickens needs further investigation.

Abstrak dissertasi yang dikemukakan kepada Senat Universiti Pertanian Malaysia bagi memenuhi syarat untuk Ijazah Doktor Falsafah

PEMENCILAN, PENCIRIAN, DAN KEPATOGENAN *MYCOPLASMA GALLINARUM* DALAM AYAM KAMPUNG

Oleh

MOHD SHAH BIN ABDUL MAJID

Pengerusi: **Profesor Abdul Latif Ibrahim, Ph.D.**

Fakulti: **Kedoktoran Veterinar dan Sains Peternakan**

Kajian ini telah dijalankan untuk menentu adanya jangkitan mikoplasma dalam ayam kampung di Malaysia. Kajian ini terdiri daripada pemencilan dan pencirian *Mycoplasma gallinarum*.

Kewujudan spesies mikoplasma dalam telur segar, tak subur dan/atau germa mati awal, mati dalam cengkerang, dan/atau embrio tebuk telah diselidik. Untuk tujuan pemencilan mikoplasma, sampel telah diperolehi daripada membran vitelin, yolka, dan kantung yolka. Mikoplasma tidak terdapat dalam semua telur dan embrio yang disampel itu. Ayam sehari dan ayam kampung dewasa telah diswab pada kawasan rekah koana, iaitu suatu tapak silih untuk pemencilan mikoplasma. Mikoplasma tidak wujud dalam ayam sehari. Bagaimanapun, daripada 550 ekor ayam dewasa yang disampel, 294 merupakan pembawa kepada mikoplasma. Kadar pemencilan ini berbeza daripada 26.3 hingga 73 peratus.

Spesies mikoplasma yang dipencil daripada ayam kampung telah dikenal pasti *M. gallinarum* berasaskan ujian biokimia dan serologi. Ujian imunoperoksidase tak langsung mudah dilakukan dan hasilnya juga mudah dibaca.

Kajian morfologi terhadap isolat *M. gallinarum* dilakukan melalui mikroskopi elektron pengimbas dan pemancar dan hasilnya menunjukkan yang *M. gallinarum* daripada ayam kampung ini serupa dengan yang telah dilaporkan sebelum ini.

M. gallinarum kekal hidup dalam air minum sehingga 48 jam apabila mengguna inokulum sebanyak 10^8 unit pembentuk koloni dan tidak dapat hidup pada 10^2 unit pembentuk koloni.

Kesan *M. gallinarum* yang dipencil daripada ayam kampung terhadap telur ayam kampung berembrio telah diselidik. Embrio tujuh-, sepuluh-, dua belas-, dan lapanbelas hari telah diinokulat dengan medium mengandung 10^6 unit pembentuk koloni *M. gallinarum* atau dengan medium tak terjangkit dan diramkan pada 37°C sehingga menetas. Tiada perbezaan bererti yang dicerap di antara embrio terjangkit yang gagal menetas dengan yang menetas atau di antara kedua-dua kumpulan ini dengan kumpulan yang diinokulat dengan medium semata-mata. Peratusan telur yang terinokulat sebagai embrio 18-hari adalah lebih tinggi daripada telur yang diinokulat pada tujuh, sepuluh, dan dua belas hari.

Dalam satu lagi kajian kepatogenan, jangkitan campuran *M. gallinarum* dan penyakit Newcastle telah dilakukan. Anak ayam kampung sehari telah divaksinkan secara intranasum dengan virus penyakit Newcastle strain F dan serentak diinokulatkan juga secara intratrakea dengan *M. gallinarum* dan perubahan pada epithelium trakea dicerap melalui mikroskopi elektron pengimbas. Pada hari ketiga pascapemvaksinan dan pascajangkitan, perubahan besar dapat dilihat pada permukaan epithelium dan permukaan epithelium ini pula nampaknya normal apabila sampai hari ketujuh.

Kajian ini telah mencapai objektifnya dalam pemencilan, pencirian, dan pengendalian kajian kepatogenan *M. gallinarum* dalam ayam kampung. Kajian kepatogenan menunjukkan *M. gallinarum* virulen sederhana dan kesan keseluruhan infeksi dalam ayam kampung pula masih perlu lebih banyak penyelidikan.

CHAPTER 1

INTRODUCTION

In recent years, there has been a rapid expansion in the list of diseases in animals, birds, insects and plants attributable to mycoplasmas. Mycoplasmas are rather inconspicuous predators. They often coexist with their host in a truce that is occasionally broken (Hayflick, 1972). Their ubiquity and subtle pathogenicity have often concealed their aetiological significance.

The study of avian mycoplasmal diseases began in 1952 with the association of mycoplasmas with infectious sinusitis of turkey and chronic respiratory disease of chicken. Since then numerous studies have been conducted and it is now generally accepted that infection with pathogenic avian mycoplasmas is essential for the occurrence of mycoplasmosis.

Both pathogenic and non-pathogenic mycoplasma are found in avian tissues. Sixteen species of the genus mycoplasma that have been isolated from chickens, turkeys, ducks and pigeons. They are *Mycoplasma gallisepticum* (*M. gallisepticum*), *M. gallinarum*, *M. pullorum*, *M. gallinaceum*, *M.iners*, *M. gallopavonis*, *M. meleagridis*, *M. iowae*, *M. synoviae*, *M. anatis*, *M. columbinum*, *M. columbinasale* *M. columborale*, *M. lipofaciens*, *M. glycophilum* and *M. cloacale*. The more important poultry pathogens are *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, *M. iowae* and occasionally *M. gallinarum*.

M. gallinarum is considered a non-pathogen (Chu, 1954; Adler *et al.*, 1957). In a comparison of the virulence of several strains of ***M. gallisepticum*** and a strain of ***M. gallinarum*** by the inoculation of chick embryos, chicks and poults, it was found that the mortality of embryos inoculated with ***M. gallinarum*** was intermediate between the mortality of embryos inoculated with a mild strain of ***M. gallisepticum*** and that of the broth controls (Power and Jordan, 1973, 1976). In a field investigation of a respiratory disease problems in North Georgia, USA, a high percentage of mycoplasma cultures from tracheas and air sac lesions yielded a pure culture of ***M. gallinarum*** (Kleven *et al.*, 1978). The consistent isolation of *M. gallinarum* from these organs suggest that this organism may have pathogenic potential. Subsequently, Kleven *et al.* (1978) reported that *M. gallinarum* when introduced by aerosol or by air sac inoculation produced air sac lesions in young chicken particularly with a vaccine combining Newcastle disease and infectious bronchitis or a field strain of infectious bronchitis virus.

The distribution of avian mycoplasmas seems to be worldwide (Jordan 1983; Bencina et al., 1987; Poveda et al., 1990). In Malaysia, mycoplasma was first isolated from a case of avian coryza in 1960 by Thuraisingham (1963). The Veterinary Research Institute, Ipoh, has reported the isolation of ***M. gallisepticum, M. gallinarum*** and ***M. gallinaceum*** from commercial chickens (Joseph et al., 1988). *M. gallisepticum* was isolated from clinical cases of chronic respiratory disease in these chickens. A poultry report on the investigation of the disease by the Selangor Regional Diagnostic Laboratory and Bukit Tengah Diagnostic Laboratory indicated the presence of *M. gallisepticum* and *M. synoviae* in chickens. Of the 7471 birds tested for *M. gallisepticum* by the rapid serum agglutination test, 32.8 per cent were positive and out of 2217

chicken tested for *M. synoviae* antibodies, 21.3 per cent were positive (Opitz, 1978). However, the report concluded that the causative agent could not be definitely identified and in some cases mycoplasmas were not isolated. The presence of mycoplasmas and mycoplasma infections in village chickens has therefore not been established.

Indigenous chickens reared by the rural farmers and suburban population are often referred to as village chickens (Spradbrow, 1993/94). In Peninsular Malaysia, three-quarters of a million rural families are estimated to keep village chickens in their backyard (Ramlah and Shukor, 1987). The estimated number of village chickens in 1985 was about 6.5 million birds which was 13 per cent of the total chicken population (Supramaniam, 1987). These chickens are usually freely ranging without proper management and health program and seem susceptible to all infectious diseases that afflict commercial chickens. However, little information is available on the overall incidence of disease among free-range village chickens in Malaysia.

Embryonated eggs used in the study of virulence of avian mycoplasmas have been either from specific pathogen free (SPF) flocks or commercial flocks maintained in isolation. The genetic constitution of the embryo has been found to influence its susceptibility to Newcastle disease virus (Reta *et al.*, 1963), infectious bronchitis virus (Purchase *et al.*, 1966) and strains of *M. gallisepticum* and *M. gallinarum* (Power and Jordan, 1973). However, the effect of mycoplasmas on embryonated village chicken eggs have not been studied.

Only an estimated 20 per cent of the existing population of village chicken have been vaccinated with "F" vaccine of Newcastle disease virus (Supramaniam, 1987). Ibrahim *et al.* (1986) recommended day-old commercial chicks to be vaccinated intranasally with F strain vaccine. A similar vaccination regime is suggested for village chickens although this is difficult to conduct as they are maintained under a free-range system.

Prevalence studies show that *M. gallinarum* is present in the village chickens. The effects of mixed infections with *M. gallinarum* and the F strain of Newcastle disease virus have not been studied.

The objectives of this study were:

1. to isolate *M. gallinarum* from eggs, embryos and the choanal cleft of village chickens.
2. to characterise *M. gallinarum* from village chickens.
3. to study the effect of *M. gallinarum* on embryonated village chicken eggs.
4. to determine the effects of mixed infections of *M. gallinarum* and lentogenic F strain Newcastle disease virus in village chickens.

CHAPTER 2

LITERATURE REVIEW

Avian Mycoplasmas

History

Mycoplasmas have been known to cause diseases in poultry for a long time. Avian mycoplasmas were first isolated by Nelson in 1936 from nasal exudates of chickens with coryza (Fabricant, 1969). The coccobacillary bodies described by Nelson are now generally thought to have been *M. gallisepticum* (Dierks *et al.*, 1967; Fabricant, 1969). In 1939, Nelson was able to grow the organisms in cell-free media which on the basis of the description were more likely mycoplasmas (Fabricant, 1969). The first clear description of a mycoplasmal disease in chickens called chronic respiratory disease was given by Delaplane and Stuart in 1943 (Adler, 1970).

Most workers referred to the agents of chronic respiratory disease as a virus, a rickettsia or an agent resembling the chlamydiaceae group of organisms until Markham and Wong (1952) successfully isolated the agents associated with chronic respiratory disease in Edward's medium (Jordan, 1979). Markham and Wong (1952) noticed that the isolates were morphologically similar to organisms of the pleuropneumonia group, which are now known as mycoplasma.