ISOLATION AND CHARACTERIZATION OF AVIAN MYCOPLASMAS IN ZARIA

BY

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THESIS APPROVAL

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DECLARATION

I hereby declare that the work in this thesis was carried out by me in the Mycoplasmology Laboratory of the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, under the supervision of Prof. D. S. Adegboye and Prof. S. O. Emejuaiwe.

No part of this thesis has been presented in any previous application for a higher degree. All sources of information are specifically acknowledged by means of references.



John [illegible]aka MOLOKWU

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ABSTRACT

Swabs were taken at post mortem from the nasal sinus, trachea, lung and airsac of 90 chickens which died after showing clinical signs of respiratory illness. The chickens were purchased from 7 different flocks in Zaria and environ. Swabs were taken from 30 clinically healthy chickens as control, to make up a total of 480 swabs from respiratory tissues. The swabs were cultured in standard mycoplasma medium, routinely supplemented with nicotinamide adenine dinucleotide (NAD), a specific nutrient requirement for Mycoplasma synoviae. Any mycoplasma-like organism encountered was characterized biochemically and serologically by standard techniques. Swabs of typical lesions at any of the respiratory sites were also cultured routinely for bacteria. Finally serum samples obtained from the birds were screened with the rapid plate agglutination test (RPAT) for M. gallisepticum and M. synoviae antibodies.

The isolation and characterization studies yielded M. gallisepticum (11.1%), M. synoviae (2.2%), M. gallinarum (16.7%), M. gallopavonis (10%), M. columbinum (1.1%) M. columborale (2.2%) and A. laidlawii (6.7%) from the clinically

ill birds, and M. gallinarum (3.3%), M. gallinaceum (3.3%) and M. columborale (3.3%) from the apparently healthy birds.

Prolonged incubation increased M. gallisepticum isolation rate. The study thus provides additional report of isolation of M. gallisepticum in Nigeria (in addition to a previous single report), and documents for the first time the isolation of M. synoviae, M. gallinaceum, M. columborale, M. columbinum and A. laidlawii from poultry in Nigeria.

Bacteria cultured were, Staphylococcus sp. (65.5% of the cases) E. coli (40%), Streptoccus sp. (32.2%), Proteus sp. (25.6%), Bacillus sp. (12.2%), (12.2%), Pasteurella sp. (10%), Pseudomonas sp. (6.7%), Haemophillus gallinarum (1.1%) from clinically ill birds and Staphylococcus sp. (40%), and E. coli (10%) from the apparently healthy birds.

While isolation rates for M. gallisepticum and M. synoviae were 11.1% and 2.2%, respectively among diseased birds, the seropositive rate was 50.0% for M. gallisepticum and 5.6% for M. synoviae. This suggests that the rate of isolation does not correlate with seropositivity. Continued efforts

should be made to improve the quality of media being used for isolating avian mycoplasma pathogens of the respiratory tract.

TABLE OF CONTENTS

		PAGE
TITLE PAGE		i
THESIS APPROVAL		ii
DECLARATION		iii
ACKNOWLEDGEMENT		iv
ABSTRACT		vi
TABLE OF CONTENTS		ix
LIST OF TABLES		xii
LIST OF PLATES		xiii
LIST OF FIGURES		xiv
CHAPTER 1. GENERAL INTRODUCTION		1
1.1	THE MYCOPLASMAS	
1.2	CLASSIFICATION	2
1.3	MORPHOLOGY, STRUCTURE AND FUNCTION	4
1.3.1.	Colonial Morphology ..	4
1.3.2	Cellular structure	5
1.3.3	Ultrastructure and related functions	6
1.3.4	Replication	9
1.3.5	Pathogenicity	8
1.4	OBJECTIVES OF PRESENT WORK	10
CHAPTER 2.	AVIAN MYCOPLASMAS: A REVIEW OF LITERATURE	
2.1	AVIAN MYCOPLASMA SPECIES	
2.1.1	Mycoplasma gallisepticum	12
2.1.2	Mycoplasma synoviae ..	15
2.1.3	Mycoplasma meleagridis ..	17
2.1.4	Mycoplasma gallinarum ..	20

		PAGE
2.1.5	Mycoplasma iowae	22
2.1.6	Other avian Mycoplasma spp.	24
2.2.	RECOVERY OF AVIAN MYCOPLASMAS	25
2.2.1	Media	26
2.2.2	Specimen collection	29
2.2.3	Cultural Techniques	29
2.3	IDENTIFICATION OF ISOLATES	32
2.3.1	Purification	32
2.3.2	Testing for Sterol Requirement	37
2.3.3	Other Biochemical Characterizations	39
2.3.4	Serological characterization	41
CHAPTER 3.	ISOLATION AND CHARACTERIZATION STUDIES	
3.1	INTRODUCTION	48
3.2	MATERIALS AND METHODS	49
3.2.1	Culture Media	49
3.2.2.	Preparation of Media	52
3.2.3	Media for glucose or arginine requirement test	54
3.2.4	Specimen	55
3.2.5	Cultural Procedure	56
3.2.6	Characterization	59
	Purification	60
	Reversion Studies	60
	Biochemical Tests	60
	Serological Tests	63
3.1	RESULTS	66
3.3.1	Growth and Trial Identification of Reference strains ..	66
3.3.2	Isolation Trials	67
3.3.3	Biochemical characterization	68

		PAGE
3.3.4	Serological Identification	69
3.4	DISCUSSION	83
3.5	SUMMARY	89
CHAPTER 4.	SCREENING OF CHICKENS WITH RESPIRATORY PROBLEMS FOR ANTIBODIES TO MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE	
3.1	INTRODUCTION	91
4.2	MATERIALS AND METHODS ..	91
4.2.1	Serum Samples	91
4.2.2	Serological test	92
4.3	RESULTS	93
4.4	DISCUSSION	93
CHAPTER 5.	BACTERIAL AGENTS (OTHER THAN MYCOPLASMA) INFECTING THE RESPIRATORY SYSTEM OF CHICKENS	
5.1	INTRODUCTION	97
5.2	MATERIALS AND METHODS ..	98
5.3	RESULTS	99
5.4	DISCUSSION	101
CHAPTER 6.	GENERAL DISCUSSION	104
	REFERENCES	129
	APPENDICES	134

LIST OF TABLES

Table		Page
2.1	Biochemical characteristics of some known Avian *Mycoplasma* species ..	43
3.1	Rate of isolation of Mycoplasma-like organisms from the respiratory tissues of clinical ill (CRD) and apparently healthy chickens	72
3.2	Biochemical characterization of isolates (arranged in groups of identical reactions)	73
3.3	Summary of biochemical and serological tests on isolates	75
3.4	Mycoplasma species yield per respiratory site	75
3.5	The frequency of recovery of *Mycoplasma* and *Acholeplasma* species from pathological lesions (Respiratory) and from cases with no gross lesion	76
4.1	Relation between Mycoplasma isolation rate and seropositivity for *M. gallisepticum* and *M. synoviae*	95
5.1	Common bacterial isolates from respiratory tract of chicken with CRD signs and apparently healthy ones	100

LIST OF PLATES

Plates		Page
3.1 (A) & (B)	Colonies of Mycoplasma Isolates from chicken identified serologically as M. gallisepticum	77
3.2	Colonies of isolate from chicken identified serologically as M. synoviae	78
3.3	Colonies of Mycoplasma isolate from chicken identified as M. columborale ..	78
3.4	Colonies of isolate from chicken identified as A. laidlawii	79
3.5	Colonies of Mycoplasma isolate from chicken identified as M. gallinaceum	79
3.6	Colonies of Mycoplasma from chicken identified as M. gallinarum. Film and spots formation is illustrated ..	80
3.7	Colonies of isolate from chicken identified as A. laidlawii showing colony growth close to digitonin paper disc.	80
3.8	Specific immunofluorescence of A. laidlawii colonies on agar piece stained with homologous fluorescent antibody	81
3.9	Specific fluorescence of M. gallisepticum colonies on agar piece stained with homologous fuorescent antibody	81
3.10	Fluorescence of M. synoviae colonies on agar piece stained with homologous fluorescent antibody	82

LIST OF FIGURES

Figure		Page
3.1	Pattern used for Mycoplasma agar plate inoculation	58
3.2	GI Test for Mycoplasma culture showing arrangement of antisera disc on inoculum	64

Chapter I

GENERAL INTRODUCTION

1.1 THE MYCOPLASMA

Mycoplasmas are the smallest organisms known to be capable of growth in cell free media (Anon, 1974). Similar to the bacteria, they have triple-layer limiting membrane, typical prokaryotic circular chromosomes which is folded, seventy-s- ribosomes and ferment glucose. Unlike the bacteria, they are devoid of muramic acid cell wall.

The mycoplasma was first recognized by Nocard and Roux (1898) from cases of Bovine Contagious Pleuropneumonia and was called the pleuropneumonia organism (PPO). Later similar organisms isolated and described were called pleuropneumonia - like organisms (Adegboye, 1981).

The taxonomic status of the pleuropneumonia-like organism remained confused for many years. Despite the increasing number of new strains isolated, there was no generally accepted nomenclature or classification.

Nowak (1929) suggested the name Mycoplasma as the first legitimate generic name for the organism. This name was finally adopted around 1956 (Edward and Freundt, 1956; Freundt, 1957).

Mycoplasmas have subsequently been isolated from man, small ruminants, rodents, canine, avian, etc. A new group of filtrable organism was isolated from sewage and named Mycoplasma laidlawii (Laidlaw and Elford, 1936). This group was later found to belong to the genus Acholeplasma and was renamed Acholeplasma laidlawii (Edward and Freundt, 1969; Edward and Freundt, 1970).

1.2 CLASSIFICATION

The disagreement regarding the relationship of the pleuropneumonia-like organism to the L-phase variants of bacteria was for many years the major obstacle to the definition and classification of the group. In 1967, the "mycoplasmas" a nomenclature proposed by Edward and Freundt (1956) was placed in a separate Class, the Mollicutes (Latin: soft and pliable skin) (Edward and Freundt, 1967). Based on their morphology, filtrability through 450nm Millipore membrane filters and absence of reversion to a bacterium in medium without penicillin and thallium acetate, the mycoplasma was placed in the Order Mycoplasmatales. Subdivision into the three major families - Mycoplasmatacea, Acholeplasmatacea, and Spiroplasmatacea - was based on nutritional and

morphological criteria and on differences in genome size (Freundt, 1983a). Cholesterol requirement (Edward, 1971), and sensitivity to digitonin and sodium polyethanol sulphate (Erno and Stipkovits, 1973) were used to separate the taxonomic description between the Acholeplasmatacea (non-sterol requiring) and other mycoplasmas that require sterol for growth.

The ability to hydrolyze urea is one major property distinguishing the genus Ureaplasma from other non urea utilizers, Mycoplasma and Acholeplasma, (Erno and Stipkovits, 1973; Shepard et al. 1974). Other biochemical and metabolic characteristics such as breakdown of carbohydrate substrate (Erno and Stipkovits, 1973), serum digestion and phosphatase production (Alluotto et al. 1970), film and spot formation (Edward, 1950; Rottem and Razin, 1964) aided speciation of the genera.

A spiral shaped and motile mycoplasma-like agent was isolated from "corn stunt" disease by Davis and Worley (1973) and named Spiroplasma. The current accepted taxonomic strata of the class Mollicutes is as follows, according to Freundt, (1983a):

Class: Mollicutes

Order: Mycoplasmatales

Family I: Mycoplasmatacea Genus 1 Mycoplasma

2 Ureaplasma

Family II: Acholeplasmataceae Genus 1. Acholeplasma
Family III: Spiroplasmatacea Genus 1. Spiroplasma
Genera of uncertain taxonomic position: Thermoplasma
: Anaeroplasma

1.3 MORPHOLOGY, STRUCTURE AND FUNCTION

1.3.1 Colonial Morphology

On agar medium mycoplasma colonies are usually (50-500nm in diameter) and show typical fried-egg shape. On first isolation many of them have aberrant colonial morphology (Hayflick, 1969; Smith, 1971; Stalheim, 1979). Corynebacterium isolated from Drosophilia paulistorum and the Treponomes, Leptospires grown on PPLO - agar produce this fried-egg appearance. This special feature was attributed to the organisms small size, lack of rigid cell wall, and osmotic movement into the medium (Ehrman and Maniloff, 1971, unpublished findings, Hardy et al. 1963). Bacterial L-phase variant (L-forms) share this fried-egg colony shape (Razin and Oliver, 1961).

Colonial appearance is dependent on growth conditions, age of culture, and agar concentration (Razin, 1983).

Majority of the mycoplasma colonies are rounded with a denser centre (nipple) and lighter periphery. Two or more colonies growing close together form a confluent growth (Yamamoto and Adler, 1958b; Smith, 1971; Tully, 1978). On nutritionally poor media or with inadequate pH or atmospheric conditions or when the medium surface is too dry, the initial central "down growth" may form without the accompaning peripheral surface growth (Razin and Oliver, 1961; Smith, 1971). Initial passages of some mycoplasma colonies lack the typical denser centre, e.g. M. gallisepticum, M. pneumoniae and M. hyopneumoniae, (Rosenfield, 1971). Also overcrowding brings about smaller colonies (Hayflick, 1969).

1.3.2 Cellular structure

Devoid of a rigid cell wall and a nuclear membrane, the mycoplasmas possess a remarkable pleomorphism. Viewed under a light microscope, they appear as granules, filaments, balloon, disc, ring and club (Adegboye, 1981). Minute forms (125-250nm) are capable of passing bacterial filters and can revert to larger forms. This phenomenon was one reason for the early classification of mycoplasmas as viruses (Adegboye, 1981).

1.3.3 Ultrastructure and related functions

The cell membrane bounds the dispersed cytoplasmic elements which are the chromosomes, ribosomes, macromolecular RNA, plasmids and cytoplasmic granules. The cell membrane has a triple layered structure composed of lipids, protein and carbohydrate residue. Beside providing the enclosure for the above mentioned structures, the cell membrane is the major immunogenic determinant of the mycoplasmas particularly the membrane protein (Razin et al. 1972). Thus the use of the membrane as vaccine is feasible and has been suggested (Razin et al. 1972). The resistance to chloramphenicol, dihydrostreptomycin and tetracycline was due to reduced permeability to the antibiotic rather than ribosomal alteration (Fraterrigo et al. 1971). Furthermore Maniloff (1971) noted that the increase antibiotic resistance of the mycoplasma was due to very close association between mycoplasma and the infected animal cell, accounted for by an inter-cellular membranes interaction.

The cytoplasm occupies most of the interior of the cell. The denser region at the periphery is made up of ribosomes. In M. mycoides members there are striated fibres called "Rho-forms" seen across the cytoplasm. Several avian Mycoplasma sp. have specialized

terminal protrusions termed "blebs" on one or both ends of the organism (Green and Hanson, 1973; Levisohn and Razin, 1973; Tajima et al. 1979). The bleb may be the site of increased protein synthesis (Green and Hanson, 1973). Unlike those of M. meleagridis, the blebs of M. gallisepticum have been found to be devoid of nucleic acid (Green and Hanson, 1973; Ghosh, Das and Maniloff, 1977) but rather provide the site of attachment of the M. gallisepticum organism to the host cells (Zucker - Franklin, Davidson and Thomas, 1966). Blebs generally are formed when the mycoplasma organism is in close contact with the host cells (Abu-Zahr and Butter, 1978; Tajima et al. 1979).

The genetic information is contained in the single chromosomes which is a circular DNA molecule. The DNA makes up (4-7%) dry weight of the cell. The RNA makes up (8-17%) of the cells mass and it is composed of 22S, 16S, 5S (rRNA), 4S (tRNA) and an unstable mRNA species. The ribosomes are seen as cytoplasmic granules in electron micrographs and they function in protein synthesis. The nucleic acid of M. gallisepticum is made up of 80% RNA and 20% DNA (Kirk and Morowitz, 1969). The plasmids are extra-nuclear genetic materials indicated by the existence of mycoplasma virus.

1.3.4 Replication

Mycoplasmas were reported to be composed of minimal reproductive units or elementary bodies, varying in size from 125 to 250nm (Hayflick, 1969). Several mechanisms of reproduction have been suggested. Freundt (1969) described one mode of reproduction in which there is filament formation followed by beading and disintegration into coccoidal elements. This is the typical mode of reproduction of M. mycoides group. Other species too adopt this method but gives rise to extrusions which resemble buds instead of long filaments. Filament formation have also been observed in A. laidlawii, M. hominis, M. fermentans and M. orale.

Binary fission has been observed in M. gallisepticum, M. arthritidis and M. pneumoniae (Bredt, 1968).

1.3.5 Pathogenicity

The pathogenicity and virulence of some mycoplasmas have been traced to many factors. Sobeslavsky et al. (1968) showed that the close attachment between M. pneumoniae and the neuraminic acid receptor sites on erythrocyte and tracheal epithelium leads to destruction of cell membrane and subsequent atypical

pneumonia. This is achieved without them being attacked by the catalase present in the extracellular fluid. Recent studies also showed that M. gallisepticum attach tightly to RBC leading to their distortion in shape. Moreover, the presence of M. gallisepticum in respiratory epithelium may lead to ciliostasis or loss of cilia as well as damage to cell membrane (Power and Jordan, 1976; Jordan, 1975; Razin et al. 1980). Thomas and Bitensky (1966) observed that the neurotoxic strain of M. gallisepticum cause ataxia, paralysis, convulsion and death within one hour in turkey poults.

The chronic respiratory disease (CRD) of chickens is typically a disease with long incubation period (1-3 weeks) and a long course caused primarily by M. gallisepticum. The disease is characterized clinically by sneezing, nasal discharges, sporadic coughing and respiratory rales (Fabricant, 1969; Rosenfield, 1971). MacOwen, Randall, Jones and Brand (1982) reported association of M. synoviae with respiratory disease of poultry. Etukudo (1982) published as Etukudo and Adegboye (1983a) could only isolate M. gallisepticum in two cases; they were not able to include Diphosphopyridine nucleotide (DPN) required by M. synoviae routinely. Clinical and postmortem observations indicate that CRD is common

among poultry in Nigeria (Adegboye, 1983). It is therefore pertinent to show by isolation work if M. synoviae occurs in CRD cases in Nigeria, and also if there could be an improvement on M. gallisepticum isolation. Recently new avian Mycoplasma species were identified (Adegboye, 1985). Therefore there is the need to fully characterize any isolate to see if newly reported species do occur in Nigeria.

1.4 OBJECTIVES OF PRESENT WORK

1. To use complete media formulation routinely in isolation studies, i.e. including DPN, so that M. synoviae which has been shown to be able to cause CRD could also be detected, if present, in addition to M. gallisepticum.

2. To confirm the single previous report of isolation of M. gallisepticum in Nigeria and improve the techniques of M. gallisepticum isolation, if possible.

3. To perform complete biochemical and serological characterization of any avian mycoplasma isolate with the hope of identifying some recently reported new species if such species occur in Nigeria.

Chapter II

AVIAN MYCOPLASMA: A REVIEW OF LITERATURE

2.1 AVIAN MYCOPLASMA SPECIES

The first isolation of an avian mycoplasma was by Nelson (1936) in the course of his studies on coryza of slow onset and long duration. Van Herick and Eaton (1945), demonstrated PPLO in chick embryo. Markham and Wong (1952) finally offered a proof that the agent of CRD in chickens was also present in infectious sinusitis of turkeys and that both were members of the PPLO. The organism was subsequently classified as Mycoplasma and named Mycoplasma gallisepticum (Edward and Kanarek, 1960). Many authors have since then detected several mycoplasmas in poultry.

To date three species are established pathogens, viz: M. gallisepticum, M. synoviae, and M. meleagridis. Other species that have been isolated are M. gallinarum, M. iners, M. anatis, M. iowae, M. lipofaciens, M. gallinaceum, M. gallopavonis, M. pullorum, M. columbinum, M. columborale, M. columbinasale, A. laidlawii and A. axanthum, (Adegboye, 1985).

2.1.1 Mycoplasma gallisepticum

For the pathogenic avian mycoplasma isolated Edward and Kanarek (1960) suggested the species name of M. gallisepticum (the serotype I by Yamamoto and Adler, 1958a, b, or Serotype A by Kleckner, 1960).

Recoveries of M. gallisepticum was possible from the trachea much earlier than from the air sac or lungs (Olson et al. 1962; Rodriguez and Kleven, 1980a). Several authors reported that the isolation of this organism, following exposure decreases with time until it becomes impossible to culture the organism from any part of the respiratory tract inspite of the positive serological evidence (Olson et al. 1962; Roberts, 1964b, Roberts et al. 1967; McMartin, 1967; Jordan and Kulasegaram, 1968). Similar difficulty in the isolation of M. gallisepticum has been reported from old birds which have been infected at an early age and are serologically positive (Roberts, 1964b; Roberts et al. 1967; Yoder, 1978). This phenomenon has been described as "Latent Infection" (McMartin, 1967; Roberts et al. 1967, Jordan and Kulasegarum, 1968; Sahu and Olson, 1976a). Roberts et al. (1967) reported that the clinical infection appears when the M. gallisepticum organism become extracellularly located and the latent infection ensues when they

take up the intracellular position and remain quiescent in a state of equilibrium with the host tissues. Following interruption of this equilibrium by some stress factors such as infectious bronchitis vaccination (Hungerford, 1969; Jordan and Kulasegaram, 1968) the latent M. gallisepticum organisms multiply, take up the extracellular position and the clinical disease is triggered off.

M. gallisepticum is a cause of a disease condition commonly designated as Chronic Respiratory Disease (CRD) of chickens and infectious sinusitis and occasionally encephalitis in turkeys (Fabricant, 1960; Jordan, 1975; Yoder, 1978, 1979).

M. gallisepticum alone produces a relatively mild air sacculitis which characteristically runs a chronic course (Fabricant, 1969, Yoder, 1978).

Experimental infection of the respiratory tract may result in neither clinical sign nor gross lesion (Dierks et al. 1967; Yoder, 1978), disease being restricted or generalised (McMartin, 1967; Grimes and Rosenfield, 1972) within the respiratory system, or haematogenous spread to many organs and tissues (Peters, Faber et al. 1966; Olson and Kerr, 1967). Even in natural infection, the disease may be subclinical and inapparent with no obvious effects on performance or production (Truscott et al. 1974).

Various strains of M. gallisepticum have been reported. Among these, strains R, S-6, PG31, 1150 were used as challenge strains by Hildebrand, Page and Berg (1983) in evaluating the safety and efficacy of an inactivated MG bacterin. They reported that MG Bacterin was capable of protecting chickens and turkeys of various ages from clinical signs of M. gallisepticum caused by intrasinus challenge with the above strains of M. gallisepticum.

Tropism for certain organs and tissues is exhibited by some strains of M. gallisepticum. The S6 strain shows affinity not only for respiratory tissues, but also for the brain of chicken following infection by intracerebral, intrathoracic and intraperitoneal routes (Yamamoto and Adler, 1958a). For chicken and turkey only S6 strain of MG was shown to cause nervous signs and lesion (Varley and Jordan, 1978a, 1978b). This strain also shows tropism for arteries in the brain following intravenous inoculation of the organism (Clyde and Thomas, 1973.

M. gallisepticum has also been isolated from various other avian species. It was isolated from chukarpatridges (Wichman, 1957), peafowl (Willis, 1955) pheasant (Osborn and Pomeroy, 1957) and from guinea fowl (Von Rockel, 1953). Recently M. gallisepticum was isolated from sinus aspirates

of 2 wild-type turkeys and 1 domestic turkey living in close contact on a farm in Tehama county, California (Jessup et al. 1983).

The first report of isolation of M. gallisepticum from Nigeria was by Etukudo and Adegboye (1983a) who out of 59 Mycoplasma isolates from a total of 580 CRD cases, positively identified only two (3.4%) as M. gallisepticum. As for serological evidence of infection, Onunkwo and Onoviran (1978) recorded a seropositive rate of 69.0% for MG, among 1,604 chicken slaughtered at a market in Jos, Plateau State of Nigeria, Etukudo and Adegboye (1983a) reported a seropositive rate of 71.3% for MG in Zaria Area and Abdu et al. (1983) reported an overall prevalence rate of 47.5% for MG also in Zaria Area, Kaduna State.

2.1.2 M. synoviae

The serologically distinct mycoplasma associated with infectious synovitis of poultry (Chalquest and Fabricant, 1960) was named M. synoviae (Olson et al. 1964a). The type strain is WVU 1853. Other known strains are H (Kume et al. 1976), and F2-CAS (Yoder, 1971).

M. synoviae is a more difficult mycoplasma to grow in vitro, requiring 10-15% heat inactivated swine serum together with the addition of 0.1g per litre of diphosphopyridine nucleotide (DPN) (or nicotinamide adenine dinucleotide-NAD) and 0.1g per litre of L-cysteine hydrochloride (Frey et al. 1968).

M. synoviae is primarily associated with synovitis mainly of the joints and foot pads of birds (Chalquest and Fabricant, 1960; Jordan, 1975). The association of M. synoviae with broiler respiratory diseases was first described in the U.S.A. by Kleven et al. (1971) and Vardaman et al. (1972). The air sacculitis produced is accompanied by pathological changes in the respiratory tracts of chickens and turkeys (Ghazikhanian, Yamamoto, Cordy, 1973; Jordan, 1975; Yoder, 1971, 1979; Hirano et al. 1978). The respiratory phase of M. synoviae was later confirmed by MacOwan et al. (1982). Bacteraemic phase (Olson and Kerr, 1967; MacOwan et al. 1982) is common sometimes without the swelling of the joints (O'Brien, 1972; Olson, 1978). M. synoviae airsacculitis is enhanced in broilers administered with live-virus vaccines of Newcastle disease (ND) and infectious bronchitis (IB) (Kerr and Olson, 1970; Kleven et al. 1972; King et al. 1973; Vardaman et al. 1973; Kume et al 1976). Springer et al. (1974) also reported a

synergism between M. synoviae, infectious bronchitis (IB) and ND viruses as well as with Escherichia coli. This disease constitute the major cause of condemnation for unwholesomeness (O'Brien, 1972).

M. synoviae organisms are more readily recovered from the respiratory tract than from swollen leg joints (Sato and Nomomura, 1973) and from the trachea than from the airsacs (Olson et al. 1964b; Kume, Hayatsu and Yoskioka, 1976). M. synoviae has been recovered from cloaca and yolksac of turkey poult (Jordan and Amin, 1980). Though the natural hosts of M. synoviae are chickens and turkeys, the organism has been isolated from a natural outbreak of synovitis in guinea fowl (Pascucci et al. 1976). The susceptability of 24 - and 180 day-old White Pekin ducks experimentally exposed to M. synoviae has been reported (Yamada and Matsuo, 1983).

To the best of our knowledge, there is no report of isolation from Nigeria, but a few workers have presented serological evidence of occurence (Onunkwo and Onoviran, 1978; Abdu et al. 1983).

2.1.3 M. meleagridis

It was Adler et al. (1958) who first established the association of a mycoplasma other than

M. gallisepticum with infections in turkeys. Later the mycoplasma was named M. meleagridis and shown to be capable of causing airsacculitis in turkeys (Yamamoto et al. 1965; Yamamoto and Ortmayer, 1966).

M. meleagridis colonies appear on Mycoplasma agar media after approximately 72 hours of incubation at 37°C under 5% C02. The colonies on initial isolation do not show characteristic fried-egg appearance (Mohan, Adegboye and Orajaka, 1979).

M. meleagridis is the cause of airsacculitis in turkeys all over the world (Dieks et al. 1967; Mohammed and Bohl, 1967; Yamamoto, 1978; Mohan, Adegboye and Orajaka, 1979). The infection occurs either as a genital or a respiratory infection, although the respiratory phase might occur in the absence of the vaginal infection which readily occurs following insemination with infected semen (Mohammed and Bohl, 1967; Kleven and Pomeroy, 1971). This organism causes airsacculitis in day-old poults (Kumar et al. 1963). In this case the embryo becomes infected with this organism in vivo (Yamamoto, 1967; Rhoades, 1971). Several investigators have associated leg abnormalities or deformities designated "Turkey Syndrome 65" (TS 65) with M. meleagridis infection of turkeys and it is suggested that generalised

infection with this organism brings about the condition (Frazer, 1966; Wannop, 1971; Wise *et al*. 1973; Mohan *et al*. 1979).

Synergism has been reported between *M. meleagridis* and *E. coli* (Yamamoto, 1978) as well as with *M. synoviae* (Rhoades, 1977) to produce, either a case or a more severe airsacculitis.

M. meleagridis, variation in virulence has been shown. Pathogenic and nonpathogenic strains have been isolated and characterised (Ghazikhanian and Yamamoto, 1974a,b). Tropism of *M. meleagridis* seems to be associated with the age of the turkey. In young stock the organism may be found in the cloaca and bursa of Fabricius (Yamamoto and Ortmayer, 1967) while in mature birds, the organism is found in the oviduct or phallus (Yamamoto, 1978).

Only the turkey seems to be naturally susceptible to the infection of *M. meleagridis*, although a number of other species such as Japanese quail, peacocks and pigeons can apparently be infected (Yamamoto, 1978).

The first report of isolation from Nigeria was by Mohan, Adegboye and Orajaka (1979). They isolated arginine - utilizing mycoplasma - like

organism from an outbreak of airsacculitis and bad-leg syndrome in turkeys on the University Farm at Nsukka, Anambra State. The organism was identified biochemically and serologically as Mycoplasma meleagridis (Mohan, Adegboye,and Orajaka, 1979; Orajaka, 1982).

2.1.4 M. gallinarum

The first avian mycoplasma species M. gallinarum, was described by Edward and Freundt (1956), which was identical with Serogroup B-M. M. gallinarum is considered non-pathogenic (Fabricant, 1969). The non-pathogenic mycoplasmas have very rapid growth rate, and their presence makes the isolation of the pathogenic ones more difficult (Rosenfeld, 1971; Mallinson and Rosenstein, 1976; Sahu and Olson, 1976a, b) or impossible (Sahu and Olson, 1976b). In either case the infection has to be diagnosed serologically (Sahu and Olson, 1976a, b). Alternatively the organism could be propagated in susceptible fowl (Roberts, 1964a; McMartin, 1976; Rosenfeld, 1971) or in chick embryo (Roberts et al. 1967) before any attempt is made to isolate it. M. gallinarum is frequently found in the respiratory tract of chickens (Rosenfeld, 1971) where it might play a protective role to the ciliated respiratory

epithelium against the damaging effect of . M. gallisepticum (Taylor-Robinson and Cherry, 1972; Cherry-and Taylor-Robinson, 1973).

However the pathogenic potentials of M. gallinarum have been demonstrated by various workers. Dierks et al. (1967) found mild airsac lesion in mycoplasma-free turkey poults inoculated in the airsacs with a broth culture of M. gallinarum. During a field investigation of a respiratory-disease problem in a concentrated broiler-producing area in North Georgia, by Kleven et al. (1978), a high percentage of mycoplasma cultures from tracheas and airsacs lesions, which yielded pure cultures of M. gallinarum was reported. Kleven, Eidson and Fletcher (1978) also demonstrated that a representative M. gallinarum isolate given by aerosol or by air-sac inoculation, produced airsac lesions in young chickens when given in combination with a vaccine combining Newcastle disease and infectious brochitis or with a field strain of infectious bronchitis virus. In this respect, M. gallinarum they suggested is similar to M. gallisepticum or M. synoviae. Stipkovits et al. (1978) reported a pathogenic synergism betweem M. gallinarum and parvovirus, resulting in the production of much more severe fibrinous airsaculitis. Kleven et al. (1978) believe that M. gallinarum could

be a factor in the respiratory disease in chickens depending on the combination of circumstances. Extra-respiratory pathological condition such as perosis have been attributed to M. gallinarum (Varley and Jordan, 1978a).

In Nigeria, particular attention has not been paid to M. gallinarum probably because it is regarded as being non-pathogenic (Fabricant, 1969). The only document available to us is that of Etukudo (1982) who reported 28 isolates of M. gallinarum (and only 2 of M. gallisepticum) out of 58 Mycoplasma sp. cultured from 580 suspected CRD cases. In this report, M. gallinarum is the predominant mycoplasma serotype isolated. This agrees with the finding of Kleven, Eidson and Fletcher (1978). The overall impact of M. gallinarum on respiratory disease in poultry is not yet known.

2.1.5 M. iowae

Avian mycoplasma serotype I (Iowa 695) was first described by Yoder and Hofstad (1962, 1964) and was established by Dierks and others (1967) as one of 19 separate avian serotypes. Later, on the basis of biochemical and serological studies (Barber and Fabricant, 1971), this mycoplasma was put into one

serological group with serotypes J, K, N, O and R. This group has recently been given species status with the name of Mycoplasma iowae (Jordan et al. 1981).

Some strains have been reported to cause airsac lesions in poults and tendovaginitis in the hock and footpad regions of chickens and turkeys following inoculation at these sites (Yoder and Hofstad, 1962, 1964, Dierks et al. 1967). Chicken embryo mortality was demonstrated by Yoder and Hofstad (1962, 1964) and turkey embryo mortality by McClenaghan et al. (1981) and Rhoades (1981). Bradbury and McCarthy (1981) reported tenosynovitis, arthritis and rupture of digital flexor tendons in chickens after infection with M. iowae. Deformities and other abnormalities such as shortening and bowing of the long bones, which, together with the stunting and poor feathering closely resembled the condition known as turkey syndrome 65, and deviation of toes were recorded at postmortem examination of turkey poults infected with M. iowae (Bradbury and Ideris, 1982).

Apparently this species has not been reported from Nigeria.

2.1.6 Other avian Mycoplasma species

Isolates from ducks were named M. anatis (Roberts, 1964b). M. anatis is known to be antigenically related to serotype B by metabolic inhibition (Barber and Fabricant, 1971). The saprophytic mycoplasma recovered from the infraorbital sinuses of chicken, were classified as A. laidlawii (Adler and Shefrin, 1964). Shimizu, Ernø and Nagatomo (1978) isolated from pigeons, arginine positive mycoplasma strains for which the name M. columbinum was proposed and glucose fermenting strains for which the name M. columborale was proposed. The type strain of M. columbinum is MMPI while that of M. columborale is MMP4. Whether M. columbinum and M. columborale are pathogenic for pigeons remains to be determined.

According to Fabricant (1969) and Barber and Fabricant (1971) serotype D (Mycoplasma gallinaceum) isolated from chicken trachea was serologically related to serotype P. Serotype D (M. gallinaceum) have been grown from turkey (Jordan and Amin, 1980)

Serotype F (Mycoplasma gallopavonis) was isolated by Adler and Yamamoto (1957). Serotype F (M. gallopavonis) has been recovered infrequently from

both chickens and turkeys (Jordan and Amin, 1980). Recently strain SA of serotype F (M. gallopavonis) was shown to belong to species, Mycoplasma gallinaceum (Talkington and Kleven, 1984).

Apparently species of avian mycoplasma such as M. anatis, M. columbinum, M. columborale, M. gallinaceum, M. gallopavonis, A. laidlawii have not been reported from Nigeria.

2.2 RECOVERY OF AVIAN MYCOPLASMA

The successful recovery of mycoplasma from clinical specimens is dependent upon a number of factors, including those host-dependent components that may condition recovery and a variety of cultural or technical factors that may either enhance or limit primary isolation of these organisms. Some of these factors as listed by (Tully, 1983c) are as follows: host factors which include, antibiotics or other drugs in tissue or body fluids; presence of enzymes or other inhibitors in ground tissues; low levels of organisms in the tissues selected, and antibody in host tissues or fluids. The cultural factors include, poor quality of growth medium due to batch variability; poor choice of culture medium supplements, pH, atmospheric conditions, and

temperature; inhibitory activities of thallium acetate or antibiotics in medium; mycoplasma strain sensitivity to growth medium components, such as yeast extract or serum (more frequently observed with fresh isolates); competing microbial flora, including other mycoplasmas; latent mycoplasmas that may occur in culture procedures employed (animal host, cell cultures, serum, chicken embryo, etc.) for primary isolation. These various factors must be considered in the development of proper cultural techniques for mycoplasma recovery.

2.2.1 Media

Because of their small genome size, mycoplasmas have limited biosynthetic capabilities. They thus require wide range of precursor molecules for macromolecular synthesis. Vitamins, nucleic acid precursors, amino acids and lipids are usually supplied as growth requirements in various forms in artificial media (Fabricant, 1958, 1959, 1969; Frey, Hanson and Anderson, 1968; Frey et al. 1973; Whittlestone, 1976). As a result of their fastidious growth requirement their isolation from field specimens could be difficult and tedious (Jordan and Amin, 1975; Adegboye, 1977; Yoder, 1979). It has been suggested that no single medium is suitable for the recovery

of all avian mycoplasma species (Barile, 1974; Yoder, 1975). With modification and variation, media that could give adequate growth have been prepared (Frey et al. 1968; Jordan and Amin, 1980). The same media may be used for the recovery of mycoplasmas and acholeplasmas while different media is used for the ureaplasmas (Jordan, 1983).

Medium Supplement

Usually, swine or horse serum is often incorporated in the media for mycoplasma cultivation (Mohammed and Bohl, 1967; Frey et al. 1968; Shimizu, Erno and Nagatomo, 1978, Mohan et al. 1979; Etukudo and Adegboye, 1983a). Serum growth promoting ability differs considerably among different lots of serum, when large amount of mycoplasma work is to be done the use of horse serum becomes costly. Thus, other materials that could replace horse serum have been sought (Jensen et al. 1965; Tamura et al. 1975; Hayatsu, 1978; Washburn et al. 1978; Johnson and Somerson, 1980). Egg yolk has been found to be one of the most suitable (Jensen et al. 1965; Tomura et al. 1975; Hayatsu, 1978). It has been found to be consistent in growth promoting ability for M. pneumoniae (Sasaki et al. 1983). It could also be preserved for 2 years at $4^{o}C$. It is inexpensive, easy to obtain and

contain large amount of lipoproteins, cholesterol and phospholipids.

Specifically M. synoviae requires 0.1gm per litre of diphosphopyridine nucleotide (DPN) or nicotinamide adenine dinucleotide (NAD) and 0.1gm per litre of L-cysteine hydrochloride (Frey et al. 1968). Various studies show that a range of 0.002-0.1% NAD is adequate for M. synoviae growth (Thorpe, 1970; Olson and Meadows, 1972; Adler et al. 1974; Jordan, 1983). Etukudo (1982) attempted to incorporate NAD routinely, but could not because the material is expensive. Worse still, some mycoplasma isolates, obtained in the NAD containing media batches were lost before they could be identified. Thus in his cultural examination of 580 cases of CRD diagnosed clinically or at postmortem, no M. synoviae was reported.

The constituents of medium that has been successfully used (Adegboye, personal communication) to propagate M. gallisepticum is as follows:

Basal medium - PPLO broth powder (5.2gm/175ml distilled deionized water) - 70%
Horse serum - 15 - 20%
Glucose, Arginine or urea (10%) solution, - 1%
Yeast extract - 10%
L-cysteine hydrochloride - 0.1%
Nicotinamide adenine dinucleotide NAD or Diphosphopyridine nucleotide (DPN) - 0.1%

Phenol Red (1% solution) - 0.2%

Penicillin solution (10^6u/ml) - 0.1%

Thallium acetate - 0.2%

2.2.2 Specimen Collection

Various methods for specimen collection have been used. These include swabs taken from cropharynx, esophagus, trachea, cloaca during life and from airsacs, joints and lungs after death (Roberts, Olesunk and VonRockel, 1967; Frey et al. 1968; Yoder, 1979; Rodriguez and Kleven, 1980a, b; Jordan, 1983). Aspiration from infraobital sinus or joints, semen or yolk and inoculum from homogenized tissue such as lungs have also been used (Jordan, 1983).

2.2.3 Cultural Techniques

Broth cultures:

Primary isolation are usually made by snapping off swabs into broth in sterile test tubes with loose fitting tops. A serial tenfold dilution of the specimen is made in the range 10^{-4} to 10^{-10}. This is because at low dilutions, some cultures are overgrown by bacterial contaminents, while the

higher dilution often yield pure mycoplasmas (Frey, 1968; Adegboye, 1981; Jordan, 1983; Tully, 1983c). Tubes are sealed airtight and incubated at 37°C. Usually, all three types of broth (glucose, arginine or urea supplemented) are inoculated, unless a specific mycoplasma with known nutritional requirement is being sought.

Glucose-supplemented broth is observed daily for colour change of the phenol red indicator from pink (pH, 7.6) to yellowish (pH, 7.0) as was suggested by Roberts *et al*. (1967); Rodriguez and Kleven (1980a,b) is used to indicate growth. Though of minor value, increased turbidity of broth is also used as an index of growth (Butler and Knight, 1960; Tourtellotte, 1960; Mohan *et al*. 1979; Yoder, 1979).

Incubation period for pH change can vary from few hours to several days, depending on the mycoplasma species (Adegboye, 1981). Cultures showing homogenous colour change (without obvious bacteria contaminants) are used for inoculating solid media.

Cultures on solid media

Routinely appropriate agar plates are inoculated and allowed to dry. Plates of glucose and arginine-supplemented media are incubated at 37°C in glass

anaerobic culture jar, in a CO_2 enriched atmosphere provided by the addition of 1N HCl to marble chips in a small beaker. The atmosphere is usually humidified by the inclusion of cotton wool soaked in water (Frey, 1968; Adegboye, 1981; Jordan, 1983). For avian mycoplasma an atmosphere of CO_2 is of no particular advantage (Amin and Jordan, 1978).

Maximum recovery of mycoplasmas by direct plating onto agar have been reported (Amin and Jordan, 1978). But the author agrees with Yoder (1979) who maintained that initial passage in broth was necessary to reduce bacterial contamination. By Tully's (1983c) evaluation, specimen inoculated directly onto agar medium are only successful with host tissues and fluids containing large numbers of mycoplasmas; also some organisms may not propagate on secondary passage.

Although swabs are usually satisfactory for the isolation of organisms from field material, it may be desirable to prepare an inoculum from homogenized tissue, such as lung. Such tissue is broken up in small quantity of appropriate liquid medium with a pestle and mortar, glass, or mechanical homogenizer (Jordan, 1983).

As field strains are occasionally not seen until after 20 days of inoculation, it is worthwhile to

incubate cultures on solid medium for 20 to 30 days before discarding as negative (Jordan, 1983).

2.3 IDENTIFICATION OF ISOLATES

2.3.1 Purification

Impediments to successful identification and characterization of the mycoplasma in both primary isolates and laboratory culture have been that of mixed cultures, rapid overgrowth by non virulent species, and L-phase of bacteria (Razin and Oliver, 1961; Al-Aubadi et al. 1971a; Sahu and Olson, 1976a, b; Rosefeld, 1971).

Sabin and Johnson (1940) were the first to describe a mixed culture of mycoplasma. They recovered three kinds of mycoplasma colonies from mice. Adler and Yamamoto (1957) reported a mixed culture of mycoplasma from sinus exudate of a turkey in a case of infectious sinusitis. Adler et al. (1958), Fabricant (1960), and Jordan and Amin (1980) found that mixtures of more than one type were often recovered in cases of chronic respiratory disease of chickens. Similarly other workers reported the isolation of more than one species from a particular site in various hosts including bovine (Hudson, 1963),

dogs (Edward and Fitzgerald, 1951), and humans (Crawford and Kraybill, 1967). Mallison et al. (1976) noted that the presence of M. gallisepticum in broth and plate cultures may be masked by the overgrowth of other mycoplasmas especially M. gallinarum. He also reported the overgrowth by M. gallopavonis as a hinderance to successful culture of M. gallisepticum. It is therefore very difficult to make proper identification without adequate culture purity.

The problem of mixed culture and overgrowth by less fastidious organism may be tackled by cloning, (Anon, 1979), filtration through membrane filters 220-450nm (Tully, 1983a), by immunofluorescence staining of mixed growth on agar (Barile and Del Giudice, 1972; Jordan and Amin, 1980) and recently by a combination of growth inhibition technique and immunofluorescence (Bradbury and McClenaghan, 1982).

Cloning as one purification procedure involves the picking of well isolated colonies and subculturing three times from terminal dilution plates. It has been noted that cultures can be hard to purify by cloning when more than one species is present within one colony (Del Giudice et al. 1967; Fabricant and Freundt, 1967). Al-Aubaidi and Fabricant (1971a) observed that cultures which appear mixed on the bases of colonial morphology may actually be mixed

or be a pure culture, while some cultures which appear morphologically homogenous are actually mixed. Fabricant (1969) reported that some mycoplasma cultures sent to his laboratory as "type cultures" or representative serotypes on which species or serotype description were based, were found to be mixed cultures. Most of these mislabelled cultures were supposed to be purified by the accepted procedure of cloning three times. He added that this method does not guarantee a pure culture of a single mycoplasma species.

As L-phase variants of bacteria share the fried egg shape colonial morphology, they could be confused with true mycoplasma. Suspected isolates are therefore transfered to penicillin and thallium acetate free medium. True mycoplasma maintain their colonial appearance, while L-phase variants will revert to the bacterial forms and will produce the typical surface colonies of wall-covered bacteria (Razin and Oliver, 1961; Adegboye, 1977; Gourlay et al. 1974; Ojo, 1976; Razin, 1983). It is useful to pretest the filtration characteristic of the organism to be cloned before actual cloning is performed (Tully, 1983a).

Filtration process is as recommended by Tully (1983a), with a sterile needle and syringe, few ml of the test mycoplasma broth culture is taken. The needle

carefully replaced with appropriate membrane filters (220nm - 450nm pore diameters) on the syringe. Gentle pressure is applied and the broth passes through the filter into a clean sterile vial. Serial dilution of the filtrate is made and the vials securely capped, incubated and observed for colour change.

Tully (1983a) on filtration characteristics stated that members of the class Mollicutes will pass through 450nm filters and that most mycoplasmas usually will exhibit passage of variable number of cells through 300 and 220nm membranes. He also observed that filtrates obtained after gentle pressure filtration through 100nm membranes are usually sterile. Bacterial L-phase variants show only minimal passage of organisms through 450nm filters, and filtrate obtained from 300nm membrane is usually sterile (Tully, 1983a). Most mycoplasmas show minimal decrease in cfu/ml on filtration through 450 and 300nm membrane filters, a few of them show a drop of 2 to 4 logs in number after passage through 300nm filters. Filtrates obtained after passage of broth culture through 220nm membrane filters demonstrate a decline of 5 to 6 logs in cfu/ml over unfiltered broth cultures, whereas 100nm filtrates are usually sterile (Tully, 1983a).

Fluorescent antibody (FA) technique has been used for the identification of mycoplasma isolates. Specific mycoplasma antisera are prepared in rabbits by the methods of Morton and Roberts (1967). The gamma globulin fraction of the serum is usually conjugated with fluorescein isothiocyanate (FITC) by the procedure of Nairn (1969). The fluorescein-conjugated antiserum is then used to stain directly, mycoplasma colony impressions (Clerke et al. 1961) or agar blocks of cultures (Al-Aubaidi and Fabricant, 1971b) for FA examination. Use of this procedure obviates the need to obtain pure cultures of each organism, as mixed cultures can readily be detected, and as assessment of the relative proportions of different mycoplasmas in the sample obtained (DelGiudice et al. 1967).

Successful detection of mycoplasma mixtures by immunofluorescence depends on the proportions present in the mixture, fastidious mycoplasma could be masked by the overgrowth of a more prolific species. Barile and DelGiudice (1972) reported practical difficulties of applying direct fluorescent antibody test to "heavy and mixed mycoplasma growth", and they referred to incorporation of growth - inhibition antiserum in their medium.

Recently, Bradbury and McClenaghan (1982) described the use of a modification of the growth inhibition technique for detecting mycoplasma mixtures from avian sources, combined with immunofluorescence. This procedure was aimed at inhibiting the growth of a predominant mycoplasma in a mixed culture and thus allowing a minor component to emerge. Rabbit antiserum to the already identified mycoplasma was used for the growth inhibition test. The resulting colonies were identified by FA test. This technique, they reported, revealed mixtures that were not detectable by immunofluorescence alone. It may also be of value for checking the purity of cultures.

2.3.2 Testing for Sterol Requirement

The test to establish the sterol requirement of mycoplasma is an essential part of the identification and taxonomic description of these organisms. Hence cloned isolates are differentiated from Acholeplasmas by any or all of the following tests: direct method of growth in serum-free medium, assessment of the growth response of the mycoplasma to increasing amount of cholesterol, and indirectly by measurement of growth inhibition by digitonin. Serum

is simply excluded from the medium components in the direct sterol requirement test. True mycoplasmas would not grow in serum-free medium, while Acholeplasmas could. But it has been reported that some mycoplasmas have very minimal need for sterols and so carry over of serum cholesterol in the inoculum may allow the organism to grow through four or five consecutive passages (Tully, 1983b).

The growth response to cholesterol technique was introduced by Razin and Tully (1970), who observed that increasing amount of growth occur with increased levels of cholesterol. The Acholeplasmas do not show this graded growth response (Razin and Tully, 1970).

The digitonin sensitivity test was adapted from a paper disc growth inhibition test by Freundt et al. (1973). Paper disc saturated with 1.5% ethanolic suspension of digitonin and placed on agar inoculated with mycoplasma broth produced zones of growth inhibition. Most Acholesplasmas give a negative result and their growth occur up to the digitonin disc. Few non-sterol-requiring acholeplasmas may show slight inhibition zones (1.3mm) to digitonin (Tully, 1983b).

2.3.3 Other Biochemical Characterization

The obligatory biochemical tests for avian mycoplasma include glucose fermentation, arginine and urea decomposition (Erno and Stipkovits, 1973; Aluotto et al. 1970; Razin and Cirillo, 1983) and film and spots formation (Edward, 1950; Fabricant and Freundt, 1967; Freundt, 1983b). Other optional tests for identification are phosphatase activity, tetrazolium reduction, and serum digestion (Aluotto et al. 1970; Williams and Wittler, 1971; Bradbury, 1977, 1983) and arbutin and esculin hydrolysis (Williams and Wittler, 1971, Rose and Tully, 1983).

The glucose fermenters are recognized by appropriate pH changes. A drop of 0.5 pH units or more in glucose containing culture, compared with a control tube indicate a positive reaction (Aluotto et al. 1970). It is worthy to note that growth medium containing horse serum and yeast extract may show slight fall in pH even in uninoculated media after several days of incubation. Also non-fermentative organisms such as M. bovigenitalium may produce small amount of acidic metabolic products from constituents othe than sugars, leading to a subsequent drop in pH (Razin and Cirillo, 1983). To circumvent these problems, it has been suggested that medium without

yeast extract and with 1% (v/v) PPLO serum fraction instead of horse serum be used. Also that the basal medium be pretreated with glucose oxidase, peroxidase, and arginine decarboxylase to remove any trace of glucose and arginine (Freundt et al. 1979). Some Acholeplasmas decolorize the indicator so fast that the acidic shift may not be observed (Razin and Cirillo, 1983).

The mycoplasmas are divided into two groups on glucose fermenting ability which are: the glucose fermenters such as, M. gallisepticum, and the non-glucose fermenters, such as M. gallinarum. Barile et al. (1963, 1966) categorized Mycoplasma species as arginine-positive or arginine-negative organisms. Most arginine positive species e.g. M. meleagridis do not ferment glucose and most glucose fermenters do not hydrolyse arginine. Few mycoplasmas ferment both, e.g. M. iowae, an avian isolate.

When arginine is hydrolysed, the resultant ammonia produce an alkaline shift in the mycoplasma culture (Shimke and Barile, 1963). The phenol red (pH indicator) added to the medium changes colour from yellow to pink in arginine positive mycoplasma culture. This test could be done on both the broth and agar medium.

The film and spots phenomenon first described by Edward (1950, 1954) is exhibited by some mycoplasmas. These organisms develop a characteristic wrinkled pearly film on the agar surface and tiny black spots beneath and around the colonies. He used a medium containing 20% heat inactivated horse serum. An enhancing effect on "film and spots" formation was reported by Fabricant and Freundt (1967) when they used swine serum instead of horse serum. Freundt (1983b) recorded more positive reaction when he used media containing 10% egg yolk emulsion in addition to swine serum. Film and spots usually appear as soon as growth is well developed. Incubation and observation made beyond 14 days may lead to false positive reaction (Freundt, 1983b). The film consists of cholesterol and phospholipids, while the spots are attributed to the deposition of calcium and magnesium salts of fatty acids liberated through the lipolytic activities of the mycoplasmas (Freundt, 1983b).

2.3.4 Serological Characterization

Use of serological procedures have made it possible to identify and speciate the mycoplasmas. Typing of isolates into species involves serological tests such as the Growth inhibition and Epiimmunofluorescent tests. Edward and Fizgerald,

(1954) first described the use of the colony-inhibition test.

These authors and Nicol and Edward (1953), produced inhibition of mycoplasma growth by incorporating antiserum to the homologous species. Huijsmans-Evers and Rugs (1956) and Herderschee et al. (1963), later found that filter paper disc saturated with specific antiserum and placed on agar previously inoculated with a homologous species, lead to zones of growth inhibition around the disc. Clyde (1964) modified this test and observed a high degree of specificity using hyperimmune rabbit antiserum. Stanbridge and Hayflick. (1967) advocated that antiserum-impregnanted paper discs could be dried and stored for long periods with full retention of activity.

Table 2.1 summarizes biochemical reactions of some known avian mycoplasma.

Table 2.1

Biochemical Characteristics of some known avian Mycoplasma Species*

Species	Glucose Catabolism	Arginine Hydrolysis	Film and Spots	Tetrazolium reduction (Ae/an)[a]	Phosphatase
M. anatis	+	-	+	-/+	+
M. columbinasale	-	+	+	-/-	+
M. columbinum	-	+	+	-/+	-
M. columborale	+	-	-	-/+	-
M. gallinaceum	+	-	-	-/-	-
M. gallinarum	-	+	+	+/+	-
M. gallisepticum	+	-	-	+/+	-
M. gallopavonis	+	-	-	w/w	-
M. iners	-	+	+	-/-	-
M. iowae	+	+	-	+/+	-
M. lipofaciens	+	+	+	-/+	-
M. meleagridis	-	+	-	-/+	+
M. synoviae	+	-	+	-/w	-
A. laidlawii	+	-	-		

*Adapted from Methods in Mycoplasmology
Vol. I, 1983, Academic Press, New York London

[a]Ae = Aerobically. An = Anaerobically
w = Weak reaction.

Some pitfalls have been pointed out with the growth inhibition test. The depth of agar medium influences the number and size of mycoplasma colonies (Bradbury and Jordan, unpublished findings); the degree of inhibition is affected by the number of organisms on solid medium used for the GI test (Clyde, 1964). Therefore the depth of the medium has effect on the GI test (Jordan, 1983). Also it has been observed that new mycoplasma isolates were less susceptible to growth inhibition and agglutination by specific antiserum till after several subcultures (Frey and Hanson, 1969).

Another test widely used for the identification of the mycoplasma including avian species is the epiimmunofluorescence test. The first of such studies was conducted by Liu (1957) to examine Eaton agent before its identification as a Mycoplasma. Chanock et al (1961) used this method to demonstrate M. pneumoniae in the lungs of chicken embryos. Two methods of fluorescent antibody staining of mycoplasma colonies are used. These are the direct and indirect tests.

For the direct method, mycoplasma cultures are plated on PPLO agar and incubated until good growth is obtained. Petri dishes (90mm) containing 15ml of 1.5% agar media are most satisfactory.

Thicker plates are optically undesirable and thinner plates resulted in manipulative problems (Bass and Jasper, 1972). For best result, mycoplasma growth on agar plate should not be confluent (Gardella et al. 1983).

Specific mycoplasma antiserum is prepared in rabbits by the methods of Morton and Roberts (1967). Mycoplasma antisera could also be prepared in horses, donkeys or mules, but such antisera contain large amounts of lipids and other substrates that interfare with FITC conjugation (Gardella et al. 1983). The gamma globulin fraction of the antiserum is labelled with FITC as described by Nairn (1969). Few drops of the working dilution of the specific conjugated antisera are placed on agar plates or agar pieces cut from agar plate containing mycoplasma colonies (Al-Aubaidi and Fabricant 1971b; Rosendal and Black, 1972; Gardella et al. 1983). The agar plates are placed in a shallow pan over moist paper towels and incubated for 30 min. at 37°C with covers slightly open (Baas and Jasper, 1972). The conjugate is then poured off the plate. The plate is washed several times with PBS, dried and examined under FA microscope Dovadola and Cancellotti (1972) reported the successful use of the direct FA test for identifying M. gallisepticum and M. meleagridis and Buys (1976)

described the use of the direct test with conjugates for 10 avian mycoplasma species for identifying such isolates "in situ" on agar plates.

For the indirect immunofluorescence test, same materials are used with the exception of a secondary antiserum. This secondary antiserum is a fluorescein-conjugated antiserum (e.g. fluorescein-conjugated anti-rabbit globulin) to the primary antiserum. The indirect procedure is performed similarly to the direct test with some variation. The unconjugated mycoplasma antiserum is placed on the agar plate with mycoplasma colonies. The plate is incubated for 30 minutes, washed twice with PBS. The working dilution of conjugated secondary antiserum is added to the plate and incubated further. The plate is then washed with PBS and examined microscopically as described earlier. Stipkovits and El-Ebeedy (1977) used indirect immunofluorescence to examine serological relationships between the avian mycoplasma serotypes. The main advantage of this procedure is the potential for screening sera for anti-mycoplasma antibodies, and only one fluorescent reagent is needed here.

Corstvet and Sadler (1964) found the direct fluorescent antibody test more valuable than the indirect test for staining colony impressions of

M. gallisepticum, M. synoviae and M. meleagridis.

Bradbury (1982) described the use of antisera produced in chicken for the identification of avian mycoplasma by indirect immunofluoresence. She reported that this indirect fluorescent antibody test was successful in identifying the majority of unknown cultures, and almost all the avian serotypes, but failed to detect isolates of M. columbinasale, M. pullorum and M. gallinaceum. She suggested that failure to detect isolates of M. columbinasale was probably because this mycoplasma appears to be confined to pigeons (Fabricant, 1969).

Chapter III

ISOLATION AND CHARACTERIZATION STUDIES

3.1 INTRODUCTION

Chronic respiratory disease (CRD) is one of the costliest disease problem confronting the poultry industry (Yoder, Jr., 1978). Economic losses result from reduced feed efficiency, decreased egg production, downgrading of carcasses and increased medication cost. The disease is caused primarily by M. gallisepticum, which belongs to a group of fastidious bacteria. A closely related mycoplasma M. synoviae which was more often associated with joint infections was shown to be able to cause respiratory disease in poultry (MaCowan, Randall, Jones, and Brand, 1982). Its exact role in CRD is not yet known. The disease as encountered in the field is very often complicated by other bacteria and respiratory viral infections, giving rise to a very serious illness (Fabricant, 1969; Jordan, 1972; Etukudo and Adegboye, 1983a, b).

CRD is generally believed to be common in Nigeria. This belief is based mainly only on clinical signs and postmortem findings of airsacculitis. Only few reports on the Rapid Plate Agglutination (RPA)

test is available (Onunkwo and Onoviran, 1978, Etukudo, 1982; Kujul, 1983; Abdu, Bishu, Adesiyun and Adegboye, 1983) in addition to clinical and postmortem observations (Mohan, Adegboye, and Orajaka, 1979; Olufarati, Ikede, Kasali and Ojo, 1981). Moreover there is one report of M. gallisepticum isolation (Etukudo and Adegboye, 1983a). These reports indicate that CRD is a major problem in Nigeria.

There appears to be no published cultural investigation on M. synoviae in Nigeria, especially on whether the organism is involved in CRD. Majority of reports had been only on serological survey. Apparently there are also no reports of investigations on the involvement of other avian mycoplasmas (e.g. M. iners, M. meleagridis, M. anatis, A. laidawii etc.) in CRD. In the study presented below attempts were made to isolate and characterize mycoplasmas associated with diseased and non-diseased respiratory tissues of chickens.

3.2 MATERIALS AND METHODS

3.2.1 Culture Media

The medium used was according to Etukudo and Adegboye (1983a) which was adapted from Frey et al. (1968) with minor modifications.

Constituents

1. Basal medium:

Bacto PPLO broth powder without crystal violet (Difco Laboratories, Detroit, Michigan) was used.

Bacto PPLO Agar (Difco Laboratories Michigan) was used directly for agar plates.

2. Horse serum:

Two horses at the Veterinary Clinic of the National Veterinary Research Institute, Vom, were bled aseptically, from time to time. Serum from the two horses had earlier been shown to be able to support the growth of different mycoplasmas. The blood was allowed to clot at +4°C overnight. Serum was aseptically separated. Pooled sera was seitz filter-sterilized and frozen at -20°C.

3. Glucose:

10% (w/v) stock solution was prepared using Dextrose (Difco Laboratories Detroit Michigan). This was dispensed in 10ml amounts into universal bottles, autoclaved at 121°C, 15lb per sq. in pressure for 15 min and then stored at +4°C.

4. Yeast extract:

Yeastolate (Difco Laboratories, Detroit, Michigan, U.S.A.) was used instead of Bakers yeast extract of Ftukudo and Adegboye (1983). 10% (w/v) solution of TC yeastolate was prepared and filter sterilized for each batch of media prepared.

5. L-Cysteine hydrochloride:

A 10% stock solution was prepared by making up 1.0gm of L-cysteine hydrochloride to 10ml with distilled deionized water. This was filter-sterilized using 450nm millipore membrane, dispensed in 2ml aliquots into sterile universal bottles, and stored at +4°C. This was used as a growth promoter according to Frey et al (1968).

6. Nicotinamide adenine dinucleotide (NAD) or Diphosphopyridine nucleotide (DPN):

A 10% stock solution was made by dissolving 0.5gm NAD (BDH Chemical, Ltd. Poole, England) in 2ml distilled deionized water. This was made up to 5ml. It was filter-sterilized through 450 nm Millipore membrane. The solution was dispensed in 1ml amounts into sterile bijou bottles and frozen. This was used as a growth promoter especially for M. synoviae.

7. Phenol Red:

1.0gm phenol red (Difco Laboratories, Detroit Michigan) was dissolved in 100ml distilled deionized water to make 1% stock solution. This was dispensed in 10ml aliquots and stored at +4°C.

8. Penicillin:

Benzyl penicillin sodium (Crystapen 600mg, Glaxo Laboratories Ltd. Greenford, England) was reconstituted with 1.0ml sterile water to give 1 x 10^6 units per ml. This was stored at +4°C until used.

9. Thallium acetate:

10% stock solution was prepared by dissolving 1.0gm thallium acetate (Fisher Scientific Co. New Jersey) in distilled deionized water to make final volume of 10mls. This was sterilized by autoclaving, dispensed in 2ml aliquots and stored at +4°C. Both the penicillin and thallium acetate were used as bacterial inhibitors.

3.2.2 Preparation of Media

A 250ml broth and a 250ml agar media were prepared from time to time as follows:

5.25gm of the PPLO broth powder was weighed into a conical flask. To this flask 175mls distilled deionized water was added and the mixture stirred and heated to boiling using a magnetic stirrer-thermost hot plate (Gallenkamp and Co. Ltd., London). The powder having dissolved completely, phenol red (0.5ml of 1% solution) was added and pH tested and adjusted to 7.6 with 1N Hydrochloric acid or 1N sodium hydroxide. The broth was then autoclaved at 121°C, 15lb per sq. in. pressure for 15 minutes. It was then transfered to water bath set at 40°C where the other components were added aseptically in appropriate amounts as follows:

1.	Horse serum	50.0ml
2.	Glucose (10% solution)	2.5ml
3.	Yeastolate	25.0ml
4.	Cysteine Hydrochloride (10% solution)	0.25ml
5.	NAD (DPN) (10% solution)	0.25ml
6.	Benzyl Peinicillin 10^6 u/ml	0.25ml
7.	Thallium acetate (10% solution)	0.5ml

Agar medium:

8.5gm PPLO Agar base (Difco Laboratories, Detroit Michigan) was weighed and dissolved in 170ml distilled deionized water. After pH adjustment and autoclaving, other materials (items 3 to 7 above) were added aseptically. The molten agar at approximately 40°C was dispensed into sterile 90mm plastic petri dishes (Sterilin Ltd. Teddington, Middlessex, England) and allowed to solidify. These plates were stored at +4°C used, usually not longer than two weeks.

3.2.3 Media for glucose or arginine requirement test

Alluottos Formula (Alluotto et al. 1977) was used. A 100ml test medium consisted of:

PPLO broth base	74ml
Horse serum	10ml
Glucose or Arginine (10% w/v)	10ml
Yeastolate (10% w/v)	5ml
Cysteine Hydrochloride (10% solution)	0.12ml

NAD (10% solution)	0.2ml
Phenol Red (0.5% w/v)	1.0ml
Benzyl Penicillin (10^6U/M)	0.1ml
Thallium Acetate (10% solution)	0.25ml

The pH was adjusted to 7.6 for glucose broth and 7.0 for arginine broth using IN NaOH and IN HCI respectively. Each broth medium was then seitz-filtered and stored at $+4^{\circ}C$.

3.2.4 Specimens

From 3 to 32 chickens were randomly purchased from each of 7 different poultry flocks with clinical CRD in Zaria area. A total of 90 chickens were so purchased. Clinical signs exhibited included catarrhal nasal discharge, coughing, sneezing, rales and respiratory distress.

As controls, 30 apparently healthy birds were purchased from 1 farm with no history of CRD for the past 2 years. Blood was collected for serum from each bird by exsanguination and a postmortem examination carried out. Swabs were taken routinely from the nasal sinus, trachea, lungs and air sacs of each bird, to provide a total of 480 respiratory specimens.

Reference strains of M. gallisepticum (PG 31), M. synoviae (WVU 1853) and M. gallinarum (PG 16) were kindly supplied by Professor E. A. Freundt of the World Health Organization/Food and Agricultural Organization Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Denmark. Moreover, Dr. Janet M. Bradbury of the Sub-Department of Avian Medicine University of Liverpool, Leahurst, Field Station, Neston, Wirral L64. 7TE, United Kingdom, supplied the above named reference strains in addition to strain S6 of M. gallisepticum. These strains were used as positive controls to check efficacy of new batches of media and in biochemical and serological characterization of new isolates.

3.2.5 Cultural Procedure

In broth: Each swab was snapped off into a screw capped test tube containing 2ml broth. The tubes were frozen at -20°C usually for not more than two weeks until a reasonable number of specimens were available to work on. The frozen specimens were thawed at room temperature and mixed thoroughly. Then 0.2ml of each specimen was transfered into a fresh 1.8ml broth. This was diluted serially in ten folds down to 10^{-3}. Tubes were tightly screwed and incubated at 37°C. Cultures were examined every

24 hours for colour change from pinkish (pH 7.6) to pink-yellow (pH 7.2 to 7.0). Tubes with good homogenous colour change (i.e. without precipitates indicative of bacteria contamination) were frozen. After 7 days, cultures that failed to show colour change were passaged into fresh broth and incubated for 7 more days. Broth culture with appropriate colour change but with heavy precipitate indicative of bacteria growth were filtered through 450nm Millipore membrane using 25mm filter holders and then subcultured. Alternatively, the contaminated broth cultures were re-diluted serially and re-incubated.

On Agar:

To check for colonies, 0.1ml of each broth culture with appropriate colour change was diluted serially to 10^{-3}. Then a drop of each dilution and the undiluted culture was inoculated onto a quadrant of an agar plate as shown in Fig. 3.1.

Broth cultures with no apparent colour change after approx. 14 days of incubation were also blind passaged by inoculating 1 drop of the undiluted culture onto agar plates. Plates were incubated at 37°C in glass anaerobic jars (Gallenkemp and Co. Ltd. London) under 5-10% CO_2 tension provided by the addition of 1N

hydrochloric acid to marble chips contained in an enclosed beaker. The atmosphere in the jar was humidified by enclosing a wet cotton wool.

Plates were examined every 48 hours for mycoplasma-like colonies using a stereoscopic microscope (Olympus Optical Co., Japan) at a magnification of X40 to X80. Plates were incubated for at least 12 to 14 days before being considered negative for Mycoplasma.

3.2.6 Characterization

The various steps used in characterization were based on standard methods earlier described (Razin and Tully, 1983), with minor modifications. They were as follows:

Purification of Isolates

Mycoplasma-like colonies on any plate were purified by cloning. A single representative colony was picked using the bevel of a hypodermic needle mounted on a syringe. This was inoculated into 0.2ml broth, which was then diluted down to 10^{-3}. The cloned culture was incubated and subcultured onto agar medium after approx. 3-4 days. If colonies were recovered the cloning process was repeated twice

more in those cases where the original plates had some bacterial contaminants. However, if the original plate yielded pure mycoplasma-like colonies and care was taken to remove only one well isolated representative colony from the plate, cloning was done only once. This was because repeated cloning had led to complete loss of some valuable avian mycoplasma isolates in the past (Adegboye, personal communication).

Reversion Studies

Each cloned mycoplasma-like isolate was plated on mycoplasma agar devoid of penicillin and thallium acetate, incubated at 37°C, and examined daily for 7 days. The mycoplasma colonies were examined for changes in typical morphology, if any. Blood agar plates were also plated with broth culture of each cloned organism and examined for bacterial colonies, if any.

Biochemical Tests

(a) Sensitivity to digitonin

To test whether an isolate was sterol-requiring and hence a true Mycoplasma sp., sterile paper discs 6.35mm diameter (Scheischer and Scheuell Inc., Keene,

N.H. 03431, U.S.A.) were soaked in 1.5% digitonin in ethanol. The discs were dried in an oven and stored for use. Cloned broth culture of each isolate was inoculated on an agar medium and allowed to form a streak line along the medium. A digitonin-impregnated disc was then placed on the inoculum, and the culture incubated by routine method. When the Scheicher and Scheuell disc were no longer available, Millipore filter absorbent pads (Millipore SA. 67120 Molshein France) were cut to shape and used with satisfactory result.

(b) Direct test for sterol-requirement

The direct broth method for quantitating the growth response to cholesterol as recommended by Razin and Tully (1970) was attempted with some modifications. About 4-5 colonies from plates of thrice cloned isolates were picked and transfered into 1.8ml broth. This was serially diluted with mycoplasma broth devoid of horse serum down to 10^{-5}. As a control, similar serial dilutions were made using the regular mycoplasma broth. Cultures were incubated for 7-14 days and then subcultured onto agar medium as follows: cultures in broth devoid of horse serum were plated onto glucose agar devoid of horse serum, while cultures in regular mycoplasma

broth was plated unto regular agar medium containing horse serum. All plates were incubated routinely and examined every 48 hours for up to 14 days for growth. The serial dilution done in broth devoid of horse serum ensured that horse serum contained in the original culture was diluted out terminally.

(c) Test for glucose and arginine utilization

1.8ml of each specially prepared test medium containing either glucose or arginine (section 3.3) was inoculated with 0.2ml 24 hours broth culture of the isolate. Control (uninoculated) test medium was similarly set up. Tubes were examined for a drop in pH (glucose-supplemented) or a rise in pH (arginine-supplemented) with reference to control test medium. A drop in pH shown as a colour change from pink to orange-yellow in the glucose broth was recorded as positive for glucose-utilization, while a rise in pH in the arginine medium shown by colour change from yellowish to pinkish was recorded as positive for arginine-utilization.

(d) Production of film and spots

Regular mycoplasma agar plates were used for this test. A cloned isolate was inoculated on a plate which was then incubated routinely for up to 12 days.

Plates were examined every 24 hours for production of film and spots.

Serological Tests

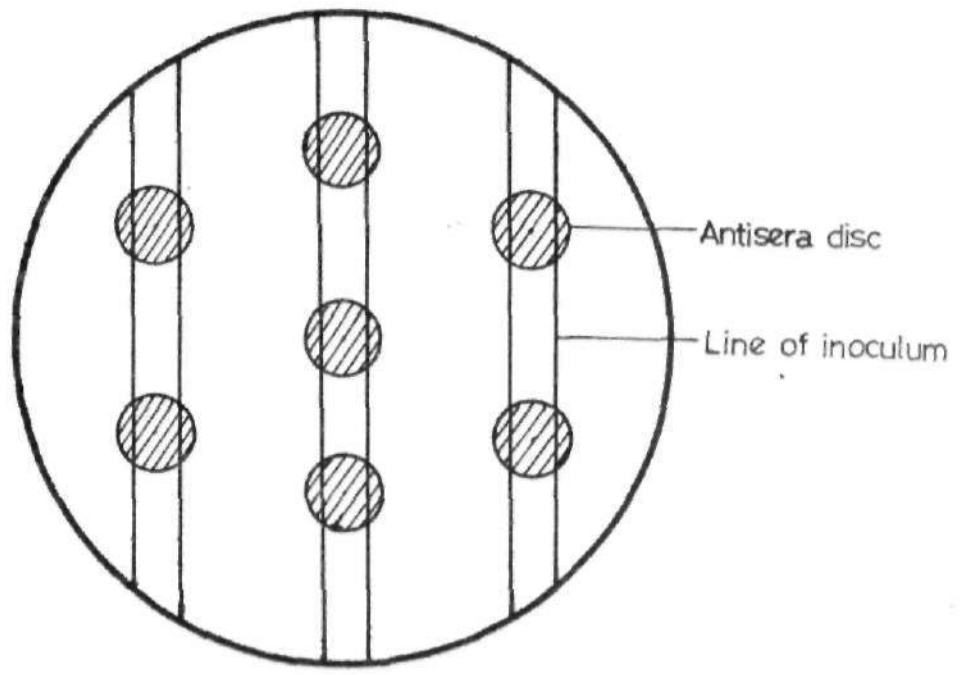
a) Growth Inhibition Test:

The running drop technique (Clyde, 1964) was used. One drop of a suitable dilution broth culture of a cloned isolate which contained approx. 10^4 colony forming units (c.f.u) per ml was allowed to run down the agar medium in a 90mm petri dish, and allowed to dry. Three lines of such inoculum could be accommodated on the 90mm petri dish and a 10^{-3} or 10^{-4} dilution of an actively growing isolate was found suitable. A 6.35 diameter paper disc (Scheicher and Scheuell) was impregnated with each suspect antiserum. Discs were arranged 20mm apart on the inoculum. Each isolate was so tested using hyperimmune sera against the following organisms: M. gallisepticum (PG 31), M. synoviae (WVU 1853), M. gallinarum (PG 16) (from Professor E. A. Freundt, FAO/WHO International Reference Centre for Animal Mycoplasma, Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark), M. gallopavonis (WRI), M. gallinaceum (DD), M. iners (PG 30), M. iowae (695), M. columborale (MMP-4), M. columbinum (MMPP-1), M. columbinasale (694),

(from Dr. J. G. Tully, Mycoplasma Section, NIAD BLDG 550, Frederick Cancer Research Centre, Frederick, MD 21701, U.S.A.).

Control tests were set up using reference strains of M. gallisepticum, M. synoviae, M. gallinarum, against homologous and heterologous antisera. Fig. 3.2 shows how the disc were often arranged.

FIG 3.2 GI. TEST FOR MYCOPLASMA CULTURE SHOWING ARRANGEMENT OF ANTISERA DISCS ON INOCULUM LINE.



b) Epiimunofluorescent (EF) Test:

Cloned and uncloned cultures of isolates were used for this test. The use of uncloned cultures was based on the recommendation of Al-Aubaidi and Fabricant (1971b). Fluorescein isothiocynate labelled hyperimmune serum against M. gallisepticum (PG 31), M. synoviae (WVU 1853), M. gallinarum (PG 16), M. iowae (695), A. laidlawii (PG 9) and M. anatis (1340) (from Dr. J. G. Tully, Mycoplasma Section, NIAD BLDG 550, Frederick Cancer Research Centre, Frederick, MD 21701, U.S.A.) were used.

Agar blocks containing few young colonies of each isolate were placed on a clean microscope slide. Reference strain of MG, MS, M. gallinarum were grown and used as controls. Slides were carefully identified in respect of the organism and conjugate to be applied to avoid mix up. Slides were arranged on an improvised glass rod rack over a laboratory sink.

Colonies were first washed two times by soaking the blocks in PBS for approx. 5 minutes and carefully sucking off the PBS with a pasteur pipette. Agar blocks were then dried by absorbing PBS from their edges with a paper towel. Then few drops of recommended working dilution of each conjugate were

placed on the surface of agar block. Colonies were thus stained for 30-45 minutes at room temperature. Excess conjugate was rinsed off with PBS, pH 7.2 and the blocks were rinsed once with PBS. Excess PBS [illegible] was removed with absorbent tissue.

Initially blocks were examined without a mountant under the Leitzlaborlux fluorescent microscope using an Osram HBO 200W mercury vapour high pressure lamp as light source.

Fluorescence considered specific was bright yellow-green marking out the colonies precisely. This was graded as +++, ++, +, -, depending on the relative intensity.

3.3 RESULTS

3.3.1 Growth and trial identification of Reference Strains

Reference strains of M. gallisepticum (PG31), M. synoviae (WVU1853), M. gallinarum (PG16) from Prof. E. A. Freundt were found to grow well on the media formulation used. The original ampoule material grew within 7 days in the broth, and when subcultured onto agar medium, good colonies were

obtained within 4-7 days. Further subculturing in the broth or agar led to more rapid colour change in broth or production of colonies on agar.

In the GI and IMF test, satisfactory results were obtained with these strains using the standard antisera from Dr. J. G. Tully and Prof. E. A. Freundt. With the GI test, clear inhibition zones were produced. Fluorescence graded as +++ was obtained in the IFA test.

3.3.2 Isolation Trials

Of the total of 90 clinically ill chickens from 7 flocks, 41 (45.56%) birds yielded mycoplasma-like colonies from a total of 360 respiratory tissue swabs. On the other hand, only 1 (3.33%) of 30 clinically healthy chickens yielded a mycoplasma-like organisms from 120 respiratory tissue swabs (Table 3.1). A total of 480 respiratory tissue swabs were examined. Each of the 7 flocks had chickens positive for mycoplasma-like colonies.

Isolates confirmed as Mycoplasma sp. did not revert to bacteria in penicillin and thallium acetate-free media, and they did not grow at all on blood agar. They were all widely inhibited by digitonin impregnated discs which confirmed their dependence on sterol. 7 of the isolates were resistant to digitonin and were not affected by decreasing gradation of horse serum. They were thus identified as Acholeplasma sp. (Plate 3.7).

3.3.3 Biochemical Characterization

The isolates were subjected to the following biochemical tests:

- digitonin and serum degradation test
- glucose and arginine metabolism and observation for film and spot production.

The result is summarized in Table 3.2.

Six distinct groups emerged as follows:-

	Glucose	Arginine	Film and Spots	Digitonin
I	+	-	-	-
II	+	-	-	+
III	+	-	+	+
IV	+	+	+	+
V	-	+	+	+
VI	-	-	++or-	+

3.3.4 Serological identification

The isolates were then subjected to GI and FA tests. The GI test result is summarized in Appendix 1. Clear inhibition zones ranging from 2.5mm to 4mm were recorded as positive inhibitions Cases of non specific reactions whereby an isolate is inhibited by many (heterologous) antisera were common but, by selecting the antisera that produce the widest inhibition zone, and with the background knowledge of each isolates biochemical reactions (Table 3.2), an inference to the following mycoplasma species were made for each isolate: M. gallisepticum, M. synoviae, M. gallinarum, M. gallopavonis, M. gallinaceum, M. columborale, and M. columbinum. 9 isolates were lost after biochemical test and as such growth inhibition test was not carried out. They were not speciated. 7 isolates were not affected by any of the 10 avian mycoplasma antisera used. They were identified as Acholeplasma species, as they were the same isolates resistant to digitonin.

Finally the isolates were tested by FA test. The result is summarized in Appendix 2. Based on the results, the following species were identified:

M. gallisepticum PG 31, M. synoviae WVU 1853, M. gallinarum PG 9 and A. laidlawii PG 9. There was no cross reaction. This confirms the species specificity of immunofluorescence test.

Table 3.3 and Appendix 3, show the main results of the biochemical and serological tests and the final identification of each isolate. On the basis of these, 12 isolates were typed as M. gallisepticum (PG31), 2 as M. synoviae (WVU 1853), 18 as M. gallinarum (PG16), 7 as A. laidlawii (PG9), 9 as M. gallopavonis (WR 1), 1 as M. gallinaceum (DD), 3 as M. columborale (MMP-4), and 1 as M. columbinum (MMP-I).

The distribution of the Mycoplasma sp. and Acholeplasma sp. per respiratory site is presented in Table 3.4. It shows that 64.1% of all isolates were made from the nasal sinus, 21.9% from the trachea, 9.4% from the air sacs and only 4.7% from the lungs.

Table 3.5 and Appendix 4 compare postmortem findings in all birds with frequency of Mycoplasma and Acholeplasma species isolation. It revealed that in all cases of airsacculitis, M. gallisepticum species had the highest frequency of occurrence followed by M. gallinarum species, then M. synoviae. In cases with pneumonitis M. gallopavonis occurred more

frequently, followed by M. gallinarum then M. gallisepticum. In cases of tracheitis M. gallinarum occurred more frequently followed by Acholeplasma sp., then M. gallopavonis. In cases of sinusitis M. gallinarum occurred more frequently, followed by M. synoviae. In birds with no gross lesion, M. gallinarum was isolated more often, followed by M. gallisepticum and M. gallopavonis.

Table 3.5 and Appendix 4 also show that large number of cases of airsacculitis (22), pneumonitis (11), tracheitis (15) and sinusitis (10) did not yield mycoplasma isolate.

Representative colonies of some isolates are illustrated on Plates 3.1 to 3.7.

Table 3.1

Rate of Isolation of Mycoplasma-like organisms from the Respiratory Tissues of Clinically ill (CRD) and Apparently Healthy Chickens

Groups	Flock Designation	No. of chickens sampled	No. chickens positive for Mycoplasma-like isolates	%
I Clinically ill Chickens (360 swabs)	A	17	12	70.59
	B	22	7	31.82
	C	32	17	53.13
	D	11	1	9.10
	F	3	2	66.67
	G	5	2	40.00
SUB TOTAL		90	41	45.56
II Apparently healthy chickens (120 swabs)	E	30	1	3.33

Table 3.2

Biochemical Characterization of isolates
(arranged in groups of Identical Reactions)

Groups	Isolate No.	Total No.	Glucose Catabolism	Arginine Hydrolysis	Film and Spots	Digitonin
I	2S 2A 64A 23Sb,	7	+	-	-	-
II	4T 6Sb 6Sc 7S 9Sc 9Sb 21Sb 22Sa 22Sb 23Sa 24T 25S 25T 25A 26A 28S 29S 48Sb 93S 93Tb 100T 102A 107S 109S 116S	25	+	-	-	-
III	15S 41T 59Sb	3	+	-	+	+
IV	(16S 41S 42S)* 87S'	4	+	+	+	+
V	4Tb 5S 6Sa 9Sa 20A 21S 31T 32S 48Sa 50S 50T 49S 89S 89T 93Ta 94T 102S 120S 86S (17L 42L 44S)*	22	-	+	+	+
VI	(31S 25L)* 60S'	3	-	-	+or-	+

* Dead before full identification

' Untyped isolates

Table 3.3

Summary of Biochemical and Serological test of Isolates

Biochemical Tests				Identification by		Final Identification	No. of Isolates
Glucose	Arginine	Digitonin	Film & Spots	GI Test	FA Test		
++	-	-	-	NT	d	A. laidlawii	7
+	-	+	-	e	NT	M. gallopavonis	9
-	+	+	+	c	c	M. gallinarum	19
++	-	+	-	a	a	M. gallisepticum	12
+	-	+	+	b	b	M. synoviae	2
+w	-	+	-	f	NT	M. gallinaceum	1
-	+	+	+	h	NT	M. columbinum	1
+	-	+	-	g	NT	M. columborale	3

a = M. gallisepticum

b = M. synoviae

c = M. gallinarum

d = A. laidlawii

e = M. gallopavonis

f = M. gallinaceum

g = M. columborale

h = M. columbinum

NT = Not Tested

Table 3.4

Mycoplasma Species Yield Per Respiratory Site

	Mycoplasma Sp	Swab Sites: Nasal Sinus	Trachea	Lung	Airsac	Total
Clinically ill chickens (90)	M. gallisepticum	7	3	-	2	12
	M. synoviae	1	1	-	-	2
	M. gallinarum	12	4	-	1	17
	A. laidlawii	2	2	-	3	7
	M. gallopavonis	7	2	-	-	9
	M. columborale	2	-	-	-	2
	M. columbinum	1	-	-	-	1
	Untyped Sp[a]	8	-	3	-	11
	Sub Total	40	12	3	6	61
Apparently healthy chickens (30)	M. gallinarum	-	1	-	-	1
	M. gallinaceum	1	-	-	-	1
	M. columborale	-	1	-	-	1
	Grand Total	41 (64.1%)	14 (21.9%)	3 (4.7%)	6 (9.4%)	64

[a]Includes nine mycoplasma isolates that were dead, before serological identification.

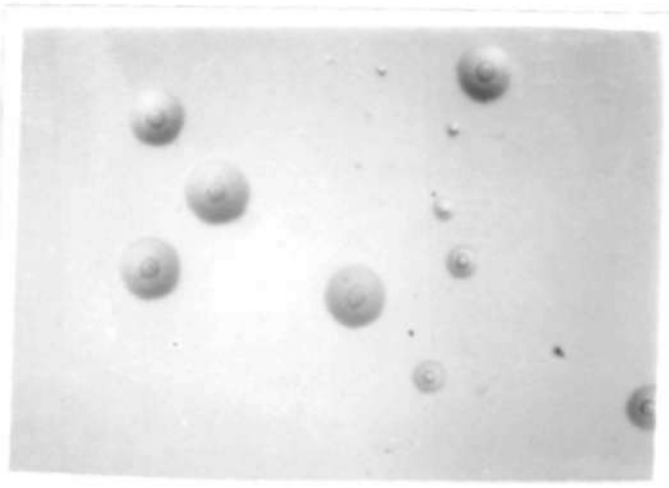
Table 3.5

The frequency of recovery of Mycoplasma and Acholeplasma species from Pathological lesions (Respiratory) and from cases with no gross lesion

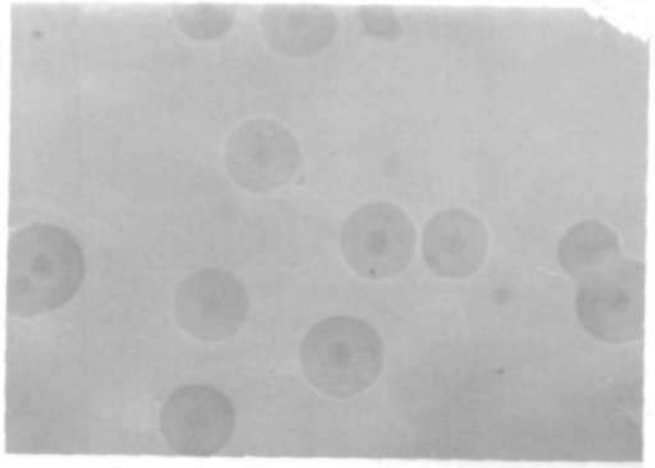
	Mycoplasma sp.	Pathological lesions				
		Sinusitis	Tracheitis	Pneumonitis	Airsacculitis	No Gross Lesions
Clinically ill birds (90)	M. gallisepticum	-	1	2	6	2
	M. synoviae	1	1	-	1	-
	M. gallinarum	2	4	3	5	4
	A. laidlawii	-	3	-	-	-
	M. gallopavonis	-	2	5	1	2
	M. columborale	-	-	-	1	1
	M. columbinum	-	1	-	4	-
	Untyped	1	2	-	4	4
	No isolate	10	15	11	22	11
Apparently healthy birds (30)	M. gallinarum	-	-	-	-	1
	M. gallinaceum	-	-	-	-	1
	M. columborale	-	-	-	-	1
	No Isolate	-	-	-	-	29

*Pathological lesions occurred singly or in combination with one another.

[d]Include mycoplasma isolates that died before serological identification.



A



B

Plate 3.1(A) and (B) Colonies of mycoplasma isolate No.'s 102A and 23S, identified serologically as M. gallisepticum x 44.

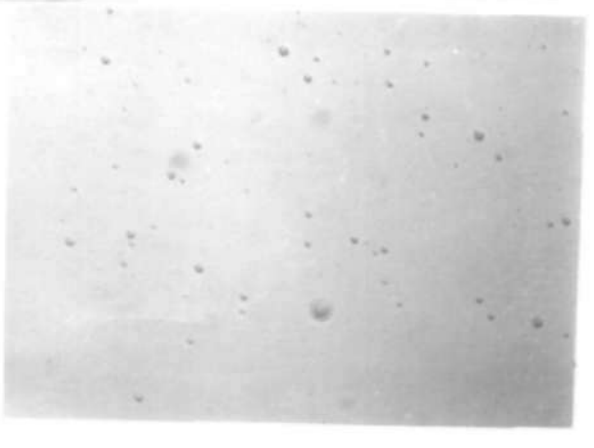


Plate 3.2 Colonies of isolate from chicken No. 41T identified serologically as M. synoviae x 79.

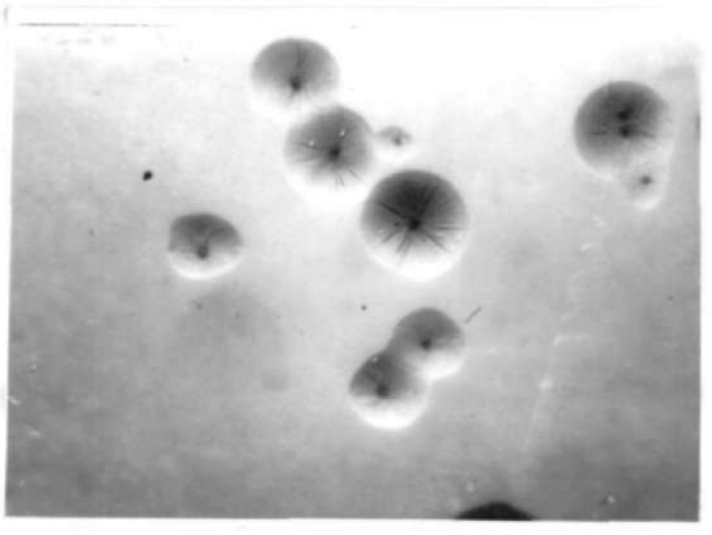


Plate 3.3 Colonies of mycoplasma isolates from chicken No. 6S identified serologically as M. columborale x 44.

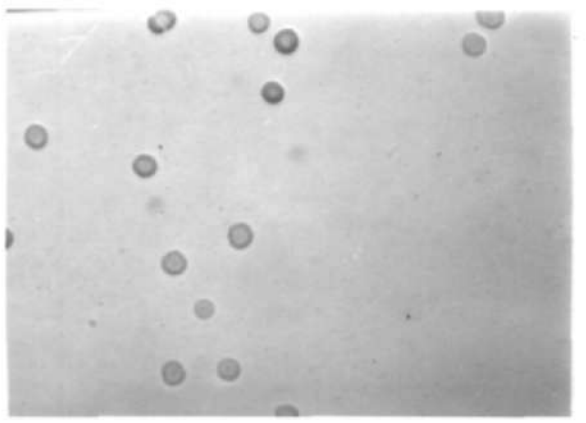


Plate 3.4 Colonies of isolates from chicken No. 2S identified as A. laidlawii x 44.

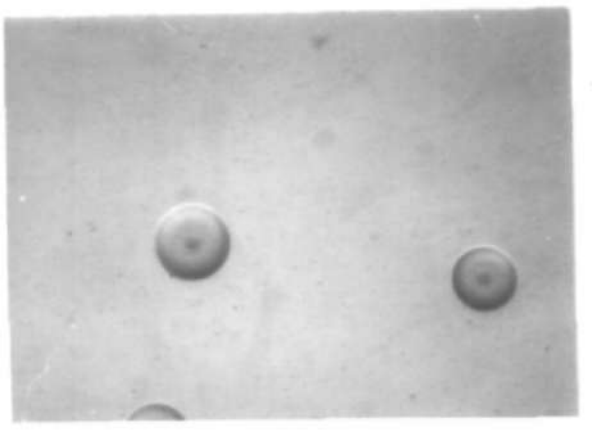


Plate 3.5 Colonies of mycoplasma isolates from chicken No. 93S identified as M. gallinaceum x 44.

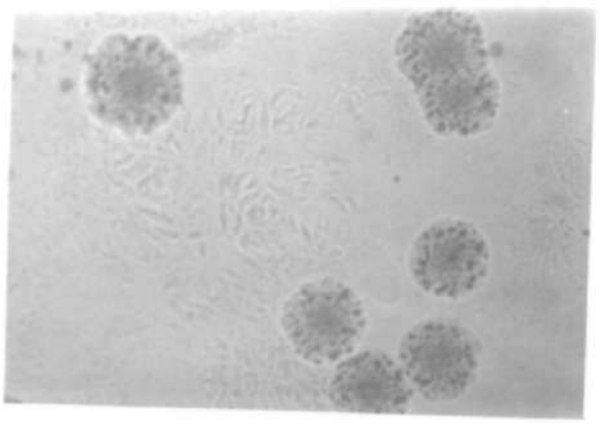


Plate 3.6 Colonies of mycoplasma isolate from chicken No. 20A identified as M. gallinarum. Film and spots formation is illustrated x 44.

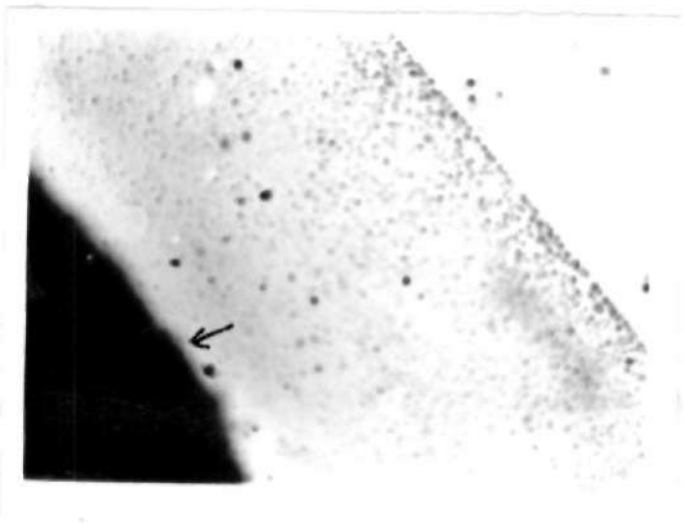


Plate 3.7 Colonies of isolate from chicken No. 25 identified as Acholeplasma laidlawii showing colony growth close to digitonin paper disc. Arrow points at digitonin paper disc.

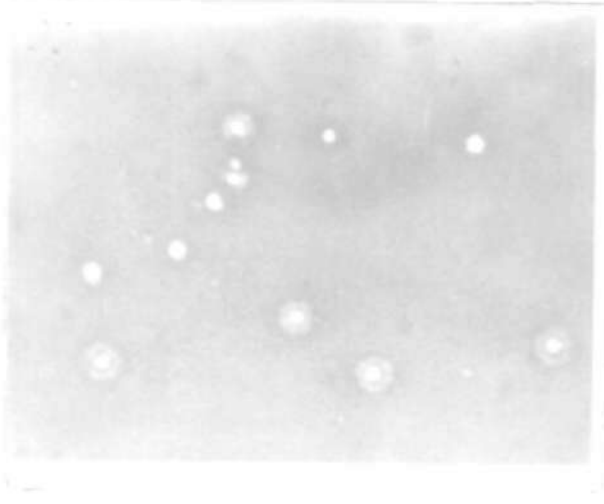


Plate 3.8 Specific Immunofluorescence of A. laidlawii colonies (isolate 2S) on agar piece stained with homologous fluorescent antibody x 79.



Plate 3.9 Specific fluorescence of M. gallisepticum colonies (isolate 7S) on agar piece stained with homologous fluorescent antibody x 125.

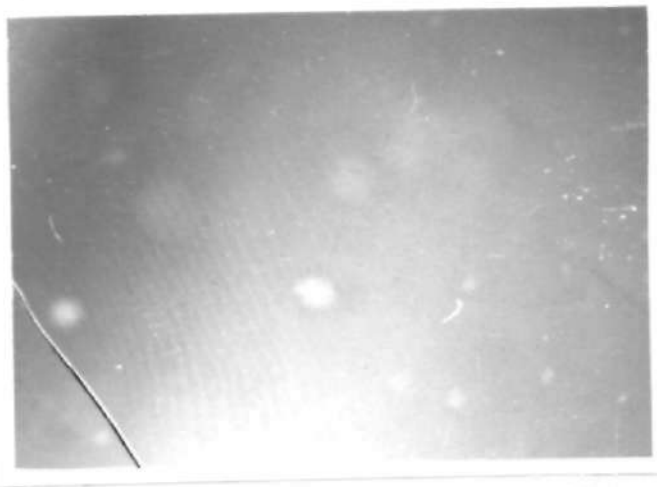


Plate 3.10 Fluorescence of M. synoviae colonies (isolate 41T) on agar piece stained with homologous fluorescent antibody x 79.

3.4 DISCUSSION

By biochemical and serological characterization the isolates consisted of M. gallisepticum (12) M. synoviae (2), M. gallinarum (17), A. laidlawii (7), M. gallopavonis (9), M. columborale (1) M. columbinum (1), all, from the 90 clinically ill chickens. Two of the isolates could not be identified while 9 were lost during cloning and storage before they were fully identified. From the 30 clinically healthy chickens the only 3 isolates made consisted of one each of M. gallinarum, M. gallinaceum, and M. columborale.

In this study the rate of M. gallisepticum (MG) isolation in sick birds was 11.1% in contrast with the findings of Etukudo who in 1982 reported a 0.3% isolation rate of M. gallisepticum in similar birds in the same environment. The medium used contained NAD routinely, which might have improved M. gallisepticum isolation rate. Perhaps, the most important factor is that primary culture tubes and plates were incubated much longer than Etukudo (1982) often as long as 14 days. Two MG isolates (9Sb, 22Sb) were actually recovered and identified from negative agar plates stored at +4^{o}C for three weeks.

Jordan (1983) also recommended the keeping of mycoplasma negative plates for up to 30 days especially for field strains before discarding. Another reason for the desparity between our findings and that of Etukudo (1982) might be the fact that our investigations included examination of the nasal sinus of clinically ill chickens from where we recorded 7 of the M. gallisepticum isolates, wereas this organ was not examined by Etukudo (1982).

M. synoviae was recovered from only 2 cases giving a 2% isolation rate, despite the routine addition of NAD to the media. This is in agreement with Jordan who reported the infrequent recovery of M. synoviae from chicken (Jordan, 1983). Apparently this is the first time M. synoviae recovery is being reported in Nigeria. Etukudo (1982) who worked in the same environment failed to isolate M. synoviae in the few instances when he incorporated NAD in his growth medium.

M. gallisepticum (MG), M. synoviae (MS) and M. meleagridis are the most wide spread pathogenic poultry mycoplasmas (Jordan, 1981). Among these three species, M. gallisepticum and M. synoviae were successfully cultured, but at a rate considered very low since CRD is common among poultry in Nigeria

(Olufarati et al. 1981; Adegboye, 1983). The apparently low isolation rate of the pathogenic species (11.1% for MG and 2% for MS) may be due to the following factors. Firstly, pathogenic mycoplasma species are very fastidious. M. gallisepticum isolation is difficult even from experimentally infected birds let alone from diseased materials (Sahu and Olson, 1976a, b; Whittlestone, 1974). Due to the difficulty encountered in the recovery and propagation of M. synoviae workers have resorted to propagating the organismsin birds (Millinson and Rosenstein, 1976; Vardaman and Droft, 1978). The pathogenic species are slow growers and as such are outgrown, masked and suppressed by fast proliferating non pathogenic species such as M. gallinarum, M. gallopavonis, A. laidlawii etc (Sahu and Olso, 1976a, b). This report which shows M. gallinarum isolation rate (15%) higher than that of the pathogenic species (11.1% for MG, 2% for MS) support the above findings of Sahu and Olson (1976a, b). Etukudo (1982) had similar result with isolation rates of MG and M. gallinarum being 0.3% and 47.5% respectively.

The methods in culturing mycoplasmas in this study may also be a limiting factor. Homogenization of tissue as practiced by some workers (Robert et al. 1967; Amin and Jordan, 1978, Jordan, 1983) which may be

a means of releasing mycoplasmas was not done in this study. This was because the possibility of the release and development of mycoplasmacidal substances during incubation was considered (Adegboye, personal communication).

Repeated freezing and thawing rapidly deplete viability (Smith and Sasaki, 1958; Kelton, 1964) and must be avoided (Leach, 1983). In this study, frequent freezing and thawing of stored cultures resulting from frequent power cuts occurred. This may have led to the low isolation rates of pathogenic mycoplasma got in this study. The process where by primary swabs in broth are stored frozen for a period before processing, as it was in this case, might have led to the loss of many isolates.

The host factor effects are very important for the recovery of pathogenic species of mycoplasmas. For example, sample early in the disease may lead to the recovery of more pathogenic species, probably due to minimal number of secondary invaders and low antibody titre (Whittlestone, 1974). In this study, only one of the flocks (A) had a history of recent infection. In others the farmers simply said CRD had been on for some time and usually they resort to antibiotics medication, with partial success.

Several authors have reported that it is difficult to isolate M. gallisepticum from older birds infected at an early age and are serologically positive (Roberts, 1964a; Yoder, 1978). Majority of the birds used in this study were old layers and broilers, and majority of them, though in different flocks, originate from a large poultry farm with hatcheries that supply day-old chicks to different categories of farmers recently reported to have CRD (Abdu, Bisha, Adesiyun, and Adegboye, 1983). The above factors may have been responsible for the low isolation rate of M. gallisepticum.

Another host factor that may have caused the low isolation rate of the pathogenic species is that of medication. Most farmers frequently rush to treat their flock as soon as CRD signs are noted, using most commonly Tylan (tylosin tartarate) and Coridrol(R) (combination of chlortetracycline, dehydrostreptomycin, chloramphenicol and vitamins). It has been reported that antibiotic treatment of M. gallisepticum infection of poultry results in partial success (Levisohn, 1981; Levisohn and Kleven, 1981) apparently by reducing to minimum the population of the organism. Thus the isolation of this organism is made difficult.

Lastly, cloning procedure may also account for loss of some isolates. This is because the technique of picking one isolated colony out of the multiplicity of other colonies for propagation, has a chance of missing out colonies of pathogenic species.

A. laidlawii, M. gallinaceum, M. gallopavonis, M. columborale and M. columbinum were successfully cultured and identified. These species are apparently being reported for the first time in poultry in Nigeria. M. gallopavonis, M. columborale and M. columbinum were recent additions to avian mycoplasma list.

M. columborale and M. columbinum are reported to be rare in domestic poultry. Several authors suggest that they appear to be restricted to pigeons (Fabricant, 1960; Gerlach, 1977; Shimizu et al. 1978) however these two species were recovered from chickens and identified in this study.

Three of the isolates that were lost before full identification, and one of the untyped isolated showed biochemical reactions resembling M. lipofaciens, a new species of avian origin recently reported by Bradbury et al (1983). The isolates were glucose positive, arginine positive and formed film and spots.

If results of this study are taken into consideration, as many as eighty mycoplasma species have now been report from poultry in Nigeria. These species are: M. gallisepticum, M. synoviae, M. gallinarum, A. laidlawii, M. gallopavonis, M. gallinaceum, M. columborale and M. columbinum. With the use of complete media formulation, and the performance of complete biochemical and serological characterizations, M. synoviae was recovered from poultry in Nigeria, M. gallisepticum isolation report by Etukudo (1982) from same environment was confirmed, and some recently reported new avian species were isolated.

3.5 SUMMARY

Swabs of the nasal sinus, trachea, lung and airsac were taken from each of 90 chickens showing clinical signs of respiratory tract infect and from 30 apparently healthy birds that were killed. The swabs were cultured for mycoplasma using standard medium containing NAD routinely. 64 mycoplasma isolates were made, out of which 41 were from the nasal sinus, 14 from trachea, 6 from airsacs and 3 from the lung. 61 of these isolates were from the clinically ill birds. Only 3 were from the healthy birds.

Biochemical and serological characterization of isolates were carried out using standard reagents to speciate them. This speciation showed 12 to be M. gallisepticum, 2 M. synoviae, 18 M. gallinarum, 9 M. gallopavonis, 2 A. laidlawii, 1 M. gallinaceum, 3 M. columborale and 1 M. columbinum. 2 Mycoplasma isolates were untyped while 9 were lost before full identification. The species M. synoviae, A. laidlawii, M. gallopavonis, M. gallinaceum, M. columborale and M. columbinum are apparently being documented culturally in poultry for the first time in Nigeria.

Chapter IV

SCREENING OF CHICKEN WITH RESPIRATORY PROBLEMS FOR ANTIBODIES TO MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE

4.1 INTRODUCTION

In order to obtain a fairly complete picture of the disease status in chickens used in Chapter 3, it was considered worthwhile to check their serum for antibodies against MG and MS, which are the two most important mycoplasmas to worry about as far as present knowledge is concerned. This is more so, since like Etukudo's earlier report (Etukudo, 1982), the rate of MG isolation did not correlate with seropositivity. While Etukudo (1982) was able to screen his chickens against only MG, efforts were made to secure MS stained antigen as well, so that just as attempts were made to isolate both organisms, the rate of seropositivity to both organisms could also be determined.

4.2 MATERIALS AND METHODS

4.2.1. Serum Samples

90 clinically ill chicken showing signs of respiratory disease and 30 apparently healthy ones from various flocks in Zaria were sacrificed and

blood collected aseptically. The blood was allowed to clot at +4°C overnight and the serum was separated and stored in sterile bijou bottles at -20°C until analysed.

4.2.2. Serological test

The Rapid Plate Agglutination test (RPA) was used as previously described (Anon, 1966). Commercial stained MG and MS antigens (Salsbury Laboratories, Charles City, Iowae, 50616, USA) were kindly supplied by Dr. A. A. Adesiyun (Veterinary Public Health and Preventive Medicine, A.B.U., Zaria). Positive and negative control sera to MG and MS (from Salsbury Laboratory, Charles City, Iowa, 50616, USA) were kindly supplied by Prof. D. S. Adegboye (Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, Nigeria).

The above reagents and the sera to be tested were allowed to warm up to room temperature. Approximately 0.03ml antigen was mixed with 0.02ml of a test serum on a ruled glass plate mounted on a box provided with an oblique light source. The plate was rocked gently for five seconds. Any clumping of the antigen i.e. precipitate formation

within 2 minutes, was noted. Trial positive test samples were run, negative and positive control sera samples were simultaneously ran to ensure that the antigens were still performing satisfactorily each day.

4.3 RESULTS

Out of 90 clinically ill birds screened, 45 (50%) were seropositive for MG, 5 (5.6%) were positive for MS. 3 (3.3%) of the 90 birds were positive for both MG and MS. Of the 30 apparently healthy birds 14 (46.7%) were positive for MG and none positive for MS. Table 4.1 shows the relationship between mycoplasma isolation and seropositivity. 10(11.1%) of the 90 birds yielded MG isolates, while 2 (2.2%) of the 90 birds yielded M. synoviae isolate as reported in Chapter 3.

4.4 DISCUSSION

The seropositivity for MG in the clinically ill birds was 50% while the isolation rate was 11.1%. For the healthy birds seropositivity was 46.7%. (Table 4.1 and Appendix 5) while the isolation rate was 0%. Thus there was no positivie correlation between the presence of antibody in the host and the rate of mycoplasma isolation. Similarly the seropositivity for MS (5.6%) was higher than the isolation rate (2.2%). The RPA test result was rather more related

to clinical and postmortem findings, which agrees with earlier report in the same environment in Nigeria (Etukudo and Adegboye, 1983a).

50% of the clinically ill birds were seropositive for MG. 46.7% of the healthy birds were seropositive for MG. This results compared favourably with that of other authors in Nigeria who reported 69.0% seropositivity for MG among chicken slaughtered at a market in Jos (Onunkwo and Onoviran, 1978); 47.5% in Zaria, Kaduna State (Abdul, Bishu, Adesiyun and Adegboye, 1983); 71.3% in Zaria Area (Etukudo and Adegboye, 1983a) and 61.5% seropositivity for MG also in Zaria market (Kujul, 1983).

The seropositive rate for MS (5.6%) reported in this study does not agree with that of earlier studies in the same environment. Kujul (1983) had a seropositive rate of 80% for MS, Abdu et al (1983) had a seropositive rate of 49.4% for MS. The reasons for the difference can be suggested as follows: perhaps the storage of sera used for this study over a period had detrimental effect on the potency of MS antibody. May be also that the exposure rate of flocks within Zaria to MS infection is on a decline. This gives room for further

Table 4.1

Relationship between Mycoplasma isolation rate and seropositivity for M. gallisepticum and M. synoviae

	Isolation Rate (%)		Seropositivity (%)	
	MG	MS	MG	MS
Clinically ill Chickens	11.1	2.2	50.0	5.6
Healthy Chicken	0	0	46.7	0

investigation. Since the RPA test is depended upon heavily in our environment to provide serological evidence of mycoplasma infection, it is suggested that fresh serum be used at all times to avoid false results.

Since clinical signs/post mortem findings have been shown to be better correlated with RPA test and not with isolation rate, the problem is obviously that of isolation. As this study also shows that, inclusion of more swab sites (e.g. nasal sinus) and longer incubation period of cultures resulted in more pathogenic species being recovered, it is recommended that until more efficient growth medium and improved techniques for avian mycoplasma isolation are introduced, diagnosis of CRD in Nigeria be based mainly on serological test and backed up by isolation where possible.

Chapter V

BACTERIAL AGENTS (OTHER THAN MYCOPLASMA) INFECTING THE RESPIRATORY SYSTEM OF CHICKENS

5.1 INTRODUCTION

The closeness of the upper respiratory tract to the external environment leads to inhalation of many micro-organisms into the body. The natural inhabitants of the respiratory tract are mainly Gram positive organisms (Bisgaard, 1977). It is mainly Gram negative in cases where infectious bronchitis and chronic respiratory disease are incriminated (Bisgaard, 1977, Fabricant, 1969).

The CRD is often of multifarious aetiology involving bacterial, viral and fungal agents (Jordan, 1972). Several bacterial organisms have been incriminated as complicating agents in the disease. *E. coli* occurs most frequently, worldwide (Fabricant, 1969; Garside, 1965; Jordan, 1972, 1975) and has been shown to be noninvasive to the tracheal mucosa unless mixed with mycoplasma or infectious bronchitis virus, producing lesions which are those of primary organism (Garside, 1965; Fabricant, 1969; Jordan, 1972, 1975).

In Nigeria, analytical data of the organisms infecting the respiratory tract of the avian species

are very rare. Apparently only two of such information are available. They are that of Olufarati et al. (1981) who reported the involvement of Staphylococcus aureus, S. epidermidis, E. coli, Proteus sp., Pasteurella gallinarum, Pasteurella haemolytica, Streptococcus sp. Pseudomonas aeruginosa, and Haemophilus gallinarum, from a survey of bacterial organisms infecting the upper respiratory tract of chickens and turkeys in South Western Nigeria (Ondo and Oyo States), and that of Etukudo and Adegboye (1983b) who reported isolating E. coli, Staphylococcus sp., Streptococcus sp., Proteus sp., Bacillus sp., and Salmonella sp. from 580 chickens with respiratory distress in Zaria area, Kaduna state. More of such information and detailed ones, are required in order to establish their possible role and pattern of mixed infections especially with Mycoplasmas in respiratory diseases in Nigeria. To this effect the group of birds used in this study were simultaneously screened for other bacterial organisms.

5.2 MATERIALS AND METHODS

Swabs were taken from the nasal sinus, trachea, lung and air sacs of the 90 clinically sick and 30

healthy birds at the same time swabs for mycoplasma cultures were taken. They were streaked on Blood agar base (Oxoid Ltd., London) supplemented with 10% defibrinated sheep blood. Colonies were identified, confirmed by various biochemical tests, using, MacConkey agar, Eosin Methylene Blue (EMB) agar, Sobitol and human plasma. Identification was by the standard procedure of Cowan and Steel (1970).

5.3 RESULTS

Ten different species of other bacterial organisms were identified from the sick chickens and three from the apparently healthy ones. These are summarized in Table 5.1 and Appendix 6. Isolation rates were as follows: E. coli 36 (40%), Staphylococcus epidermidis 37 (41.1%), Staphylococcus aureus 22 (24.0%), Streptococcus zooepidermicus 29 (32.2%), Proteus sp. 23 (25.6%), Bacillus sp. 11 (12.2%), Pasteurella haemolytica 6 (6.7%), Pseudomonas sp. 6 (6.7%), Pasteurella multocida, 3 (3.3%), and Haemophilus gallinarum 1 (1.1%) for clinically ill birds, and Staphylococcus epidermidis 10 (33.3%), E. coli 3 (10%), and Staphylococcus aurea 2(6.7%). Staphylococcus sp. add up to 59 isolates (65.1%) in sick birds and 12 (40.0%) in healthy birds.

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Table 5.1

Common Bacterial Isolates from Respiratory tract of Chicken with CRD signs and Apparently Healthy ones

Bacterial Isolate	Clinically ill (Total of 90)						Apparently healthy (Total of 30)					
	Nasal sinus	Trachea	Lung	Airsac	Total	%	Nasal sinus	Trachea	Lung	Airsac	Total	%
Staphylococcus epidemidis	10	7	10	10	37	41.1	2	8	-	-	10	33.3
Staphylococcus aureus	7	3	5	7	22	24.4	2	-	-	-	2	6.7
E. coli	16	6	0	5	36	40.0	2	1	-	-	3	10
Streptococcus zooepidemicus	12	7	4	5	29	32.2	-	-	-	-	-	
Proteus sp.	14	5	2	2	23	25.6	-	-	-	-	-	
Bacillus cerus	4	3	2	2	11	12.2	-	-	-	-	-	
Pasteurella haemolytica	1	3	2	-	6	6.7	-	-	-	-	-	
Pseudomonas sp.	4	-	-	2	-	6.7	-	-	-	-	-	
Pasteurella multocida	1	-	2	-	3	3.3	-	-	-	-	-	
Haemophillus gallinarum	-	1	-	-	1	1.1	-	-	-	-	-	

The table shows that Staphylococcus sp. predominantes with 65.6% and 33.3% in both sick and healthy birds, respectively, followed by E. coli with 36 (40%) and 3 (10%) respectively.

Appendix 6 shows the mixed infection pattern of Mycoplasma and other bacteria species isolated from the sick and healthy birds. The table shows that M. gallisepticum occurred more frequently in combination with E. coli (5 cases) than with any other bacteria, followed by the combination of MG and Staph aureus (4 cases).

E. coli occurred alone in 5 cases and in combination with Staph aureus in 7 cases.

5.4 DISCUSSION

Ten different bacteria species were recovered from 90 birds with respiratory disease and 30 healthy ones. Staphylococcus sp. (65.6%) occurred more frequently followed by E. coli (40%). This result is at variance with that of Etukudo and Adegboye (1983b) who reported a predominance of E. coli (55.7%) followed by Staphylococcus sp. (25.2%) from ill chickens from the same environment, and that of Olufarati et al (1981) who also reported

a predominance of E. coli (50.0%) followed by Staphylococcus sp. (25.9%) from diseased birds in Ondo and Oyo states. Few explanations to this differences can be given as follows: Etukudo and Adegboye (1983b) did not include nasal sinus in their study. 30% of the Staphylococcus sp. got in this study was from the nasal sinus. Olufaranti et al. (1981) took swabs from only the trachea and sinus. These workers did not sample the lung and airsac. In this study the sinus, trachea, lung and air sacs were sampled and as such more Staphylococcus sp. may have resulted from sampling the nasal sinus. From this result it may be concluded that staphylococcosis which is reported to be a common problem in turkeys (Domermuth are Gross, 1975; Olufarati et al. 1981) may also be a common problem in chicken in Nigeria.

Appendix 6 shows that the E. coli occurred predominantly with Mycoplasma species especially with M. gallisepticum in diseased birds. A case of synergism may thus be suggested here. This is in agreement with the reports of earlier investigators (Bidde and Cover, 1957; Garside, 1965; Gross, 1961; Olufarati et al. 1981, Etukudo and Adegboye, 1983b). Garside (1965) specifically

reported that E. coli was unable to invade the respiratory tract unless the latter was previously primed by the presence of Mycoplasma or infectious bronchitis virus. He was able to make only two isolates of MG from the 227 batches of birds examined, irrespective of the fact that most of the birds were seropositive for mycoplasma.

M. synoviae in the two cases it occurred were in combination with Pasteurella haemolytica in one instance and with Streptococcus zooepidermicus in the other. This may not be of any significance.

The multiplicity of various other bacteria recovered from the respiratory system had no definite pattern of occurrence. This mixed aetiological agents of respiratory disease makes the diagnosis very difficult. As much as 50% of clinically ill birds were seronegative for MG and as much as 88.9% of them were culturally negative for MG, yet they all showed CRD signs suggesting that other bacteria agents must be very much involved. E. coli has been reported to be capable of causing airsacculitis (Gross, 1957; Harry, 1964). Caution should therefore be exercised in clinical diagnosis and treatment of diseases affecting the respiratory tract of chickens, since Mycoplasma sp. is not always involved.

Chapter VI

GENERAL DISCUSSION

This study has provided further cultural evidence of the involvement of M. gallisepticum in poultry respiratory disease in the Zaria area, i.e. in addition to the first report of isolation of the organism in Nigeria (Etukudo and Adegboye, 1983a). While the latter workers reported a rate of 0.3% by isolation, a rate of 11.1% by isolation, i.e. 10 strains from 90 clinically ill birds, is hereby reported. It was noted that if the culture medium is satisfactory, patience with the duration of incubation is a major factor for successful culture of MG from clinical cases. None of the M. gallisepticum strains encountered in this study produced colonies on primary agar plates under 4 days of incubation. Infact, with the medium used, if colonies were obtained on primary plates within 4 days, it was possible to predict that the Mycoplasma growing was not M. gallisepticum. Jordan (1983) had earlier emphasized the need for long incubation periods.

The seropositive rate obtained for M. gallisepticum in the present study was 50%. Etukudo and Adegboye (1983a) recorded 71.3%, Abdul et al (1983) recorded 47.5% in clinically affected flocks,

while Onunkwo and Onoviran (1978) recorded 69.0% and Addo (1976) recorded 32.4% in randomly slaughtered chickens. Thus for the purpose of policy formulation, it appears that one can put the rate of infection of poultry population in northern part of Nigeria at approximately 65% by rapid plate agglutination test. Moreover, one can only conclude, like Etukudo (1982) that, with currently used media formulations, rate of mycoplasma isolation is not a true reflection of rate of M. gallisepticum and M. synoviae infection in a flock.

In view of the usual poor isolation rate of MS and the reports that M. synoviae (MS), traditionally associated with poultry arthritis, might play a pathogenic role in respiratory illness in poultry, efforts were made to ensure that the NAD required by MS was routinely added. Even then, it was disappointing that only 2 isolations were made from 90 clinically affected birds, even though rate of seropositivity by the RPAT was 5.6%. A reference strain was also rapidly lost after first passage. Thus it seems the same type of conclusion drawn on MG isolation is applicable to MS. Efforts should be intensified to improve the isolation of these pathogenic mycoplasmas since the problem of non-specificity or cross-reactivity often encountered

with serodiagnosis, even with the sensitive ELISA test (Whittlestone, 1984) obviously does not pose as much problem to diagnosis by culture.

The pursuit of one of the objectives of this study, i.e. to isolate and fully characterize any Mycoplasma sp. encountered led to documentation of M. synoviae, A. laidlawii, M. gallopavonis, M. columborale, M. columbinum and M. gallinaceum, apparently for the first time in Nigeria. M. columborale, M. columbinum, M. gallopavonis were avian mycoplasma species recently accepted by the subcommittee on Taxonomy of Mycoplasmas (Anon, 1979). The pathogenic potential of these isolates for poultry is unknown. However another new member, M. iowae was reported to be able to cause airsacculitis in poults, tenosynovitis, and rupture of digital flexor tendon if inoculated into foot pads of day old chicks and turkeys.

Finally, it is gratifying that the project has exposed the author to problems associated with the diagnosis of poultry mycoplasma infection. The general procedure involved with the culturing and characterization of avian mycoplasmas, or any mycoplasma infection for that matter, has also

been mastered, so are some serodiagnostic techniques. The knowledge thus acquired would be of immense use to the author in his job as a Research Officer at the Mycoplasma Laboratory, National Veterinary Research Institute, Vom, Plateau State, Nigeria.

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APPENDICES

Serological characterization of isolates
by the Growth Inhibition test ..... Appendix 1

Serological characterization of isolates
by the Immunoflourescent test ..... Appendix 2

Summary of biochemical and serological tests
on isolates ..... Appendix 3

Pathological lesions and *Mycoplasma*
species recovered from various sites ..... Appendix 4

Relation between Mycoplasma isolation and
seropositivity for *M. gallisepticum* and
M. synoviae ..... Appendix 5

Mixed infection pattern of *Mycoplasma*
species and other bacteria species
isolated from CRD cases and from
apparently healthy chickens ,..... Appendix 6

APPENDIX 1

Serological Characterization of Isolates by the Growth Inhibition Test*

Isolate No.	Zone of inhibition of each isolate with Reference Mycoplasma Antisera*										Tentative Identification
	M. gallisepticum	M. synoviae	M. gallinarum	M. gallopavonis	M. gallinaceum	M. columboorale	M. columbinum	M. columbinasale	M. iowae	M. iners	
2B	0	0	0	0	0	0	0	0	0	0	Acholeplasma sp
2A	0	0	0	0	0	0	0	0	0	0	Acholeplasma sp
4Ta	"	"	2.5	2.5	"	"	"	"	"	"	M. gallopavonis
4Tb	"	"	2.5	2.5	"	"	"	"	"		M. gallinarum
5S	"	"	1.5	2.0	"	"	"	"	"	"	M. gallinarum
6Sa	"	"	2.5	2.0	"	"		"	"	"	M. gallinarum
6Sb	"	"	0	2.5	"	2.5	"	"	"	"	M. gallopavonis
6Sc	"	"	0	0	"	3.0	"	"	"	"	M. columborale
7S	3.0	"	0	2.9	"	0	"	"	"	"	M. gallisepticum
9Sa	0	"	3.5	0	0	0	0	0	0	0	M. gallinarum
9Sb	3.0	"	0	2.9	"	"	"	"	"	"	M. gallisepticum
9Sc	0	"	2.0	2.0	"	2.5	"	"	"	"	M. columborale
15S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
16S	"	"	"	"	"	"	"	"	"	"	
17L	"	"	"	"	"	"	"	"	"	"	
20A	0	0	3.0	0	0	0	0	0	0	0	M. gallinarum
21Sa	0	"	2.9	"	"	"	"	"	"	"	M. gallinarum
21Sb	2.5	"	0	"	"	2.0	"	"	"	"	M. gallisepticum
22Sa	0	"	3.0	2.0	2.0	2.0	"	"	"	"	M. gallopavonis
22Sb	2.9	"	0	0	0	0	0	0	0	0	M. gallisepticum
23Sa	3.0	3.0	0	0	0	0	0	0	0	0	M. gallisepticum
23Sb	0	"	"	0	0	0	0	0	0	0	Acholeplasma sp
24T	3.0	"	"	2.0	"	"	"	"	"	"	M. gallisepticum

*Number indicate zone of inhibition in mm.

Appendix 1 (cont.)

25S	4.0	"	"	2.0	"	"	"	"	"	"	M. gallisepticum
25T	3.0	"	"	2.0	"	"	"	"	"	"	
25L	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
25A	3.0	0	0	2.0	0	0	0	0	0	0	M. gallisepticum
26A	3.0	0	0	0	"	2.0	"	"	"	"	M. gallisepticum
28S	0	0	2.9	2.0	"	3.0	"	"	"	"	M. gallopavonis
28T	"	"	0	0	"	0	"	"	"	"	Acholeplasma sp.
29S	"	"	3.0	2.0	"	"	"	"	"	"	M. gallopavonis
31S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
31T	0	0	3.0	2.0	0	3.0	0	2.0	0	0	M. gallinarum
32S	"	"	4.0	2.5	"	3.0	"	0	"	"	M. gallinarum
41S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
41T	0	3.0	0	3.0	0	0	0	0	0	●	M. synoviae
32S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	
42L	"	"	"	"	"	"	"	"	"	"	
44S	"	"	"	"	"	"	"	"	"	"	
48Sa	0	0	3.0	2.0	0	0	0	0	0	0	M. gallinarum
48Sb	"	"	3.0	2.0	2	3.5	"	"	"	"	M. gallopavanis
49S	"	"	4.0	"	"	"	"	"	"	"	M. gallinarum
50S	"	"	3.0	1.5	"	2.0	"	"	"	"	M. gallinarum
50T	"	"	3.0	1.5	"	2.0	"	"	"	"	M. gallinarum
52A	"	"	0	0	"	0	"	"	"	"	Acholeplasma sp.
59Sa	"	"	2.5	2.5	"	"	"	"	"	"	M. gallinarum
59Sb	"	3.2	0	0	"	"	"	"	"	"	M. synoviae
60S	"	"	2.5	3.0	"	2.5	"	"	"	"	Untyped
64A	"	"	0	0	"	0	"	"	"	"	Acholeplasma sp.
86S	"	"	"	3.5	0	0	2.5	"	"	"	M. columbinum
87S	"	"	"	2.9	"	2.2	0	"	2.5	"	Untyped
89S	"	"	3.0	4.0	3.0	0	"	"	0	"	M. gallinarum
93T	"	"	2.5	3.0	0	3.2	"	"	"	"	M. gallinarum

Appendix 1 (cont.)

93Tb	"	"	0	0	0	3.2	"	"	"	"	M. columborale
93S	"	"	0	"	2.0	0	"	"	"	"	M. gallinaceum
94T	"	"	3.0	"	0	"	"	"	"	"	
100T3.1	"		0	"	"	"	"	"	"	"	M. gallisepticum
102S 0	"		3.0	2.5	"	2.0	"	"	"	"	M. gallinarum
102A2.9	"		0	2.8	"	0	"	"	"	"	M. gallisepticum
107S 0		0	0	3.5	0	3.8	0	0	3.5	0	M. gallopavonis
108T	"	"	"	0	"	0	0	0	0	0	Acholeplasma
109S	"	"	"	3.5	"	3.0	"	"	3.0		M. gallopavonis
116S	"	"	3.5	3.5	"	3.0	"	"	3.0		M. gallopavonis
PG16	"	"	3.0	2.4	"	2.5	"	"	2.6		Reference strain
PG312.9	0	0	2.9	"	2.9	"	"	2.7			Reference strain
WVU1853	3.0	0	0	"	2.9	"	"	0			Reference strain

NT = Not Tested, lost before full identification.

APPENDIX 2

Serological Characterisation of Isolates by the Immunofluorescent test

Isolate No.	Fluorescein labelled conjugate						Inference
	M. gallisepticum (PG31)	M. synoviae WVU1853	M. gallinarum PG16	M. iowae 695	A. laidlawii PG9	M. anatis 1340	
2S	-	-	-	-	++	-	A. laidlawii
2A	-	-	-	+	+	-	A. laidlawii
4Ta	-	-	-	-	-	-	M. gallopavonis
4Tb	-		++	-	-	-	M. gallinarum
5S	-	-	+	-	-	-	M. gallinarum
6Sa	-	-	+	-	-	-	M. gallinarum
6Sb	-	-	-	-	-	-	M. gallopavonis
6Sc	-	-	-	-	-	-	M. columborale
7S	++	-	-	-	-	-	M. gallisepticum
9Sa	-	-	+	-	-	-	M. gallinarum
9Sb	+	-	-	-	-	-	M. gallisepticum
9Sc	-	-	-	-	-	-	M. columborale
15S	NT	NT	NT	NT	NT	NT	
16S	"	"	"	"	"	"	
17L	"	"	"	"	"	"	
20A	-	-	+	-	-	-	M. gallinarum
21Sa	-	-	+	-	-	-	M. gallinarum
22Sa	-	-	-	-	-	-	M. gallopavonis
22Sb	+	-	-	-	-	-	M. gallisepticum
23Sa	+	-	-	-	-	-	M. gallisepticum
23Sb	-	-	-	-	+	-	A. laidlawii

Appendix 2 (cont.)

24T	+	-	-	-	-	-	M. gallisepticum
25S	+	-	-	-	-	-	M. gallisppticum
25A	+	-	-	-	-	-	M. gallisepticum
25T	+	-	-	-	-	-	M. gallisepticum
25L	NT	NT	NT	NT	NT	NT	
26A	+	-	-	-	-	-	M. gallisepticum
28S	-	-	-	-	-	-	M. gallopavonis
28T	-	-	-	-	+	-	Acholeplasma Laidlawii
29S	-	-	-	-	-	-	M. gallopavonis
31S	NT	NT	NT	NT	NT	NT	
31T	-	-	+	-	-	-	M. gallinarum
41T	-	+	-	-	-	-	M. synoviae
42S	NT	NT	NT	NT	NT	NT	
42L	"	"	"	"	"	"	
44S	"	"	"	"	"	"	
48Sa	-	-	+	-	-	-	M. gallinarum
48Sb	-	-	+	-	-	-	M. gallopavonis
49S	-	-	+	-	-	-	M. gallinarum
50S	-	-	+	-	-	-	M. gallinarum
50T	-	-	+	-	-	-	M. gallinarum
52A	-	-	-	-	+	-	A. laidlawii
59Sa		+	++				M. gallinarum
59Sb	+	+					M. synoviae
60S	-	-	-	-	-	-	Untyped
64A	-	-	-	-	+	-	A. laidlawii
86S	-	-	-	-	-	-	M. columbinum
87S	-	-	-	-	-	-	Untyped
89S	-	-	++	-	-	-	M. gallinarum
89T	-	-	++	-	-	-	M. gallinarum
93S	-	-	-	-	-	-	M. gallinaceum

Appendix 2 (cont.)

93Ta	-	-	+	-	-	-	M. gallinarum
93Tb	-	-	-	-	-	-	M. columborale
94Tb	-	-	-	-	-	-	M. gallinarum
100T	++	-	-	-	-	-	M. gallisepticum
102A	+	-	-	-	-	-	M. gallisepticum
102S	-	-	+	-	-	-	M. gallinarum
107S	-	-	-	-	-	-	M. gallopavonis
108T	-	-	-	-	+	-	A. laidlawii
109S	-	-	-	-	-	-	M. gallopavonis
116S	-	-	-	-	-	-	M. gallopavonis
120S	-	-	+	-	-	-	M. gallinarum
PG31	++	-	-	-	-	-	Reference strain
PG16	-	-	++	-	-	-	Reference strain
WVU1853	-	+	-	-	-	-	Reference strain
PG9	-	-	-	-	++	-	Reference strain

Immunofluorescence was graded from

0 (-) to 2+ in comparison with homologous control.

APPENDIX 3

Biochemical and Serological tests of isolates

Isolate No.	Biochemical Tests				Identification by		Final Identification
	Glucose	Arginine	Digitonin	F&S	GI Test	FA Test	
2S	++	-	-	-	-	d	Acholeplasma laidlawii
2A	+	-	-	-	-	"	"
4Ta	+	-	+	-	e	-	M. gallopavonis
4Tb	-	+	+	+	c	c	M. gallinarum
5S	-	+	+	+	c	c	M. gallinarum
6Sa	-	+	+	+	c	c	M. gallinarum
6Sb	+	-	+	-	e	NT	M. gallopavonis
6Sc	+	-	+	-	g	-	M. columborale
7S	++	-	++	-	a	a	M. gallisepticum
9Sa	-	+	+	+	c	c	M. gallinarum
9Sb	+	-	+	-	a	a	M. gallisepticum
9Sc	+	-	+	-	g		M. columborale
15S	+	-	+	+	NT	NT	DI
16S	+	+	+	+	"	"	DI
17L	-	+	+	+	"	"	DI
20A	-	+	+	+	c	c	M. gallinarum
21Sa	-	+	+	+	c	c	M. gallinarum
21Sb	+	-	+	-	a	a	M. gallisepticum
22Sa	+	-	+	-	e	-	M. gallopavonis
22Sb	+	-	+	-	a	a	M. gallisepticum
23Sa	++	-	+	-	a	a	M. gallisepticum
23Sb	++	-	-	-	NT	d	A. Laidlawii
24T	+	-	+	-	a	a	M. gallisepticum
25S	++	-	+	-	a	a	M. gallisepticum

Appendix 3 (conti.)

25A	++	-	+	-	a	a	M. gallisepticum
25T	++	-	+	-	a	a	M. gallisepticum
25L	-	-	+	-	NT	NT	DI
26A	+	-	+	-	a	a	M. gallisepticum
28S	+	-	+	-	e	-	M. gallopavonis
28T	+	-	-	-	NT	d	A. laidlawii
29S	+	-	+	-	a	a	M. gallopavonis
31S	-	-	+	+	NT	NT	DI
31T	-	+	+	-	c	c	M. gallinarum
32S	-	+	+	+	c	c	M. gallinarum
41S	+	+	+	+	NT	NT	DI
41T	+	-	+	+	b	b	M. synoviae
42S	+	+	+	+	NT	NT	DI
42L	=	±	±	+	"	"	DI
44S	-	+	+	±	"	"	DI
48a	-	+	+	+	c	c	M. gallinarum
48Sb	+	-	-	+	e	-	M. gallopavonis
50S	-	+	+	+	c	c	M. gallinarum
50T	-	+	+	+	c	c	M. gallinarum
49S	-	+	+	+	c	c	M. gallinarum
52A	++	-	+	-	NT	d	A. laidlawii
59Sa	-	-	+	-	c	c	M. gallinarum
59Sb	-	-	+	-	b	b	M. synoviae
60S	-	-	+	+	-	-	Untyped
64A	+	-	-	-	NT	d	A. laidlawii
86S	-	+	+	+	h	-	M. columbinum
87S	±	+	+	+	-	-	Untyped
89S	-	+	+	-	c	c	M. gallinarum
89T	-	+	+	-	c	c	M. gallinarum
93S	+W	-	+	-	f	-	M. gallinaceum
93Ta	-	+	+	+	c	c	M. gallinarum

Appendix 3 (cont.)

93Tb	+	-	+	-	g	-	M. columborale
94T	+W	+	+	+	c	c	M. gallinarum
100T	+	-	+	-	a	a	M. gallisepticum
102S	-	+	+	+	c	c	M. gallinarum
102A	+	-	+	-	a	a	M. gallisepticum
107S	+	-	+	-	e	-	M. gallopavonis
108T	++	-	-	-	NT	d	A. laidlawii
109S	+	-	+	-	e	-	M. gallopavonis
120S	-	+	+	+	c	c	M. gallinarum
116S	+	-	+	-	e	-	M. gallopavonis

a = M. gallisepticum
b = M. synoviae
c = M. gallinarum
d = A. laidlawii
e = M. gallopavonis
f = M. gallinaceum
g = M. columborale
h = M. columbinum
NT = Not Tested
DI = lost before full identification

APPENDIX 4

Pathological lesions and Mycoplasma species recovered from various sites

Bird Serial No.	Pathological lesions	Mycoplasma species recovered per site			
		Nasal sinus	Trachea	Lung	Air Sac
1	Tracheitis	d			d
2	Tracheitis	-	-	-	-
3	Tracheitis, Airsacculitis		c,e,		
4	NGL	c			
5	"	c,e,g			
6	"				
7	"	DI			
8	"	DI			
9	Tracheitis, pneumonitis air-sacculitis	a			
10	Tracheitis	-	-	-	-
11	Pneumonitis	e	-	-	-
12	"				
13	"				
14	Tracheitis, Air-sacculitis	c,			
15	Tracheitis, Air-sacculitis	Un			
16	Tracheitis, pneumonitis				
17	Airsacculitis	c			
18	Tracheitis				
19	NGL	a			
20	Airsacculitis				
21	NGL				

Appendix 4 (cont.)

22	Fibrinous Air-sacculitis	c,a,g	
23	Tracheitis, Pneumonitis		
24	Airsacculitis		
25	NGL		
26	Sinusitis		DI
27	Tracheitis, Sinusitis		
28	Tracheitis, Airsacculitis		
29	Tracheitis, Pneumonitis	a,e	
30	Tracheitis, Pneumonitis		
31	Tracheitis, Pneumonitis		
32	NGL		
33	NGL		
34	Tracheitis, Pneumonitis		
35	Tracheitis, Pneumonitis		
36	Tracheitis, Pneumonitis		
37	NGL		e
38	Tracheitis, Airsacculitis		
39	Pneumonitis		
40	Airsacculitis		
41	Airsacculitis	,a	
42	Pneumonitis, airsacculitis	a,e	
43	Tracheitis, Peritonitis	a,d	

Appendix 4 (cont.)

44	Airsacculitis		a		
45	NGL	a	a	a	DI
46	NGL	DI	•		
47	NGL	c			
48	Airsacculitis, Peritonitis				
49	Airsacculitis				
50	Tracheitis, Sinusitis				
51	Airsacculitis				
52	Airsacculitis				
53	Airsacculitis				
54	Airsacculitis				
55	Airsacculitis				
56	Airsacculitis, Peritonitis	DI	b		
57	Airsaccultis, Peritonitis	DI			DI
58					
59	Airsacculitis, Peritonitis	DI			
60	Sinusitis, Tracheitis	b,c			
61	Tracheitis	Un			
62	Tracheitis				
63	Pneumonitis, Airsaccultis	•,		a	
64	Airsaccultis				
65	NGL				
66	NGL				
67	Airsacculitis, Sinusitis				
68	Pneumonitis	e	d		
69	Pneumonitis				
70	Tracheitis	e			

Appendix 4 (cont.)

71	Sinusitis, Pneumonitis	
72	Pneumonitis, Airsacculitis	
73	Pneumonitis, Airsacculitis	
74	Pneumonitis, Airsacculitis	
75	Tracheitis	
76	Tracheitis	d
77	Sinusitis, Tracheitis	
78	Sinusitis, Tracheitis	
79	Pneumonitis, Airsacculitis	
80	Haemorhagic syndrome	
81	Haemorhagic syndrome	
82	Haemorrhagic syndrome	
83	Tracheitis, airsacculitis	d
84	Sinusitis Tracheitis	
85	Tracheitis	
86	Peritonitis	c
87	Sinusitis, pneumonia, airsacculitis	
88	Airsacculitis	a
89	NGL	
90	NGL	
91	NGL	
92	NGL	
93	NGL	
94	NGL	

Appendix 4 (cont.)

95	NGL		
96	NGL		
97	NGL		
98	NGL		
99	NGL		
100	NGL		
101	NGL		
102	NGL		
103	NGL		
104	NGL		
105	NGL		
106	NGL		
107	NGL		
108	NGL		
109	NGL		
110	NGL		
111	NGL		
112	NGL		
113	NGL		
114	NGL		
115	NGL		
116	NGL		
117	NGL	f	g,c
118	NGL		
119	NGL		
120	NGL		

a = M. gallisepticum
b = M. synoviae
c = M. gallinarum
d = A. laidlawii
e = M. gallopavonis
f = M. gallinaceum
f = M. columborale
h = M. columbinum
NGL = No gross lesion
DI = Dead before full identification
Un = Untyped isolates.

APPENDIX 5

Relationship between Mycoplasma Isolation and Seropositivity for M. gallisepticum and M. synoviae

	Serial No	Mycoplasma Isolated MG OR MS	Seropositivity (Rapid Plate Agglutination Test) MG	MS
	1		-	-
	2		-	-
	3		+	+
	4		+	-
Clinically ill	5		-	-
	6		+	-
	7		+	-
birds	8		+	-
	9	M. gallisepticum	+	-
	10		+	-
	11		-	-
	12		+	-
	13		-	-
	14		+	-
	15		+	-
	16		++	-
	17		++	-
	18		-	-
	19	M. gallisepticum	+	-
	20		+	-
	21		NT	NT
	22	M. gallisepticum	+	-
	23		-	-
	24		-	-
	25		-	-
	26		+	-

Appendix 5 (cont.)

Serial No.	Mycoplasma Isolated	Seropositivity (Rapid Plate Agglutination Test)	
	MG or MS	MG	MS
27		+	+
28		++	-
29		-	-
30		+	-
31		-	-
32		-	-
33		-	-
34		+	-
35		+	-
36		-	-
37		-	-
38		+	-
39		±	-
40		+	-
41	M. gallisepticum	+	-
42	M. gallisepticum	++	-
43	M. gallisepticum	++	-
44	M. gallisepticum	+	+
45	M. gallisepticum	+	-
46		-	-
47		-	-
48		+	-
49		-	-
50		+	-
51		+	-
52		-	-
53		-	-
54		-	-

Appendix 5 (cont.)

Serial No	Mycoplasma Isolated	Seropositivity (Rapid Plate Agglutination Test)	
		MG	MS
55		-	-
56	M. synoviae	-	+
57		-	-
58		+	-
59		+	-
60	M. synoviae	-	+
61		-	-
62		+	-
63	M. gallisepticum	NT	NT
64		-	-
65		-	-
66		-	-
67		+	-
68		+	-
69		±	-
70		-	-
71		-	-
72		-	-
73		-	-
74		-	-
75		-	-
76		-	-
77		-	-
78		-	-
79		-	-
80		+	-
81		-	-
82		-	-
83		+	-

Appendix 5 (cont.)

	Serial No	Mycoplasma Isolated MG or MS	Seropositivity Rapid Plate Agglutination Test MG	MS
	84		-	-
	85		+	-
	86		+	-
	87		++	-
	88		+	-
	89	M. gallisepticum	+	-
	90		+	-
		Subtotal =	44	5
Apparently	91		++	-
Healthy	92		+	-
birds	93		+	-
	94		-	-
	95		++	-
	96		-	-
	97		++	-
	98		+	-
	99		-	-
	100		-	-
	101		-	-
	102		-	-
	103		-	-
	104		-	-
	105		-	-
	106		-	-
	107		++	-
	108		+	-
	109		++	-
	110		+	-
	111		+	-
	112		++	-

Appendix 5 (cont.)

Serial No	Mycoplasma Isolation MG or MS	Seropositivity (Rapid Plate Agglutination Test)	
		MG	MS
113		-	-
114		-	-
115		-	-
116		-	-
117		+	-
118		-	-
119		+	-
120		-	-
	Subtotal	14	0
	Grand total	58	5

NT = Not tested

Appendix 5 (cont.)

Infection pattern Mycoplasma sp. and Bacterial agents	No. of Cases III Chickens (90)	No. of Cases Healthy Chickens (30)
M. gallinarum + Staph aureus + Strep zooepidermicus	1	
M. gallinarum + Strep zooepidermicus	4	
M. gallinarum + Staph aureus + Pseudomonas acruginosa	1	
M. gallinarum + Proteus sp.	1	
M. gallinarum + Bacillus sp.	1	
A. laidlawii + E. coli	2	
A. laidlawii + E. coli + Past. haemolytica	1	
A. laidlawii + staph. aureus	1	
A. laidlawii + Staph aureus + Strep. Zooepidermicus	1	
A. laidlawii + Strep. zooepidermicus + Proteus sp.	1	
M. gallopavonis + E. coli	-	
M. gallopavonis + Staph aureus	3	
M. gallopavonis + Staph. aureus + Bacillus sp.	1	
M. gallopavonis + Staph. aureus + Pasteurella multocida	1	
M. gallopavonis + Step. zooepidermicus	1	

Appendix 6 (Contd)

Infection pattern *Mycoplasma* sp. and Bacterial agents	No. of Cases ill Chickens (90)	No. of Cases Healthy Chickens (30)
Untyped Mycoplasma + *Pseudomas aeruginosa*	1	
Untyped Mycoplasma + *E. coli* + *Bacillus* sp. + *Strep. zooepidermis*	1	
E. coli alone	5	
E. coli + *Staph aureus*	7	
E. coli + *Strep. zooepidermicus*	1	
E. coli + *Pasteurella haemolytica*	1	
Staph aureus alone	8	
Staph aureus + *Strep zooepidermicus*	9	
Staph aureus + *Proteus* sp.	2	
Pasteurella multocida + *Staph. aureus*	2	
Pasteurella multocida alone	1	