CULTURAL AND SEROLOGICAL STUDIE3

ON CHRONIC RESPIRATORY DISEASE

IN SOME POULTRY FLOCKS

BY

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D E C L A R A T I O N

I hereby declare that this piece of work was carried out by me in the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria under the supervision of Dr. D. S. Adegboye, Prof. C. N. Chineme and Dr. J. M. Olomu.

The work is original and has not already been submitted in any form to any other university. Where use has been made of the published work of others, this is acknowledged in the appropriate parts of the text and in the list of references at the end of the dissertation.

.....
Bassey O. Etukudo

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C H A P T E R I

GENERAL INTRODUCTION

1.1. CHRONIC RESPIRATORY DISEASE (CRD):

The CRD of chickens is typically a disease with a long incubation period (1 - 3 weeks) and a long course caused primarily by Mycoplasma gallisepticum. The disease is characterized clinically by sneezing, nasal discharges, sporadic coughing and respiratory rales (Fabricant, 1969; Rosenfeld, 1971). In young chicks, the disease is usually mild and often clinically inapparent. In adult birds, CRD tends to be of longer duration and greater severity and may result in lowered egg production, high embryo mortality and lowered hatchability (Fabricant, 1969; Yoder, 1978).

1.2. MYCOPLASMAS IN GENERAL:

Mycoplasmas are the smallest free-living microorganisms known (Anon, 1974). They represent a group of small prokaryotes which do not have a rigid cell wall (Levisohn and Razin, 1973) but are rather bounded by a limiting tripple membrane (Uppal and Chu, 1977; Tajima, Nunoya and Yagihashi, 1979). Mycoplasmas exhibit considerable pleomorphism and are usually spherical or coccoid (Rosenfeld, 1971; Tully, 1978) as well as coccobacilliform (Abu-Zahr and Butler, 1978) bodies.

Ring forms, globles or filaments are sometimes present (Stalheim, 1979). The largest diameters range between 300 and 800 n m (Tully, 1978; Tajima et al.1979). These organisms appear to have a predilection for moist mucosal surfaces and are most often isolated from the respiratory tract, oral cavity, urogenital tract, and joints of man and animals (Fabricant, 1969).

1. 3. HISTORICAL CONCEPT:

Nocard and Roux (1898) made a history through the discovery of the etiological agent of a highly infectious disease of cattle - The Contagious Bovine Pleuropneumomia. The causative agent was then referred to as the Pleuropneumonia Organism (PPO). Repeated attempts to cultivate the infectious agent on the common bacteriological media known then met with failure. Successful cultivation finally was achieved by inoculating bouillon with infectious fluid, placing this in a colloidion sac and inserting it into the peritoneal cavity of rabbits. This led to the isolation and description of similar organisms with cellular and colonial morphology of the agent of bovine pleuropneumonia(Borrel, Dujardin - Beaumetz, 1900; Bordet, 1910; Dujardin - Beaumetz, 1900).

The lack of an acceptable classification scheme led to their being called Pleuropneumonia - like Organisms (PPLO). Unfortunately, much confusion arose from the use of these terms and symbols; therefore, Nowak (1929) proposed the now accepted generic name Mycoplasma, which was not officially adopted until after 1956 (Edward and Freundt, 1956; Freundt, 1957).

Consequently, the isolation or cultivation of Mycoplasma was later reported from other animal species including the sheep (Bridre and Donatien, 1923), dog (Shoetensack, 1934), guinea pig (Klieneberger, 1935), fowl (Nelson, 1936), rats (Ito, Imaizumi, Tajima, Endo and Koyama, 1957), duck (Roberts, 1964c), pig (Goodwin et al, 1965; Whittlestone, 1967), and cat (Cole, Golightly and Ward, 1967). Laidlaw and Elford (1936) reported the isolation of a saprophytic mycoplasma from sewage; this was named M. laidlawii, but has now been identified as belonging to a related genus, Acholeplasma and hence renamed A. laidlawii.

The first human Mycoplasma was isolated by Dienes and Edsall (1937) and much later came the reports of the cultivation of M. pneumoniae by Marmion and Goodburn (1961) and Chanock, Hayflick and Barile (1962).

In 1956, Shepard recognized the T-strains (T meaning tiny colonies) which affected the genitourinary tract of man (Shepard, 1956).

1. 4. CLASSIFICATION:

The current basis for the classification and nomenclature of mycoplasmas was proposed in 1956 (Edward and Freundt, 1956), and this scheme has been modified and extended with international cooperation as new information became available. In 1967, a recommended extension of the system placed the mycoplasmas in a new and separate class,the Mollicutes (Latin, meaning "soft and pliable skin") (Edward and Freundt, 1967) of the order Mycoplasmatales. The generally accepted taxonomic position of the mycoplasmas is as follows (Tully, 1978):

Class: Mollicutes

Order: Mycoplasmatales

Family: Mycoplasmataceae .. Genus: 1. Mycoplasma
2. Ureaplasma

Family: Acholeplasmataceae.. Genus: Acholeplasma

Family: Spiroplasmataceae .. Genus: Spiroplasma

The criteria for consideration as members of the Order Mycoplasmatales is based on morphology, ability to pass through a 450 nm membrane and absence of reversion

to a bacterium in medium without penicillin and thallium acetate (Fabricant, 1969; International Committee, 1972).

Classfication within the families of Mycoplasmataceae and Acholeplasmataceae has been based on cholesterol requirement (Edward, 1971), resistance to digitonin and sodium polyethanol sulphonate (Erno and Stipkovits, 1973 b), ability to decompose glucose, mannose, galactose, cellobiose, xylose, sorbitol, arginine and urea (Erno and Stipkovits, 1973 b), phosphatase production (Aluotto, Wittler, Williams and Faber, 1970), film and spot formation (Edward, 1950; Rottem and Razin, 1964), serum digestion (Aluotto et al. 1970) as well as haemagglutinating activity (Erno and Stipkovits, 1973 a).

The genus Ureaplasma was created to provide more adequate status for those mycoplasmas previously referred to as T - strain or T-mycoplasmas (Shepard et al. 1974). They represent unique mycoplasmas which are able to hydrolyze urea but under the usual culture conditions do not attain colony sizes on agar much larger than 10 µm (± 5) in diameter.

The designation "Spiroplasma" was first given to helical, motile, wall - free prokaryotes observed in corn

plants infected with the insect - borne corn stunt disease (Davis and Worley, 1973). Before then, a mycoplasma-like agent had been visualized and eventually cultivated from citrus plants infected with "Stubborn disease" (Saglio, La Fleche, Bonissol, and Bove, 1971). More lately, a Spiroplasma sp. was isolated from ticks and found to exhibit pathogenicity for vertebrates (Tully, Whitcomb, Clark and Williamson, 1977).

1.5. AIM OF PRESENT WORK:

In the present work, attempts had been made to isolate and identify Mycoplasmas from poultry flocks with clinical evidence of CRD with particular reference to M. gallisepticum, and to compare the rate of isolation with serological evidence of infection. Other bacterial agents (other than mycoplasmas) that could contribute to the overall problem of CRD in this environment were also determined.

It was hoped that the study could unfold the multiplicity of agents that clinicians and poultry owners need be aware of when handling outbreaks of CRD on their farms and lead to improved diagnostic and control measures.

CHAPTER 2

AVIAN MYCOPLASMAS:
A REVIEW OF LITERATURE

2.1 HISTORICAL BACKGROUND

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. The organisms were later propagated in cell-free media (Nelson, 1938, 1939). The first clear description of the C R D of chickens was by Delaplane and Stuart (1943), who attributed the disease to a virus - like agent which he successfully cultivated in embryonated chicken eggs. Van Herick and Eaton (1945), similarly reported isolation of PPLO from chick embryo. It was Markham and Wong (1952) who finally offered a proof that the agent of C R D in chickens was also present in infectious sinusitis of turkeys and both were members of the PPLO. The organism was subsequently classified as a Mycoplasma and named Mycoplasma gallisepticum (Edward and Kanarek, 1960).

Adler, Fabricant, Yamamoto and Berg (1958) showed that airsacculitis in day - old poults was caused by a different Mycoplasma sp. Dierks, Newman and Pomeroy (1967) demonstrated that the airsacculitis caused by this mycoplasma was milder than that produced by M. gallisepticum.

This mycoplasma was designated the N strain (Adler et al. 1958) and later named M. meleagridis (Yamamoto, Bigland, Ortmayer, 1965).

Infectious synovitis of chicken was first reported simultaneously and independently by Olson, Bletner, Shelton, Munro and Anderson (1954) and Wills (1954). Snoeyenbos and Olesiuk (1955) described a similar condition in turkeys. The causative organism was named M. synoviae by Olson, Kerr and Campbell (1964a).

The above three serotypes of Mycoplasmas (M. gallisepticum, M. meleagridis and M.synoviae) are considered pathogenic for the avian species (White et al. 1954, Fabricant, 1969; Jordan, 1975). Other avian Mycoplasmas which are apparently non - pathogenic include M. gallinarum (Edward and Freundt, 1956), M. anatis (Roberts, 1964c), M. iners (Edward and Kanarek, 1960) and A. laidlawii (Laidlaw and Elford, 1936).

2. 2. STRUCTURE AND MORPHOLOGY

2. 2. 1. Ultrastructural Features

Thin sections of avian mycoplasma cells reveal simple ultrastructure consisting essentially of the limiting unit membrane and cytoplasm

containing electron - dense granules, presumably ribosomes, and the characteristic prokaryotic nuclear material or genome (Tajima et al. 1979). Moreover in several avian Mycoplasma sp. specialized terminal protrusions termed "blebs" can be seen on one or both ends of the organism (Green and Hanson, 1973; Levisohn and Razin, 1973; Tajima et al. 1979) and these may be the sites 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 Butler, 1978; Tajima et al. 1979).

2. 2. 2. Colonial Morphology.

The colonial morphology of mycoplasmas varies considerably, depending upon species and strain variability, culture medium components, aerobic or anaerobic incubation and a number of other factors, both known and unknown (Smith, 1971; Tully, 1978; Snell, 1981). Generally,

mycoplasma colonies grown on agar are classically described as looking like "fried egg" (Hayflick, 1969; Smith, 1971; Stalheim, 1979) although several of them do grow with aberrant colonial morphology when first isolated (Stalheim, 1979). A typical mycoplasmal colony is round with a dense centre often referred to as the "nipple" and a translucent periphery. The greater density of the central area is due primarily to the penetration of the organisms into the interstices of the agar gel (Smith, 1971, Tully, 1978). Confluent growth of the colonies results when two or more cells lie close together (Yamamoto and Adler, 1958b; Smith, 1971).

Rosenfeld (1971) observed irregular Mycoplasma gallisepticum colonies that lacked the characteristic central portion. Smith (1971) explained that agar concentration greater than 1.5% impedes penetration by the organism giving rise to surface growths and smaller colonies which can also be brought about by overcrowding (Hayflick, 1969) and nutritionally deficient culture medium (Smith, 1971).

Very often mycoplasmas grow on glucose agar with a wrinkling of the surface, referred to as "film and spot" production (Rotten and Razin, 1964) due partly to sterol accumulation and partly to precipitation of insoluble soaps (Smith, 1971). The colonial

appearance sometimes is characteristic of the species or strain, but the degree of variation is usually too great to permit identification (Yoder and Hofstad, 1964; Dierks et al. 1967; Razin, 1978).

2. 2. 3. Mode of Reproduction.

Ultrastructural studies of mycoplasmas have been undertaken to determine their mode of life. Hayflick (1969) reported that mycoplasmas were composed of minimal reproductive units or elementary bodies varying in size from 125 to 250 μ m. Dutta, Dierks and Pomeroy (1965) noted that part of the growth cycle of M. gallisepticum resembled budding which resulted in the production of small and large daughter cells each with a nucleus - like structure. But Green and Hanson (1973) rather found morphological evidence suggestive of binary fission as the mode of replication in M. meleagridis. What Ibrahim and Yamamoto (1977) observed, however, confirmed the existence of sequential metamorphosis in the course of M. meleagridis development during which the single rounded forms in the lag phase gave way to filaments in the log phase which fragmented into coccus forms in the late stationary phase. So far, no consensus has been reached on the mode of reproduction in this organism (Freundt, 1960; 1969; Dienes and Bullivant, 1967; Razin et al. 1967, Uppal, Wise and Boldero, 1972). Yoder (1978) has suggested that

more than one mode of replication could be applicable.

2. 3. ISOLATION

2. 3. 1. Media

Mycoplasmas are very fastidious in their growth requirements and their recovery from avian tissues is sometimes difficult and protracted (Jordan and Amin, 1975 Adegboye, 1977; Yoder, 1979a). One of the important factors influencing recovery is the provision of optimal growth requirements in artificial media (Fabricant, 1958, 1959, 1969; Frey, Hanson and Anderson, 1968; Frey, Thomas and Hale, 1973; Whittlestone, 1976a). No single medium formulation has been accepted as suitable for the isolation of the various avian mycoplasmas (Yoder, 1975, 1979a). However, Frey et al. (1968), using the fourth French Medium (FM-4) formula, described and prepared a medium, which, with some modifications and variations, has been found capable of supporting the growth of a wide range of avian mycoplasmas.

2. 3. 2. Medium Supplements.

Both M. gallisepticum and M. meleagridis will grow satisfactorily in a medium supplemented with 10 - 15% heat - inactivated horse or swine serum (Mohamed and Bohl, 1967;

Frey et al. 1968). M. synoviae, on the other hand, is more difficult 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).

Matsuo, Kuniyasu, Yamada, Susumi and Yamamoto (1978) successfully cultivated M. gallisepticum in a medium supplemented with 20% unheated horse serum. Similarly, Adegboye (1977) has obtained satisfactory growth of mycoplasmas in media with 20% unheated horse serum. But a concentration of 20% horse serum has recently been reported by Snell (1981) to inhibit the growth of M. gallisepticum particularly in broth culture. The author rather found a broth containing 10% serum the most efficient.

In the case of M. synoviae, Vardaman and Drott (1978) reduced the concentration of NAD to 0.01g per litre and found this concentration less efficient for growth and isolation of this organism. But Thorpe (1970) using a concentration of 0.002% NAD obtained good growth. Olson and Meadows (1972) similarly found that 0.00125% to 0.01% NAD was satisfactory. From their studies, Adler, Da Massa and Field (1974) summarised that 0.01% NAD did not provide a better yield of M. synoviae than did 0.002%

thus supporting the findings of Olson and Meadows (1972) and Thorpe (1970). It was found, moreover, that nicotinamide could be used to replace the more expensive NAD without any growth disadvantage (Adler et al. 1974). M. meleagridis was reported to grow slightly better in autoclave - sterlilized than filter - sterilised media (Frey et al. 1968) whereas Thorpe (1970) obtained earlier growth of M. synoviae in a filtered medium than in an autoclaved one.

2. 3. 3. Specimen Collection

Several techniques have been used to obtain specimens for avian mycoplasma isolation. These include swabbing the lungs, airsacs or tracheas (Roberts, Olesiuk and Van Roekel, 1967; Frey et al. 1968; Rodriguez and Kleven, 1980 a, b; Yoder, 1979a), removal and pooling of respiratory organs (Amin and Jordan, 1978a), and sonic oscillation, freezing and thawing (Roberts et al. 1967).

2.3.4 Cultural Techniques

Mycoplasma cultures are conventionally incubated at 37^{o}C in a moist atmosphere and plated on mycoplasma agar after the phenol red indicator changes colour from red to orange or yellow (Roberts et al. 1967; Rodriguez and Kleven, 1980a, b) or after increased turbidity is observed (Mohan et al. 1979; Yoder, 1979a).

Sometimes subculturing into a fresh broth without apparent colour change would enhance the growth of mycoplasma (Mc Martin, 1967; Roberts et al. 1967; Yoder, 1979a). The technique of incubating the inoculated agar plates in an atmosphere of increased carbon dioxide has been considered unnecessary (Amin and Jordan, 1978a; Vardaman and Drott, 1978), optional (Yoder, 1979a) or obligatory (Fabricant, 1959; Mc Garrity, Sarama and Vanaman, 1979).

Amin and Jordan (1978a) reported maximum recovery of mycoplasma from direct plating onto agar together with subcultures from overlay. Broth culture method was inferior. Adler, Yamamoto and Bankowski (1954) and Klieneberger - Nobel (1962) made similar observations and Lemcke (1965) reasoned that there was some impairment of the mycoplasma surface membrane in the broth medium. However, Yoder (1979a) has maintained that initial broth passage tends to give better results than direct streaking onto agar medium and also reduces bacterial contamination.

2.3.5. The role of non-pathogenic Mycoplasmas in isolation

Pathogenic mycoplasmas are much more difficult to isolate than those which have a commensal role (Furr, Cooper and Taylor-Robinson, 1977) and the presence of nonpathogenic ones makes the isolation of the pathogenic ones even more difficult (Rosenfeld, 1971;

Mallinson and Rosenstein, 1976; Sahu and Olson, 1976a,b) or impossible (Sahu and Olson, 1976 b). In either case the infection has to be diagnosed serologically (Sahu and Olson, 1976 a,b). Alternatively the organism could be propagated in susceptible fowl (Roberts, 1964 b; Mc Martin, 1967; Rosenfeld, 1971) or in chick embryo (Roberts et al. 1967) before any attempt is made to isolate it.

2.4. PATHOGENICITY

2.4.1. Pathogenic Species

Avian mycoplasmas considered pathogenic are M. gallisepticum, which causes a disease condition commonly designated as Chronic Respiratory Disease (C R D) of chickens and infectious sinusitis and occasionally encephalitis in turkeys (Fabricant, 1960; Jordan, 1975; Yoder, 1978, 1979a), M. synoviae, the etiologic agent of infectious synovitis mainly of the leg joints and foot pads of chickens and turkeys (Chalquest and Fabricant, 1960; Dierks et al. 1967; Yoder, 1972; Olson, 1978), and M. meleagridis, a turkey pathogen, causing airsacculitis (Dierks et al. 1967; Mohamed and Bohl, 1967; Yoder and Hofstad, 1964; Yamamoto, 1978).

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

Experimental infection of the respiratory tract may result in neither clinical sign nor gross lesions (Dierks et al. 1967; Yoder, 1978), disease being restricted (Mc Martin, 1967) or generalised (Grimes and Rosenfeld, 1972) within the respiratory system, or haematogenous spread to many organs and tissues (Peters, Faber, Keenum and De Volt, 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, Ferguson, Ruhnke, Pettit, Robertson and Speckman, 1974).

2.4.2. Experimental Infections

Intravenous inoculation of chickens (Lamas da Silva and Adler, 1969) and turkeys (Clyde and Thomas, 1973) with M. gallicepticum results in arthritis and nervous signs respectively, and occasionally both, but no respiratory disease. Similarly, inoculation into the foot pad gives rise to lesions of the synoviae and bursae of the foot and hock in chickens and turkeys(Kerr and Olson, 1967 a, b). Corresponding changes have been reported in chicken embryo (Van Roekel, Olesiuk and Peck, 1952; Chute and Cole, 1954 ; **Whittlestone, 1972).**

Following intranasal or infra-orbital sinus inoculation of M. gallisepticum the organism could be recovered from the trachea (Olson, Heishman and Shelton, 1962;

Mc Martin, 1967). Mc Martin and Adler (1961) reported that in experimental intranasal infection of chickens with M. gallisepticum, the organism readily invaded the nasal mucosa, but rarely penetrated the lower respiratory tract. In another study, Mc Martin (1963) made a similar observation, but Roberts (1964 b) was able to isolate M. gallisepticum, though sporadically, from the lower respiratory tract following infra-orbital sinus injection.

2.4.3. Latent Infections

Recoveries of M. gallisepticum organisms become possible from the trachea much earlier than from the airsacs or lungs (Olson et al. 1962; Rodriguez and Kleven, 1980 a). Nevertheless, 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, 1964 b; Roberts et al. 1967; Mc Martin, 1967; Jordan and Kulasegaram, 1968b). A 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, 1964 b; Roberts et al. 1967; Yoder, 1978). This phenomenon has been described as "Latent Infection" (Mc Martin, 1967; Roberts et al. 1967; Jordan and Kulasegaram, 1968b; Sahu and Olson, 1976 a).

According to Roberts et al. (1967), the clinical infection appears when the M. gallisepticum organisms 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, 1968b) the latent M. gallisepticum organisms multiply, take up the extracellular position and the clinical disease is triggered off.

2.4.4. Location of M. gallisepticum

There are several reports to show that mycoplasmas are present both intracellularly and extracellularly (Edwards and Fogh, 1960; Hayflick and Stinebring, 1960; Shedden and Cole, 1966). Some authors, however, contend that the organisms predominantly and exclusively reside in an extracellular environment, or in close association with the cell surface (Abu-Zahr and Butler, 1978; Aldridge and Cole, 1978; Stanbridge and Weiss, 1978; Tajima et al. 1979; Meloni et al. 1981) and only rarely in phagocytic vacuoules (Tajima et al. 1979).

This contention has been supported by the more recent transmission and scanning electron microscopic studies (Abu-Zahr and Butler, 1978; Phillips 1978; Stanbridge and Doersen, 1978).

2.4.5. Mechanism of Pathogenicity

Although the mechanisms involved in the pathogenicity are not well defined at present, it has been postulated that the association between mycoplasmas and the host cells, whether from the intracellular or extracellular position, invariably results in cellular damage or dysfunction (Jordan, 1975). This fact has also been supported by the recent studies by several investigators who have noted that the attachment of M. gallisepticum to the RBC becomes so tight that the shape of the latter becomes distorted, the surface idented at the point of attachment and detachment from the host cells cannot be easily achieved even after prolonged washing (Banai, Kahane, Razin and Bredt, 1978; Razin, Gamliel, Polliack, Bredt and Kahane, 1980). Some authorities have suggested the actual fusion of mycoplasmas with the host cells (Apostolov and Windsor, 1975) followed by the exchange of membrane antigens (Wise, Cassell and Acton, 1978).

Although Tajima *et al.*(1979) could not demonstrate fusion, they observed intimate contact, which is the prerequisite for the development of the respiratory illness in *M. pneumoniae* (Collier and Clyde Jr. 1971; Tully, 1981).

In the respiratory apithelium, the presence of *M. gallisepticum* may lead to ciliostasis (Butler, Ellaway and Hall, 1972; Cherry and Taylor - Robinson, 1970, 1971, 1973; Power and Jordan, 1976) or actual loss of cilia (Tajima *et al.* 1979) as well as injury to cell membrane or impairment of cell nutrition (Wise, Boldero and Thornton, 1973; Jordan, 1975). It is believed that *M. gallisepticum*, in addition, produces toxic substances (Whittlestone, 1972; Jordan, 1975) including hydrogen peroxide (Manchee and Taylor - Robinson, 1970) and a neuraminidase - like enzyme (Roberts, 1967) that undoubtedly contribute to its pathogenicity.

2.5. COMPLICATED CRD

Mixed infections of mycoplasmas and bacteria, mycoplasmas and viruses and all three agents have been studied in birds (Blake, 1962; Fabricant, 1969; Stipkovits *et al.* 1978). The enhancement or inhibition by mycoplasmas of the growth of viruses and their cytopathic effects in tissue cultures has also been reported (Machpherson, 1966; Reed, 1971; Stanbridge,1971).

While M. gallisepticum is considered to be the primary cause of CRD, other organisms frequently cause complications (Biddle and Cover, 1957; Gross, 1961; Garside, 1965; Jordan, 1972) resulting in severe air-sacculitis. This condition, usually referred to as "airsac disease" is undoubtly the more commonly encountered condition in the field (Wasserman, Yates and Fry 1954, Fahey, 1955; Gross, 1956). The lesions in the airsac disease are more severe than in uncomplicated infections (Kerr and Olson, 1967a) and the variations in pathology apparently could be dependent on the complicating organisms, their virulence and routes of infection as well as environmental factors (Jordan, 1972, 1975).

2.5.1. E. coli Complication

There are several reports to show that E. coli is by far the most frequently isolated and therefore the most frequently involved complicating bacterial organism in the airsac disease (Biddle and Cover, 1957; Gross, 1956, 1958, 1961; Glantz, Narotsky and Bubash, 1962; Olufarati, Ikede, Kasali and Ojo, 1981). Fahey (1955) reviewed 187 cases of CRD with airsac complications and found E. coli to be involved in 70% of the cases. Bisgaard (1977) noted that in the later stages of infection, the bacterial flora was dominated by E. coli.

Olufarati *et al*. (1981) identified from the upper respiratory tract of both normal and sick birds, 9 species of bacterial organisms prominent among which were *E. coli* and *Staphylococcus sp*. There is however a strong indication that the bacterial organisms in the respiratory tract might be the normal inhabitants, causing disease only in the presence of stress (Bisgaard, 1977; Olufarati *et al*. 1981).

2.5.2. *Mycoplasma - Virus Synergism*

It has been observed that *Mycoplasmas* synergise with viruses to produce more severe diseases (Reed, 1971) and *M. gallisepticum* outbreakes are precipitated by respiratory viruses (Fahey, 1956; Johnson and Domermuth, 1956; Van Roekel, Gray, Shipkowitz, Clark, Luchini, 1957; Blake, 1962; Jordan and Kulasegaram, 1968b).

2.5.3 *Mycoplasma - Virus - E. coli Synergism*

Synergism of *Mycoplasma*, *E. coli* and the respiratory viruses of Newcastle disease, infectious bronchitis, infectious laryngotracheitis is also common (Gross, 1956; Van Roekel *et al*. 1957; Blake, 1962; Fabricant and Levine, 1962; Besrukava, 1965) and the resultant disease is more severe than that caused by individual agents singly (Fabricant, 1969; Jordan, 1972). The mycoplasma - virus presence in the airsacs has a great predisposing effect

to subsequent invasion of the airsacs by E. coli (Gross, 1961; Fabricant and Levine, 1962).

However, E. coli infection alone is known to cause marked pathological respiratory lesions including airsacculitis (Gross, 1957). In the presence of M. gallisepticum, a synergism is established which enhances the severity of CRD (Gross, 1956, 1961; Glantz et al. 1962; Harry, 1964; Garside, 1965; Jordan, 1975). Fabricant (1969) noted that in a flock of chickens, mortality due to E. coli alone, E. coli and infectious bronchitis virus combined and E. coli and M. gallisepticum combined was 13%, 20% and 45% respectively. A combination of all three agents produced 66% mortality. Neither M. gallisepticum alone nor infectious bronchitis alone caused any mortality.

Other reports of synergism with M. gallisepticum involve Haemophilus gallinarum (Adler and Yamamoto, 1956, 1957; Kato, 1965; Rimler, Davis, Page and Kleven, 1978) and moulds (Matuka, Aganovic and Forsek, 1968). Certain debilitating factors such as scarification of the trachea (Corstvet and Sadler, 1966) and excess ammonia in the atmosphere exacerbate M. gallisepticum infection (Sato, Shoya and Kobayashi, 1973).

2.5.4. Infection with more than one Mycoplasma Serotype

Little information is available on the effect of infection with more than one mycoplasma serotype although more than one is occasionally isolated from field material (Jordan, 1975). It has been reported that a pathogenic synergism occurs between M. synoviae and M. meleagridis to produce sinusitis in turkeys whereas exposure to either organism alone produced no sinusitis (Rhoades, 1977). Similarly, Amin and Jordan (1978 b) produced airsacculitis in ducklings with a combination of M. gallisepticum and M. anatis, neither of which was capable of producing the clinical disease.

2.5.5. The Role of M. gallinarum

M. gallinarum (Edward and Freundt, 1956) is considered non-pathogenic (Fabricant, 1969) or slightly pathogenic (Stipkovits, Kisary and El-Ebeedy, 1978) and 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 (Cherry and Taylor-Robinson, 1973; Taylor-Robinson and Cherry, 1972).

However, Dierks et al. (1967) found mild airsac lesions in mycoplasma - free turkey poults inoculated in the airsacs with a broth culture of M. gallinarum.

Kleven, Eidson and Fletcher (1978) demonstrated that the pathogenic potential of M. gallinarum when combined with infectious bronchitis and/or Newcastle disease viruses was similar to that of M. gallisepticum or M. synoviae. A pathogenic synergism has been reported between M. gallinarum and parvovirus (Stipkovits et. al. 1978) resulting in the production of much more severe fibrinous airsacculitis. One of the extra-respiratory pathological conditions that might be associated with M. gallinarum infection is perosis (Varley and Jordan, 1978). Kleven et al. (1978) believ that M. gallinarum could be a factor in the respiratory disease in chickens depending on the circumstances, and Boughton (1980) has some evidence to show that this organism is involved in turkey airsacculitis.

2.5.6. M. synoviae Airsacculitis

M. synoviae is primarily associated with synovitis mainly of the leg joints and foot pads of birds (Chalquest and Fabricant, 1960; Jordan, 1975). It also has a respiratory phase (Olson, Adler, Da Massa and Corstvet, 1964b) and the resultant airsacculitis is accompanied by pathological changes in the respiratory tracts of chickens and turkeys (Ghazikhanian, Yamamoto, and Cordy, 1973; Jordan, 1975; Yoder, 1971, 1979a; Hirano et al. 1978).

Generalized infection is common, sometimes without the swelling of the joints (O'Brien, 1972; Olson, 1978) and M. synoviae organisms are more readily recovered from the respiratory tract than from swollen leg joints (Sato and Nonomura 1973) and from the trachea than from the air sacs (Olson et al. 1964b; Kume, Hayatsu and Yoshioka, 1976). M. synoviae airsacculitis is particularly enhanced in broilers administered with live - virus vaccines of newcastle disease and infectious bronchitis (Kerr and Olson, 1970; Springer, 1971; Kleven et al. 1972; King, Kleven, Wenger and Anderson, 1973; Vardaman, Reece and Deaton, 1973; Kume, Hayatsu and Yoshioka, 1976) and constitutes the major cause of condemnations for unwholesomeness (O'Brien, 1972).

2.6. M. MELEAGRIDIS AIRSACCULITIS

M. meleagridis is the cause of airsacculitis in turkeys all over the world (Dierks et al. 1967; Mohamed 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 (Mohamed and Bohl, 1967; Kleven and Pomeroy, 1971). M. meleagridis causes airsacculitis in day - old poults (Kumar et al. 1963). In this case the embryo becomes infected with this organism in ovo (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, Butler and Pearson, 1971; Wise et al. 1973; Mohan et al. 1979). Synergism has been reported between M. meleagridis and E. coli (Yamamoto, 1978) as well as M. synoviae (Rhoades, 1977) to produce, in either case, a more severe airsacculitis.

2.7. SEROLOGY.

Information gained from serology tests is an important tool in establishing the presence of avian mycoplasmal infections. The Rapid Plate Agglutination (RPA), the Tube Agglutination (TA) and the Haemagglutination - Inhibition (HI) tests, (Adler, 1954; Olson, Kerr and Campbell, 1963; Anon, 1966; Roberts and Olesiuk, 1967; Vardaman and Yoder 1969 b; Timms and Cullen 1974; Sahu and Olson, 1976 a,b) are commonly used.

2.7.1. Agglutination Tests:

The agglutination tests have the advantages of speed and simplicity for which reasons they have been used for screening flocks for mycoplasmal infections, to be followed by HI test for confirmation (Anon, 1966; Sahu and Olson, 1976 a,b; Rhoades, 1978).

The dependability and sensitivity of aglutination tests, in general, for the detection of infected flocks have been questioned because of the nonspecific agglutination occasionally produced (Boyer, Fabricant and Brown, 1960; Adler and DaMassa, 1964; Olson et al. 1964 a; Roberts and Olesiuk, 1967; Cullenand Timms, 1972; Windsor and Thornton, 1973; Cullen and Anderton, 1974) with the result that field diagnosis of the actual infection could become very difficult, unreliable or confused (Olson et al. 1964 b; Roberts and Olesiuk, 1967; Sahu and Olson, 1974, 1976 b; Villegas, Kleven and Anderson, 1976).

Nonspecific reactions have been associated with serum contamination (Roberts et al. 1967; Sahu and Olson, 1976 a), serum freezing (cover, Benton and Whelan, 1960; Thornton, 1969), holding serum at + 4^{o}C for a long period (Wright and Meneely, 1972; Sahu and Olson, 1976a; Villegas et al. 1976), infection with Staphylococcus sp. or Streptococcus sp. (Thornton, 1973; Sahu and Olson, 1976 a), or M. synoviae (Olson et al. 1964 a; Roberts and Olesiuk, 1967; Sahu and Olson, 1976 a) as well as serum obtained from chickens inoculated with Erysipelothrix rhusiopathiae bacterin (Boyer et al. 1960; Roberts and Olesiuk, 1967).

Nonspecific serological reactions with M. gallisepticum antigen have also been reported in birds vaccinated with killed poultry virus vaccines of Newcastle disease, infectious bronchitis and avian encephalomyelitis, singly or in combination (Jordan and Kulasegaram, 1968 a, b; Roberts, 1970; Cullen and Timms, 1972;). Moreover, adjuvants alone (Cullen and Timms, 1972) have been known to cause nonspecific reactions. M. synoviae antigen (Olson et al. 1964 b; Roberts and Olesiuk, 1967; Sahu and Olson, 1974) and M. meleagridis antigen (Snell and Cullen, 1978) have similarly been reported to elicit nonspecific agglutination reactions. However, Sahu and Olson (1976a) have demonstrated that M. synoviae antigen reacts nonspecifically to a greater degree than does the M. gallisepticum antigen.

Another main cause of nonspecific reaction is the swine or horse serum which is usually incorporated in the mycoplasmal growth medium for antigen production (Wills, 1954; Roberts, 1970; Wright and Meneely, 1972; Thornton, 1973).

It is thought that the globulin content of the horse or swine serum coats the mycoplasma cells and the anti-globulin (rheumatoid) factor present in the chicken serum causes the agglutination of the coated cells (Roberts and Olesiuk, 1967; Thornton, 1969; Roberts, 1970; Bradbury and Jordan, 1971, 1972; Timms and Cullen, 1972).

2.7.2. H I Test

The HI test has been considered the most accurate, most specific and most reliable test but because of its complexity, is used only for confirmation of avian mycoplasma infections (Anon, 1966; Roberts, 1970; Vardaman and Yoder, 1969 b, 1970; Sahu and Olson, 1975; Hinz, 1977; Rhoades, 1978). However, investigations have shown that the HI test alone should not be relied upon since it might fail to detect all infected birds unless carried out on fresh sera (Timms, 1971). It might fail also to give significant titers in cases of outbreaks due to isolates of low pathogenicity (Villegas et al. 1976). HI antibodies have been known to disappear faster from the serum than the agglutinin or precipitin antibodies (Sahu and Olson, 1976 b) and some investigators have reported nonspecific reactions in the HI test (Roberts and Olesiuk, 1967; Sahu and Olson, 1976a; Hinz, 1977).

2.7.3. Complement - Fixation (CF) Test

The CF test (Gianforte, Jungherr and Jacobs, 1955; Lemcke, 1964; Marguardt and Newman, 1971)had been successfully used to detect antibodies to mycoplasmas. Similarly, Frey and Hanson (1969) pointed to the possibility that the CF test might be used in the detection of mycoplasmal antigens in tissues.

2.7.4. Elimination of Nonspecific Reactions

Nonspecific reactions can be removed by treating the serum with metaperiodate or receptor - destroying enzyme of Vibrio cholerae (neuraminidase) (Hinz, 1977; Roberts and Olesiuk, 1967), by diluting the serum with phosphate-buffered saline (PBS) (Sahu and Olson, 1976a; Timms and Cullen, 1972), with 3 M NaCl, or with an equal volume of guinea-pig serum inactivated at 56°C for 30 minutes (Wright and Meneely, 1972). Thornton (1978) demonstrated that the RPA antigen containing 7% albumen gave fewer nonspecific reactions and had better specificity. The protein (albumen) was thought to suppress the speed of nonspecific reactions rather than inhibit it completely. Roberts (1970) and Windsor and Thornton (1973) observed that many of the nonspecific agglutinins were transitory.

Nevertheless, most of the nonspecific reactions in the RPA test disappeared in the HI test (Roberts and Olesiuk, 1967; Vardaman and Yoder, 1969a, 1970; Sahu and Olson 1976a; Vardaman and Drott, 1977).

Bradbury and Jordan (1971) suggested the culture of mycoplasma in globulin-free media if nonspecific reactions were to be eliminated. Edward (1971) successfully cultured M. gallisepticum in media without gloublin but supplemented with bovine serum albumen, palmitic acid, and cholesterol. Windsor and Thornton (1973) tested the globulin-free antigen and found that it eliminated all nonspecific reactions due to M. synoviae and most nonspecific reactions due to viral vaccines.

2.7.5 Mycoplasmal Membrane as Antigens for Serologic Tests

In a continued search for better antigens, some authorities have resorted to the production of membrane antigens (Williams and Taylor - Robinson, 1967; Kahane and Razin, 1969; Goel, 1973; Levisohn and Razin, 1973; Villegas et al. 1976). These antigens have been reported to be highly immunogenic (Levisohn and Razin, 1973), superior to the whole-cell antigens in the RPA and TA tests (Goel, 1973; Villegas et al. 1976), and capable of eliminating the nonspecific reactions (Villegas et al. 1976) and hyperimmune reactions (Levishon and Razin, 1973).

2.7.6. Immunoglobulins involved in Serologic Reactions

Some work has been done and published to show that the effectiveness of both the agglutination and HI tests for detecting antibodies against mycoplasmas is related, at least in part, to the predominating immunogloblin of which the specific antibody consists. For example, studies of antibody response to M. meleagridis (Kleven and Pomeroy, 1971) and M. gallisepticum (Roberts, 1969; Villegas et al. 1976) indicated that the HI test demonstrated IgG but not IgM and that the agglutination tests demonstrated IgM but were relatively insensitive for demonstrating IgG. However, low level HI activity due to IgM was clearly demonstrated following intravenous injection of turkeys with M. synoviae (Rhoades, 1975) and of chickens and turkeys with M. gallisepticum and M. meleagridis respectively (Rhoades, 1978). These early HI antibody titers induced by intravenous inoculation were lower than antibody titers demonstrated by the agglutination tests (Kuniyasu, 1969; Kleven and Pomeroy, 1971; Rhoades 1975,1978).

2.7.7. Influence of Physical factors on Serological Tests

Certain physical and environmental factors have been found to markedly influence the quality of the

antigens and the sensitivity of the agglutination tests. Adler and Da Massa (1968) reported that killing the organism with formalin reduced its effectiveness. Razin (1964) and Rottem and Razin (1967) found that various substrates used for the growth of mycoplasmas become incorporated within the growing organism and thus influence the antigenic sensitivity. Hromatka and Adler (1969) reported that phenol and formalin were unsatisfactory preservatives.

Opinions however differ on the pH values most suited for antigen production. While Razin (1964) observed that neutral pH values were optimal, Newman (1967) obtained a greater sensitivity at lower pH values. On the other hand, Cullen and Anderton (1974) reported that antigens grown in alkaline medium had superior specifi city and Villegas *et al*. (1976) working with alkaline pH throughout, observed only a few nonspecific reactions. Butler and Knight (1960) and Roberts *et al*. (1967) reported that phosphate buffer and physiologic saline solutions were toxic for mycoplasmas. It was, moreover, observed that *M. synoviae* agglutinating antigen would not withstand lyophilization (Kume, Hayatsu and Yoshioka, 1977) whereas *M. gallisepticum* agglutinating antigen was stable at various storage conditions (Dierks *et al*. 1967; Kume *et al*. 1977).

Sonication led to the disappearance of haemagglutinating activities of both M. synoviae and M. gallisepticum antigens (Kume et al. 1977).

Medication has been found to affect some serologic reactions (Kuniyasu, Matsui, Ando and Yoshida, 1967; Olson and Sahu, 1976). Olson and Weiss (1972) observed that chlortetracycline and tylosin reduced the immune response of chickens to M. gallisepticum infection. Sahu and Olson (1976 a) reported the reduction of agglutinin antibody of birds on chlortetracycline whereas the HI and precipitin antibodies were completely inhibited.

2.7.8. Use of Serologic Techniques in Identification

Serologic techniques have proved useful in the identification of mycoplasmal isolates. Yamamoto and Adler (1958 a) and Moore, Grumbles, Beasley (1960) used the RPA test. Noel et al. (1962) and Yoder and Hofstad (1964) used the colony-inhibition test that was first described by Edward and Fitzgerald (1954). Kleckner (1960) employed a combination of TA, haemagglutination and HI tests. Kelton and Van Roekel (1963) employed both the colony-inhibition and TA tests while Dierks et al. (1967) employed both the RPA and colony-inhibition tests. Frey, Hawk and Hale (1972) used the CF test.

Nicol and Edward (1953) and Edward and Fitzgerald (1954) demonstrated that the incorporation of antiserum into culture media inhibited the growth of the homologous species. It was later found (Herderschee et al. 1963) that filter-paper discs saturated with specific antiserum and placed on agar previously inoculated with a homologous species resulted in a zone of inhibited growth surrounding the disc. The high degree of specificity of the reaction was confirmed by Clyde (1964) using hyperimmune rabbit antiserum, and refined by Stanbridge and Hayflick (1967) who found that antiserum-impregnated paper discs could be dried and stored for long periods with full retention of activity.

New mycoplasma isolates were however found to be less susceptible to growth inhibition and agglutination by specific antiserum until after several subcultures (Frey and Hanson 1969).

More recent studies have demonstrated the application of other serologic tests or their combinations for effective avain mycoplasma identification. Aycardi, Anderson and Hanson (1971) devised a relatively simple agar-gel precipitin (AGP) test and classified various serotypes of mycoplasma, and Nonomura and Yoder (1977) readily typed M. gallisepticum and M. synoviae isolates

by this technique. Grebe (1977) employed the haemadsorption and the haemadsorption-inhibition tests and Sahota, Balwant and Sodhi (1977) used the Flourescent antibody technique which Aycardi *et al*. (1971) thought was rather cumbersome. Stipkovits and El-Ebeedy (1977) used a combination of agglutination, growth inhibition, growth precipitation and immunofluorescence tests.

For the avian mycoplasmosis, emphasis is presently being shifted from the mycoplasmal isolation, as a means of diagnosing and confirming the infection, to serological manifestations. In a series of studies, Sahu and Olson (1974, 1975, 1976a, b) have successfully and convincingly demonstrated the correlation between the AGP, HI and RPA tests for the early diagnosis and confirmation of mycoplasmosis in flocks. According to the authors, confirmation of infection by isolating mycoplasma is unsatisfactory. Alternatively, they have handed down a simple formula for instant confirmation of infection even in the absence of mycoplasma isolation and the formula is to consider the flock positive if any two of the three different serologic tests are positive.

2.8. VACCINATION AND IMMUNITY:

Mycoplasma gallisepticum infection, both naturally occurring and experimentally induced produces demonstrable immunity in chickens (Nelson 1935; Delaplane and Stuart, 1943; van Roekel and Olesiuk, 1953; Olesiuk and Van Roekel, 1960 a, b;) and turkeys (Hart, 1940; Groupe and Winn, 1949; Gross, 1954; Olesiuk and Van Roekel, 1960b). Similarly, it has been shown that day - old chicks from M. gallisepticum infected hens possess maternal antibodies to this organism (Carlson and Howell, 1967; Osbaldiston and Wise, 1968; Snell and Cullen 1977) although these antibodies disappear very early in life (Snell and Cullen, 1977).

2.8.1. Use of Bacterins

Reports are contradictory on the efficacy of different types of M. gallisepticum bacterins. Adler (1958) found no protection against M. gallisepticum airsacculitis from direct air sac challenge following vacination.Whittlestone (1976 b) suggested protection through killed vaccine in suitable adjuvant and Warren, Senterfit and Sieiro (1968) reported protection in day-old chicks from formalin - inactivated bacterins with aluminium hydroxide gel as adjuvant.

But Adler (1976), repeating the experiments, could not demonstrate efficacy. Other investigators similarly obtained disappointing results (Adler and Lamas da Silva, 1970; Takemitsu, Hiramatsu, Sasaki, 1970; Kuniyasu and Yoshida, 1971) but Stone, Brugh, Hopkins, Yoder and Beard (1978) reported that M. gallisepticum vaccines in oil emulsion adjuvants were more immunogenic than those without adjuvants or with aluminium hydroxide adjuvants. Rimler et al. (1978) explained that the differences in the reports could be attributed to several factors including the amounts of formalin used, the adverse effects of which Davis, Rimler and Kleven (1976) had earlier observed on H. gallinarum. Similarly, washed M. gallisepticum cells gave poorer protection (Adler, Mc Martin and Shifrine, 1960).

2.8.2. Routes of Vaccination

On the route of vaccination, Hayatsu, Sugiyama, Kume, Kawakubo, Kimura, Yoshioka, Kaneko, Kobayashi, Yamasaki and Nishiyama (1975) and Hayatsu et al. (1974) noted that protection against M. gallisepticum infection, whether experimental or natural, was obtained through aerosol vaccination.

Similar studies by Domermuth (1962), Mc Martin (1963), and Adler (1976) indicated that the inoculation of culture intranasally induced greater immune reactions. On the other hand, Rodriguez and Kleven (1980 b) found the eye-drop method of live M. gallisepticum vaccine satisfactory. Levisohn (1977) successfully immunized one-day-old chicks by nebulization with live culture of M. gallisepticum S6 but Whittlestone (1976 b) has condemned the use of live virulent or attenuated strains of mycoplasmas. Yoder (1979 b) obtained good results with doses of 0.5 ml of formalin or beta - propiolactone inactivated M. gallisepticum vaccine given at 2 doses, 8 weeks apart subcutaneously or intra - foot - pad.

2.8.3. The F Strain for Vaccine Production

Several laboratory trials have confirmed that chicks inoculated with M. gallisepticum at one day of age respond immunologically (Grimes and Rosenfeld, 1972; Papageorgieu, 1974; Rodriguez and Kleven, 1980 b) and the F strain (unknown passage level) has been recommended as a vaccine strain because of its low virulence and transmissibility (Lunginbuhl, Tourtellotte and Frazier 1967; Fabricant, 1975, 1977; Adler, 1976; Cunningham and Olson, 1978; Nahay, Adler and Farver, 1979; Rodriguez and Kleven, 1980a, b).

2.8.4. Vaccination and Carrier Status

The general problem with avian mycoplasma live vaccines is the production of carrier birds. Many investigators have convincingly demonstrated the carrier state by reisolating the organism from vaccinated birds (Grimes and Rosenfeld, 1972; Adler, 1976; Vardaman, 1976; Levisohn, 1977; Rimler et al. 1978; Rodriguez and Kleven, 1980 b). Although many authors recommend vaccination only of multi-age flocks already infected with M. gallisepticum (Luginbuhl et al. 1967; Fabricant, 1977; Cunningham and Olson, 1978; Rodriguez and Kleven, 1980b), Rimler et al. (1978) consider the recommendation hazardous and warn that in the event of concurrent H. gallinarum infection, the resultant synergism would precipitate a more serious condition. It has been reported that commercial egg producers have defied this warning and resorted to vaccination of replacement stock with the F strain (Luginbuhl et al. 1967; Fabricant, 1975, 1977).

In conclusion, we have to reason with Whittlestone (1976 b) that animals might be protected against respiratory mycoplasmosis by the use of killed vaccines in suitable adjuvants or by the development of temperature - sensitive mutants of mycoplasmas,

without ignoring the most recent report of Levisohn and Kleven (1981) that persistent vaccination with nonvirulent M. gallisepticum strain gradually leads to the displacement of the pathogenic strains.

2.9. AVIAN MYCOPLASMAS IN NIGERIA:

2.9.1. General Consideration

Although much work has been done and published by various authors on Mycoplasmas in other animal species, including man, in Nigeria (Ojo, 1976 b; Windsor, Onoviran, Philpott, Masiga and Adegboye, 1978; Adegboye and Addo, 1978; Adegboye, Addo, Ogunkoya and Rose, 1979; Adegboye, 1981), a similar attention has not been given to the avian species. In fact, most of the diagnosis of chronic respiratory disease (C R D) in the field or even in the Research Institutions have been based solely on the clinical manifestations and the post mortem findings (Mohan, Adegboye and Orajaka, 1979; Olufarati, Ikede, Kasali, and Ojo, 1981). The failure to routinely check for mycoplasmas may not be unconnected with the lack of facilities coupled with the complexity of the organisms in their growth requirements (Adegboye, 1977; Yoder, 1979a).

2.9.2. Historical Evidence:

Nevertheless, there cannot be any doubt about the existence of mycoplasmas in this country both in the indigenous and exotic poultry flocks. Hill and Davis (1962) reported sporadic outbreaks of "Chronic respiratory condition" in birds at Ibadan University farm with the acute form coinciding with the rainy season. Kaschula (1961), in the Northern part of Nigeria, identified the old - fashioned respiratory infection known as "roup" (or infectious coryza) with the village poultry and the "modern" C R D with the improved breeds.

2.9.3. Cultural and Serological Evidence:

Mohan et al. (1979) appear to be the first investigators to conclusively document the presence of Mycoplasma meleagridis in Nigeria when they sucessfully isolated and characterized this organism during an outbreak of airsacculitis and bad - leg syndrome amongst turkeys in Nsukka University farm. Serological investigations have also revealed that a high percentage of our chickens, both local and exotic breeds, show positive agglutinins for M. gallisepticum (Addo, 1976; Onunkwo and Onoviran, 1978)and M.synoviae(Onunkwo and Onoviran,1978).

Similarly, in the most recent survey carried out by Olufarati et al. (1981) on the bacterial organisms infecting the upper respiratory tract of chickens and turkeys in South Western Nigeria, the authors incriminated M. gallisepticum in a mixed infection involving this organism, Haemophilus gallinarum and Newcastle disease virus and postulated a possible synergism among the three organisms as earlier suggested (Raggi, Young and Sharma, 1967; Rimler, Davis, Page and Kleven, 1978). Although diagonis of M. gallisepticum was based on macroscopic pathology and clinical signs, the survey was significant in that it showed the possible involvement of other bacterial organisms, notably Escherichia coli, Staphylococcus aureus and Proteus sp. among others in the development and manifestation of complicated M. gallisepticum infection or "airsac disease" as earlier described (Gross, 1956, 1961; Jordan 1972, 1975).

2. 10. ECONOMIC IMPORTANCE:

Avian mycoplasmosis has been reported all over the world. It continues to pose one of the major economic problems to the poultry industry (Rodrigues and Kleven, 1980a). As observed by Kerr and Olson (1967a), the morbidity and condemnations caused by mycoplasmas result in tremendous economic losses in both meat - and egg - producing flocks.

The economic losses are due to reduced feed and egg production efficiency, down-grading of carcasses and increased medication costs. Treatment can only alleviate the symptoms and lesions but does not eliminate the organisms, thereby leaving the birds to become carriers. Where success has been achieved through medication, it is always too costly to justify its use. According to Yoder (1978), this is one of the costliest disease problems confronting the poultry industry. Fortunately, the disease, which is as costly to treat as it is difficult to eliminate (Hayatsu, Sugiyama, Kawakubo, Kimura, Yoshioka and Nishiyama, 1974), is of little or no public health significance.

CHAPTER 3

INCIDENCE OF MYCOPLASMA GALLISEPTICUM IN SOME POULTRY FLOCKS BASED ON ISOLATION AND IDENTIFICATION OF MYCOPLASMAS

3.1. I N T R O D U C T I O N

Mycoplasma isolation as a means of diagnosing mycoplasmal disease is not routinely undertaken by many laboratories as mycoplasmas require special general techniques of isolation and identification (Whittlestone, 1974; Jordan and Amin, 1975; Adegboye, 1977). Even when optimum growth conditions have been provided, some pathogenic strains cannot still be isolated with ease, particularly from diseased materials (Whittlestone, 1974). In the study presented below attempts were made to isolate and identify the Mycoplasmas participating in C R D of chickens.

3.2. MATERIALS AND METHODS

3.2.1. Media Components

The components were as follows:

PPLO Broth Powder (Difco Laboratories, Detroit, Michigan);

Agar - Initially Ionagar No.2 (Oxoid Ltd; London) was used. When this was no longer available, Agar Noble (Difco Laboratories, Detroit, Michigan) was substituted;

Distilled deionized water

Horse Serum - Initially, this was obtained by aseptically bleeding a local horse, whose serum, experience has shown,

would support the growth of several mycoplasma species. The blood was allowed to clot at+4°C and the serum was seperated aseptically. Later on, mycoplasma - screened commercial horse serum (Gibco Bio - Cult, Glasgow) was used. Both types of sera were filter - sterilized before use with either 0.45 µm Millipore membrane filter (Gelman Instrument Company, Ann Arbor, Michigan) or 0.22 µm (Millipore Corporation, Bedford, Mass). Serum was stored at - 30°C before use.

Glucose - A 10% stock solution was prepared by making up 10g dextose (Difco Laboratories Detroit, Michigan) to 100ml using distilled deionized water. This was dispensed in 10 ml amount into sterile universal bottle, autoclaved at 121°C under 15lb per sq. in. pressure for 15 minutes and stored at +4°C.

Yeast Extract - 250g of baker's yeast (Engedura, Gist - brocades NV. Delft - Holland) was dissolved in 750 ml of distilled deionized water by boiling on a magnetic stirrer thermostat hot plate (Gallenkamp and Co. Ltd, London) for 15 minutes. The product was frozen over-night. The next day, it was thawed and centrifuged twice to remove sediments. The supernatant was then filter-sterilized using a 0.45µm or 0.22µm millipore membrane. Filtered yeast extract was stored at +4°C.

Phenol Red - A 1% stock solution was prepared by dissolving 1.0g of phenol red (Phenolsulfonphthalein) (Difco Laboratories, Detroit, Michigan) in a little quantity of distilled deionized water and making up to 100 ml. It was dispensed in 10 ml aliquots and stored at +4°C

Thallium acetate - A 10% stock solution was prepared by dissolving 1.0g thallium acetate (Fisher Scientific Co. New Jersey) in distilled deionized water to make a final 10 ml volume. This was autoclave-sterilized as usual. It was dispensed in 2 ml aliquots and stored at +4°C.

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

Nicotanamide adenine dinucleotide (NAD) or Diphosphopyridine nucleotide (DPN)

- A 10% stock solution was prepared by first dissolving 0.5g NAD in a little quantity of distilled de-ionized water and making up to 5 ml. The solution was filter - sterilized with 0.45 µm Millipore membrane and dispensed in 1 ml amounts into sterile bijou bottles and frozen.

L - cystein hydrochloride - A 10% stock solution was prepared by making up 1.0g to 10ml with distilled deionized water. The solution was filter - sterilized with 0.45 µm Millipore membrane and dispensed in 2ml aliquots into sterile universal bottles. It was stored at +4°C.

3.2.2. Media Preparation

Basically, the formulation used was based on the FM-4 medium of Frey et al.(1968) but with slight modification. A 250 ml broth was often prepared as follows: 5.25 g of the PPLO broth (powder) was weighed into a conical flask into which was added 175 ml of distilled deionized water. The mixture was stirred and heated to boiling for 15 minutes by means of the magnetic stirrer thermostat hotplate (Gallenkamp and Co. Ltd., London) until the powder dissolved completely, phenol red (0.5 ml of 1% solution) was added and the pH was tested and adjusted to 7.6 with 1 N hydrochloric acid or 1 N sodium hydroxide. The broth was then autoclaved at 121°C under 15lb per sq. in. pressure for 15 minutes. The flask was transferred to a water bath where the remaining components were added aseptically in the correct amounts (Table 3.1). The broth was dispensed in 50 ml aliquots and stored at +4°C before use. The thallium acetate and penicillin were bacterial inhibitors while DPN and cysteine hydrochloride were growth promoters.

Agar medium was prepared in a similar way to the broth except that 2.5 g Ionagar No. 2 (or Agar Noble) was weighed into the PPLO broth powder and both products dissolved.

After autoclaving, the medium was maintained at 45-50°C in a water bath while the other components were added. The molten agar was dispensed into sterile 90mm petri dishes (Sterilin Ltd; Teddington, Middlesex, England) to solidify. The agar plates were stored at +4°C.

Agar overlay was prepared by pouring 1 ml of agar medium into a bijou bottle to form a slope and later adding 1 ml of mycoplasma broth so that a portion of the agar slope was above the surface of the broth.

Each batch of newly-prepared broth and agar was tested and found to support the growth of known mycoplasmas including M. gallisepticum Reference strain P G 31.

3.2.3. Specimens

Birds

From 5 to 30 chickens were often purchased from each of 14 different flocks in which a CRD -type infection was clinically diagnosed. The individual chickens exhibited catarrhal nasal discharge and respiratory distress; in all, 230 chickens were so purchased.

Table 3.1

The Composition of a 250 ml Mycoplasmal Broth

Distilled deionized water	-	175.0ml
PPLO Broth (Powder)	-	5.25g
Yeast extract	-	25.0ml
Horse Serum	-	50.0ml
Glucose (10% Solution)	-	2.5ml
Phenol red (1% Solution)	-	0.5ml
Benzyl Penicillin (10^6 U/ml)	-	0.25ml
Thallium acetate (10% Solution)	-	0.5ml
Cysteine hydrochloride (10% Solution)	-	0.25ml
*NAD (DPN) (10% Solution)	-	0.25ml
Final pH (adjusted as necessary)	-	7.6

*NAD (or DPN) was added to the first few batches of Broth and Agar to ensure that M. syroviae, if present in the specimens, would be able to grow.

Birds, whether dead or living, coming into the diagnostic laboratory for routine post mortem examination and having a history suggestive of CRD were also examined; in all 350 chickens were so examined. After blood samples were taken for serum from live birds, they were sacrificed. Such birds and the already dead ones were opened up and swabs were taken aseptically from the trachea; air sacs or lungs of each bird for mycoplasma culture.

Eggs

On one occasion following an outbreak of CRD-type infection in a large poultry farm, 16 eggs were collected from the farm and cultured for mycoplasmas.

Known M. gallisepticum strain

Known M. gallisepticum isolate, Reference strain PG 31 was kindly supplied by Dr. J. G. Tully of National Institute of Health, Bethesda, Maryland, U. S. A. It was propagated in the media formulation used, and frozen in 1.0 ml aliquots for use at different stages.

3.2.4. Cultural Procedure

Culture in Broth

Each swab was snapped off into a test tube containing 2 ml broth.

The tube was screwed tight and frozen until a number of specimens had been collected over a period of approximately one week.

In the case of the egg, a crack was made at the larger end of the egg after the site had been disinfected. A Sterile Pasteur pipette was pushed into the interior of the egg and about 0.2 ml of the albumen was withdrawn and inoculated into 1.8 ml broth. The yolk material was similarly inoculated.

Eventually, the frozen broth cultures were allowed to thaw. Approximately 0.2 ml of each broth culture was transferred into a fresh 1.8 ml broth, followed by a serial ten-fold dilution down to 10^{-3}. The tubes were tightly screwed and incubated at $37^{o}C$. Examination for growth of mycoplasma, indicated by a change in the pH of the broth from alkaline (pinkish or reddish colour) to acid (orange of yellowish colour), was made after every 24 hours. After 5-7 days cultures without obvious colour change were passaged blindly by inoculating 0.2 ml into a fresh broth and incubating at $37^{o}C$. Broth cultures were not discarded until after 14-21 days of incubation.

It was common to have broth cultures with good colour change suggestive of the growth of mycoplasmas but with heavy sediments suggestive of bacterial contaminants. In such cases, the broth cultures were filtered through 0.45 µm membrane and recultured in mycoplasma broth.

Culture on Agar

Approximately 0.05ml of any broth culture showing a good homogenous colour change was inoculated, sometimes after diluting to 10^{-3}, onto the agar plate and allowed to dry. Broth cultures without obvious colour change after prolonged inoculation were similarly inoculated. Plates were incubated in a glass anaerobic culture jar (Gallenkamp and Co. Ltd; London) under 5 - 10% carbon dioxide tension provided by the action of IN hydrochloric acid on marble chips contained in a small beaker. Plates were examined every 48 hours for mycoplasma-like colonies using a stereoscopic microscope (Olympus Optical Co., Japan) at a magnification of x 40 to x 80.

Occasionally, swabs were directly streaked onto agar surface in addition to the propagation in Broth described above.

Similarly, on occasions approximately 0.2 ml of the Primary broth culture was inoculated onto an agar overlay containing 1 ml broth and the culture was handled the same way as those inoculated onto mycoplasma broth. The procedure was not used routinely.

3.2.5. Characterization and Identification of Isolates

Mycoplasma - like colonies were first cloned thus: a small piece of agar block on which there was a single, well isolated, colony was transferred into 0.2 ml broth, followed by a ten-fold dilution of the colony in broth to 10^{-3}. This was incubated and later subcultured onto agar as previously described. The procedure was often repeated once or twice, especially if the isolate appeared to grow fairly readily. Requirement for glucose was confirmed by reculturing in glucose - supplemented broth and checking for acidic colour change (pH<7.6). Requirement for serum and hence confirmation as a member of genus Mycoplasma was done by checking effect of digitonin on isolate, (Friis, 1975; Ojo, 1976a). Briefly, small paper discs (Schleicher and Scheuell Inc; Keene, N. H 03431, USA), presoaked in 1.5% digitonin in ethanol and dried were placed over newly inoculated agar cultures incubated as usual and examined after 48 hours.

This was run simultaneously with serological identification by disc growth-inhibition (GI) test (See below).

G I. Test

The procedure used was based on that described by Clyde Jr, (1964). The running drop method which had been used to identify M. meleagridis in Nigeria (Mohan et al. 1979) was used. One drop of a 10^{-2} cloned broth culture dilution which often contained approximately 10^4 colony forming units was inoculated onto an agar plate and allowed to run down the plate. Two or three such streaks were often made per 90mm petri dish. A 6.35 mm diameter paper disc (Schleicher and Schuell Inc., Keene, N. H. 03431 USA) impregnanted with each antiserum against M. gallisepticum (PG 31), M. gallinarum (PG 16), M. meleagridis (Strain 886), M. anatis (Strain 1340) A. laidlawaii (PG 9) and digitonin was placed on each inoculum. The discs were approximately 20 mm apart. The inoculated plates were incubated in an anaerobic jar under 5-10% carbon dioxide and a humid atmosphere for 48 hours or until mycoplasma colonies appeared. Any inhibition of growth around a disc was noted. Sometimes each disc was re-saturated with corresponding antisera 24 hours after inubation.

The known M. gallisepticum (PG 31) was similarly tested in the first series of tests to serve as control.

Epiimmunofluorescent (EF) Test

Isolates positive in the GI test were subjected to the EF test as described by DelGuiudice et al. (1967), with slight modifications. Approximately 0.2 ml of cloned broth cultures of these mycoplasmas were diluted to 10^{-3} and incubated as previously described. The 10^{-2} was inoculated onto agar and incubated in a humid anaerobic jar in an atmosphere of 5-10% carbon dioxide until colonies appeared, usually by 48-96 hours or earlier for M. gallinarum. Known M. gallisepticum organism was similarly grown for use as positive controls, while a non-suspect mycoplasma was used as a negative control (See fig. 3.1).

The test was performed on such young mycoplasma colonies as follows: an agar block containing a few, well scattered colonies was cut out. The colonies were washed by flooding the agar surface with sterile phosphate-buffered saline (PBS) pH 7.4. The PBS was decanted after 20 minutes. The recommended working dilution of the fluorescein - conjugated antiserum was poured onto the agar block to soak it.

The dye was left on the block for approximately one hour at room temperature after which it was rinsed off with PBS. The agar block was rinsed 3-4 times, 10 minutes each time, with PBS. Finally the block was dried and examined with a vertical fluorescence microscope equipped with incident illuminator (or epi-illuminator) (American Optical, Buffalo, N.Y.). Colonies were first located under the visible light with a x 10 magnification. Then they were examined under the transmitted ultraviolet (UV) light for evidence of fluorescence.

3.3. RESULTS.

3.3.1 Growth of known M. gallisepticum

Known M. gallisepticum, Reference strain PG 31 (Plate 3.2) and other mycoplasmas were found to grow very well in each batch of broth and agar media. In the GI test, there was a clear and conspicuous inhibition of the growth of the known M. gallisepticum PG 31 by the homologous antiserum up to 4mm. (Plate 3.1.a). The epi-immunofluorescence test likewise demonstrated specific greenish-yellow fluorescence with the known M. gallisepticum (MGK) colonies stained with M. gallisepticum conjugated antiserum, but not with the M. gallinarum antiserum.

3.3.2 Culture of specimens

The results are summarized in tables 3.2; 3.3; 3.4; 3.5; and 3.6. Of the 14 poultry flocks examined, mycoplasma - like isolates were obtained from 9 flocks. Altogether, 59 isolates were made from 580 chickens made up of 230 live but clinically ill chickens and 350 dead ones.

The trachea alone yielded 40 isolates from a total of 514 swabs (7.8%), the air sac yielded 13 isolates from 449 swabs (2.9%), and the lungs yielded 6 isolates from 420 swabs (1.4%). Mycoplasmas were more commonly isolated from the trachea than from the air sacs or lungs.

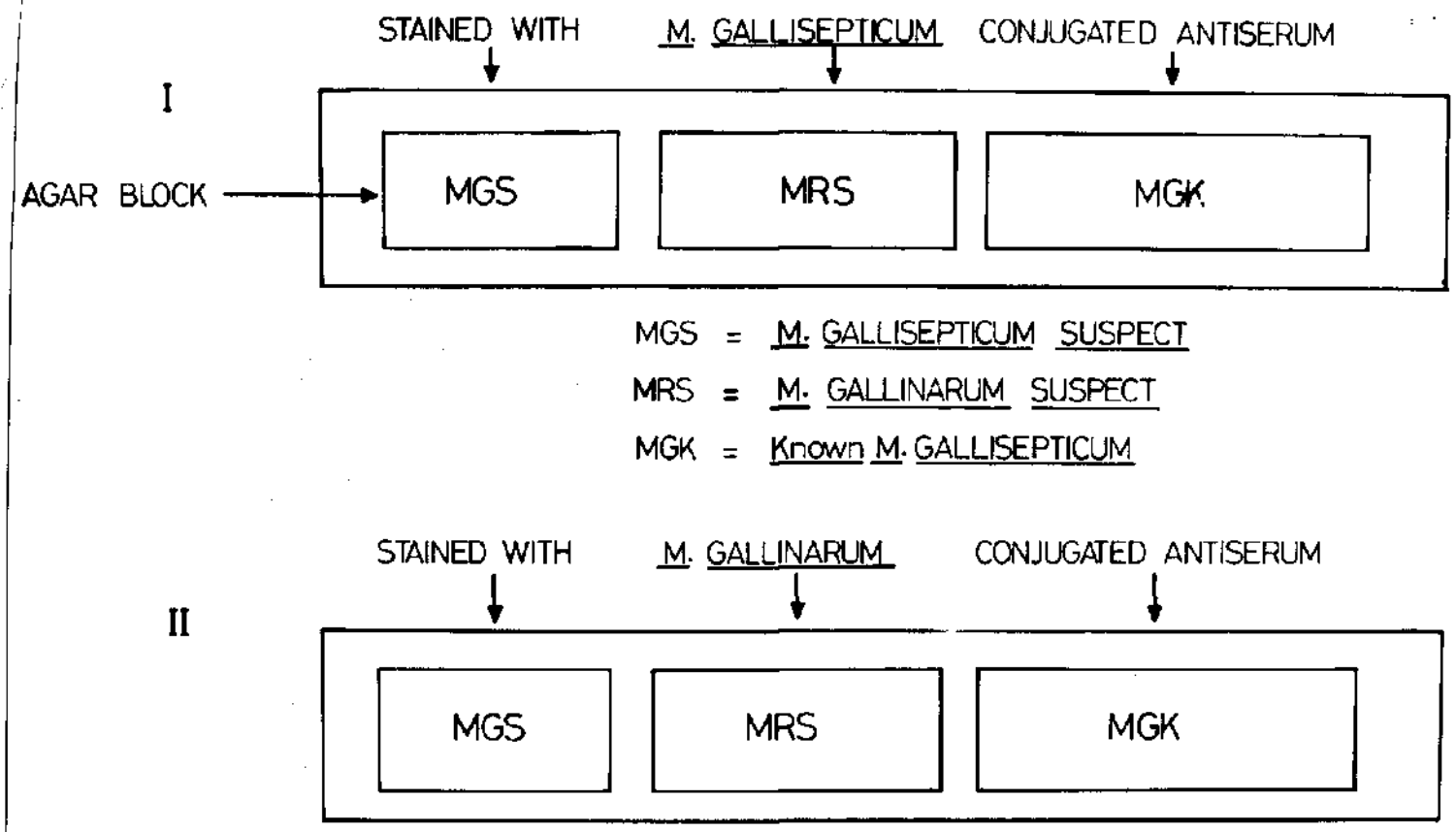


FIG. 3.1

PATTERN USED FOR EPI—IMMUNOFLUORESCENT STAINING OF AVIAN MYCOPLASMA SUSPECTS WITH POSITIVE CONTROL.



Plate 3.1.a. Disc GI Test: Inhibition of growth of *M. gallisepticum* PG 31 by *M. gallisepticum* antiserum X 12.5.

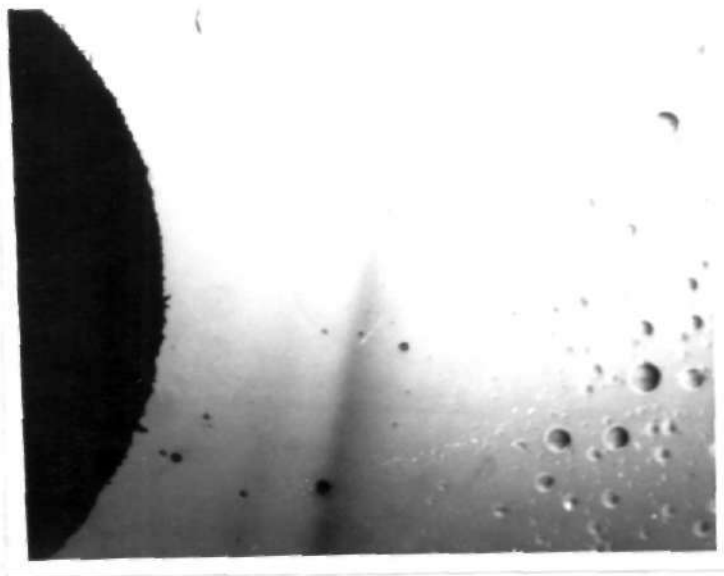


Plate 3.1.b. Disc GI Test: Inhibition of growth of *M. gallisepticum* isolate by *M. gallisepticum* antiserum. Dark spots close to the disc are most likely inhibited (dead) colonies. X 36.

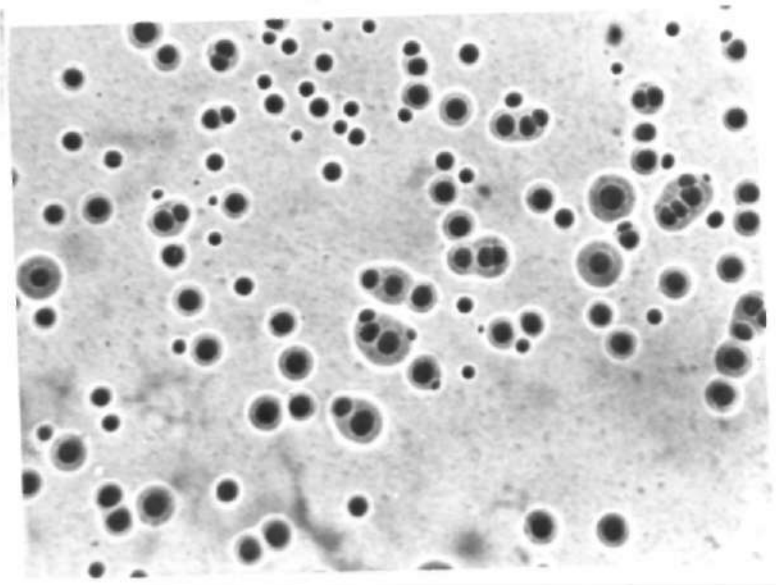


Plate 3.2. M. gallisepticum reference strain PG 31 showing typical colonial Morphology X 44.

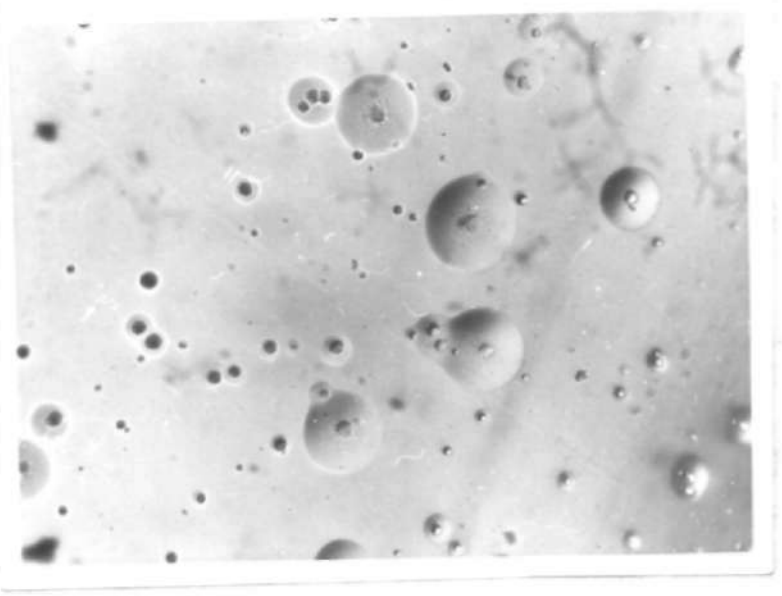


Plate 3.3. M. gallisepticum isolate showing typical colonial Morphology X 44.

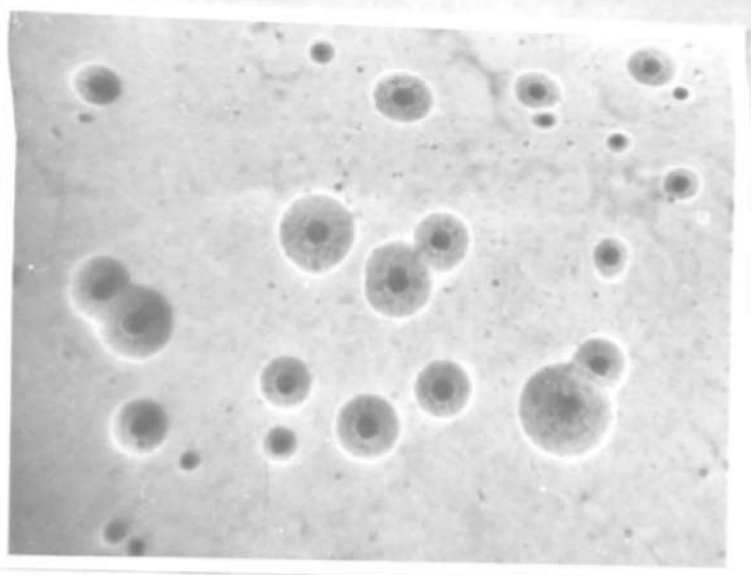


Plate 3.4. M. gallinarum isolate showing typical colonial Morphology X 44.

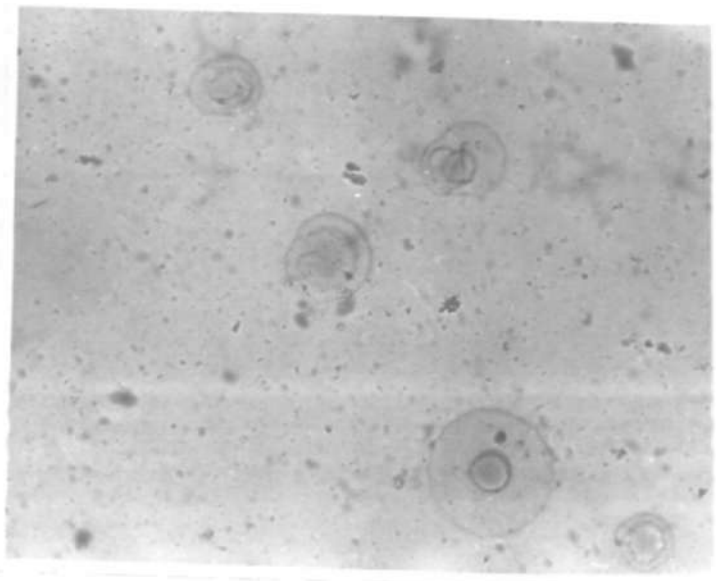


Plate 3.5. A Mycoplasma - like isolate from air sac of chicken that could not be typed X 44.

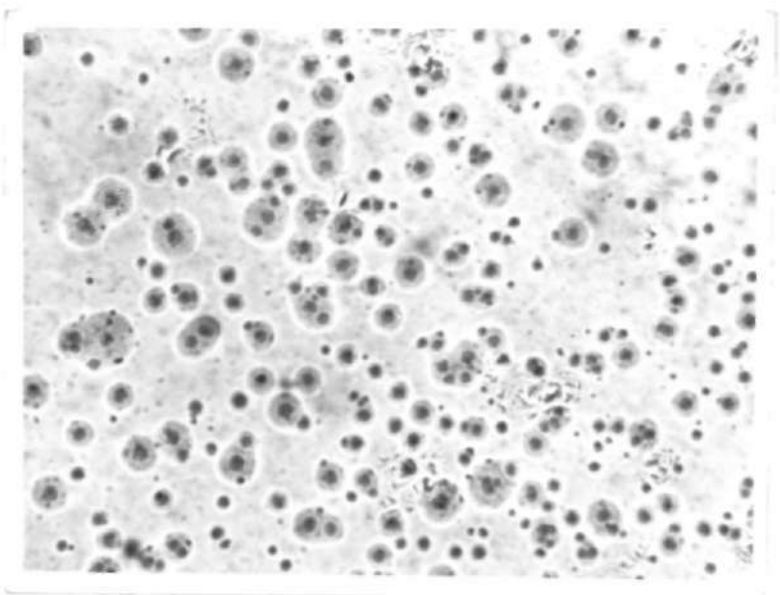


Plate 3.6. An untyped Mycoplasma - like isolate from egg X 44.

Two of the isolates (one from the trachea, and the other from the lung) from live birds were positively identified as M. gallisepticum (Plate 3.3). M. gallinarum (Plate 3.4) was positively identified in 28 cases while 8 mycoplasmas could not be typed (Plate 3.5) by any of the antisera used in the GI test, but were inhibited by digitonin. The remaining 21 mycoplasma isolates (cultured earlier from both the clinically ill as well as dead birds when the media were enriched with NAD and L-cystein hydrochloride) were lost before they could be identified

Of the 16 eggs cultured 14 (87.5%) were positive for mycoplasmas. Of this number, there was slight GI activity with M. gallinarum antiserum in 7 (50%) cases out of which 2 (14.3%) were positively identified as M. gallinarum in the epi-immunoflourescence test. Altogether 7 (50%) egg mycoplasmas could not be typed by any of the available antisera (Plate 3.6) but were inhibited by digitonin.

3.4. DISCUSSION

A total of 59 Mycoplasma sp. were successfully cultured from 580 suspected CRD cases, out of which 28 were eventually identified as M. gallinarum and only 2 as M. gallisepticum. Eight mycoplasmas could not be identified, and 21 isolates were lost.

The apparently low isolation rate of M. gallisepticum could have been due to several factors. M. gallisepticum is a very fastidious organism. Its isolation even from experimentally infected birds is never easy let alone from disease materials (McMartin, 1967; Roberts et al. 1967; Rosenfeld, 1971; Sahu and Olson, 1976 a,b; Whittlestone, 1974).

The fact that the only two M. gallisepticum organisms cultured came from live, but clinically ill chickens (and none from the dead chickens) may either indicate the ease with which M. gallisepticum dies under unfavourable environmental conditions as reported by Furr et al. (1977) and Kume et al. (1977) or re-emphasize the fact that the proliferation of the saprophytic, fast-growing mycoplasmas, which would have been the case here, greatly hinders the isolation of the pathogenic M. gallisepticum as also observed by Rosenfeld (1971), and Sahu and Olson (1976a,b).

Contamination of broth cultures was common and might have resulted in failure to make isolations from some specimens. Contamination was more common with the post mortem specimens. In many instances, filtering the contaminated broth cultures with 0.45 µm Millipore membrane removed the contaminants and allowed the mycoplasmas to be recovered from the broth filtrate.

In this study, broth culture method was conventionally used for cultivating the mycoplasmas. Direct streaking procedure and culture from agar overlay (Amin and Jordan, 1978a) were occasionally employed. These techniques were not found to be any more helpful than the conventionally used one, and contamination was common, as also observed by Whittlestone (1974) and Yoder (1979a).

Several workers prefer subjecting the disease materials to more intensive and sophisticated treatment than ordinary swabbing. McMartin (1967) removed and pooled the respiratory organs in broth, whereas Roberts et al. (1967) and Amin and Jordan (1978a) homogenised tissues in broth. It is possible therefore that under these conditions, more mycoplasmas could be freed from the tissues and made available for isolation. In this study it was feared that homogenization of tissues could lead to the release of toxic extracts or substances which might be lethal to mycoplasmas as some authors have expressed (Adegboye, personal communication).

Whittlestone (1974) noted that in order to isolate more primary mycoplasma pathogens from animal diseases, the chances of success can be increased by

sampling early in the disease when the primary mycoplasmas are many, the contaminants and the secondary organisms minimal and antibody absent or present in only low titre. Unfortunately the history of chickens covered by this study did not suggest a recent infection: the chickens had come down with the infection for quite some time and generally were not fully recovered inspite of treatment. In fact, it is only in the circumstance of the peristence of the infection resulting in considerable losses in chickens and eggs that the farmers could be persuaded to part with some of the chickens for sampling. These late samplings, no doubt, encouraged the growth of secondary invaders as well as the proliferation of nonpathogenic Mycoplasma species.

The low isolation rate of M. gallisepticum can also be attributed to "Latent Infection". This is the condition whereby the chickens remain serologically positive but culturally negative for M. gallisepticum (McMartin, 1967; Roberts et al. 1967). Since the infections were not recent, it follows that attempts to isolate the organism were made after the host immune responses were activated and under this condition, very

little success was achieved. This would probably explain why most of the chickens sampled, even though serologically positive for M. gallisepticum (chapter 4), failed to yield same culturally. These findings are in agreement with those of other workers (Roberts, 1964b; Sahu and Olson 1976 a,b).

It is a common practice among the farmers to medicate their flocks as soon as signs of CRD appear. The two most popular antibiotic preparations used by the farmers in the flocks visited were TYLAN (tylosin tartrate) and CORIDROL (containing chlortetracycline, dehydrostreptomycin, chloramphenicol and vitamins). Antibiotic treatment of M. gallisepticum infection of poultry results in partial success (Levisohn, 1981; Levisohn and Kleven, 1981) apparently by reducing to the bearest minimum the M. gallisepticum population. Under this situation, isolation of the organism is made very difficult.

The cloning procedure has been very much criticised as a means that encourages the loss of pathogenic species (Hessling, Miller and Levy, 1980). The selection of a single colony from hundreds of morphologically identical colonies on the primary agar plate presupposes that there is only one serotype present. This supposition underscores the true position in the field where mixed infection is the rule rather than the exception.

Thus in a primary agar culture of mixed serotypes the possibility that M. gallisepticum colony will be picked for cloning has very little probability and the trial-and-error method of picking and subculturing every mycoplasma colony in the primary agar culture is as tedious as it is impossible. In the same way, the proliferation of M. gallinarum and other fast-growing nonpathogenic serotypes greatly suppresses the growth of the slowgrowing serotypes thereby making the isolation of the latter increasingly difficult (Sahu and Olson, 1976 a,b). Finally, it stands to reason that the M. gallisepticum organism could be positively, though slowly, growing amongst the other serotypes, but inadvertently missed during cloning.

Inspite of the most recent report by Snell (1981) that the horse serum concentration of 20% suppresses the growth of M. gallisepticum, the media (containing an equal amount of horse serum) used in this study, supported very well the growth of M. gallisepticum reference strain PG 31. Therefore, it could be assumed that the media would have equally supported the growth of more M. gallisepticum organisms if these were present. It should, however, be remembered that growth and propagation of the already isolated and

adapted strains is less laborious with the presently available media formulations. The problem of making isolations directly from diseased materials using these conventional culture techniques has not been fully overcome (Whittlestone, 1974).

A total of 21 mycoplasma-like isolates obtained with the DPN-supplemented media were lost through unfavourable storage conditions, notably among which was the frequent power failure resulting in frequent freezing and thawing. Maybe some of the dead isolates would have included not only M. gallisepticum but also M. synoviae which requires NAD(DPN). Pathogenic species are known to be very sensitive to freezing and thawing and are likely to be the first to die under unfavourable environmental condition (Furr et al. 1977; Kume et al. 1977). Finally, the low isolation rate of M. gallisepticum could also indicate a low rate of its involvement in the respiratory diseases of chickens in this part of the country. Perhaps, other bacterial (chapter 5) and viral agents are more often involved than M. gallisepticum in cases commonly diagnosed as CRD in this environment.

The study shows that the diagnosis of avian mycoplasma infection through mycoplasma isolation may be unreliable. There is therefore the need for continuous search for more reliable diagnostic methods.

3.5. SUMMARY.

A total of 514 tracheal swabs, 449 air sac sawbs, and 420 lung swabs from a total of 580 chickens comprising 230 clinically ill chickens and 350 dead ones with previous symptoms of CRD, were cultured routinely for mycoplasmas. Fifty-nine mycoplasma-like colonies were obtained from the 580 chickens (10.2%) of which only 2 (3.4%) were identified as M. gallisenticum. On the other hand, M. gallinarum was confirmed from 28 (47.5%) cases, and 8 (13.6%) isolates could not be typed by any of the antisera used. The remaining 21 (35.6%) isolates were lost before they could be identified.

On the whole, 40 (6.9%) out of 580 chickens yielded mycoplasmas from their tracheas, 13 (2.2%) others from the air sacs, and 6 (1.0%) from the lungs. More isolates (71.2%) were made from the live, but clinically ill birds than from the dead ones (28.8%). Of the 16 eggs cultured, 14 (87.5%) were positive for mycoplasma-like organisms of which 2 (14.3%) were identified as M. gallinarum. The rest could not be typed using the available antisera. The low isolation rate of M. gallisepticum is the main subject of the discussion.

CHAPTER 4

INCIDENCE OF MYCOPLASMA GALLISEPTICUM IN SOME POULTRY FLOCKS BASED ON SEROLOGICAL SURVEY.

4. 1. INTRODUCTION.

Serological studies have become very popular and reliable in recent years as a means of not only typing mycoplasmal isolates, but also of diagnosing and confirming infection in the flock since culturing the mycoplasma organisms can be very elusive (Sahu and Olson, 1976 a, b; Thomas and Dierks, 1980): There is a consensus of opinion that the agglutination tests should be used for the preliminary screening of the infected flocks. Reactors to the agglutination tests are thereafter subjected to other more sensitive serologic tests for confirmation (Anon, 1966; Sahu and Olson, 1976a).

Several authors have used and recommended several serologic tests or their combinations for use in serodiagnosis. Sahu and Olson (1976a,b) used a combination of RPA, HI and AGP tests with remarkable success.

In the present studies, the RPA test was used for screening the sera of birds whose specimens were cultured for mycoplasmas as reported in the preceding chapter.

4. 2. MATERIALS AND METHODS

4. 2. 1. Serum Samples

All the 230 live chickens (chapter 3) were bled aseptically through the wing vein for 2-5ml blood. The serum was separated and stored at - 30°C before testing.

4.2.2. M. gallisepticum Reagents for the RPA Test.

These were kindly supplied by Dr. G. M. Brown, Reagents Section, Scientific Services Laboratory, USDA, Ames, Iowa.

The reagents included:

M. gallisepticum agglutination test stained antigen
M. gallisepticum positive control serum and Normal chicken serum which served as negative control serum. The positive control serum supplied had HI titres ranging from 1:40 to 1:640. These reagents were stored at +4°C.

4.2.3 RPA Test

The positive and negative control sera as well as the stained antigen were allowed to warm up to room temperature. A ruled glass plate having squares of 2.5 x 2.5cm was thoroughly cleaned and dried. The test was conducted on each occasion by first running the negative and positive control sera samples before the test samples. This was to ascertain that the reagents were in a good condition during each running. With the help of 2ml disposable syringes and 21G 1½ needles (B-D; Bacton, Dickinson and Co. Ltd; Dublin, Ireland) one drop of the positive control serum

was placed in one of the squares on the glass plate. One drop of the normal chicken serum (negative control) was similarly placed in another square. One drop of the stained M. gallisepticum antigen was placed alongside each serum and mixed with a sterile wooden applicator. The glass plate was then rocked from side to side for 30 seconds. The final result was read within 2 minutes. A positive result was indicated by the formation of clumps or precipitates in the mixture, made readily visible with the stained antigen. The test serum samples were each similarly run and the reaction compared with the known positive and negative samples.

4.3. RESULTS

Results from the controls in the RPA test were very satisfactory. M. gallisepticum antigen was found to be very potent in readily forming clumps with the positive control serum. There were no precipitates or clumps formed with the normal chicken serum even after more than 2 minutes.

Of the 230 test serum samples, 164(71.3%) were positive for M. gallisepticum in the RPA test.

4.4. DISCUSSION

The RPA test used in this study clearly demonstrated that 164 (71.3%) out of a total of 230 serum samples tested were positive for M. gallisepticum agglutinin.

This is in contrast to only 2 of the 230 live birds being positive for M. gallisepticum by mycoplasma isolation (chapter 3). Thus there was no correlation between mycoplasma isolation and serological manifestation by the RPA test. In fact the results of the serological test correlated much better with the clinical and post mortem findings. This report is in agreement with other previous reports, (Roberts, 1964b; Mcmartin, 1967; Roberts et al. 1967; Rosenfeld, 1971; Sahu and Olson, 1976a, b; Yoder, 1978) that it is not always easy to culture M. gallisepticum from CRD cases.

In the present study, it was not possible to confirm the results of the RPA test with the HI test although this was originally planned. Difficulties in obtaining M. gallisepticum HI antigen maintained at - 70°C to retain its haemagglutin activity was the main obstacle. Nevertheless, it is believed the RPA test was a better correlate of the true situation even though some non-specific reactions might have been recorded. Measures were taken to reduce to the bearest minimum, or eliminate, the incidence of nonspecific reactions. The chickens were bled aseptically to avoid contaminating the sera; the RPA test was often carried out on fresh sera. The only avian respiratory virus vaccine routinely used in the flocks was Newcastle disease vaccine.

In the circumstance, therefore, the results of the RPA test remain very significant with the rate of sero-positivity (71.3%) being higher than the rate of M. gallisepticum isolation (0.3%). As already pointed out, the problem is basically that of isolation. The inability to isolate an appreciable number of M. gallisepticum organisms from CRD cases is not peculiar to this study but a common problem amongst workers in this field (Rosenfeld, 1971; Grimes and Rosenfeld, 1972; Truscott et al. 1974; Sahu and Olson, 1976a).

In view of the better correlation between the RPA test and the clinical signs/post mortem findings, it is recommended that the diagnosis of CRD in Nigeria should be based, not on mycoplasma isolation, but on serologic tests, and efforts should be made to set up an HI system in the country for the confirmation of suspected cases.

4.5. SUMMARY

A serological survey to determine M. gallisepticum antibodies using the Rapid Plate Agglutination (RPA) test was conducted on the 230 serum samples obtained from clinically ill birds from poultry flocks with symptoms of CRD.

The results showed that 164 (71.3%) serum samples were positive for M. gallisepticum. This rate of sero-positivity (71.3%) was at wide variance with the isolation rate of M. gallisepticum (0.3%), but correlated better with the occurrence of clinical signs and with post mortem findings. It is therefore recommended that the diagnosis of CRD in Nigeria should be based on serological tests rather than on mycoplasma isolation.

CHAPTER 5

BACTERIAL AGENTS (OTHER THAN MYCOPLASMAS) COMMONLY ASSOCIATED WITH CHRONIC RESPIRATORY DISEASE OF POULTRY

5.1. INTRODUCTION

The CRD is often complicated by bacterial, viral or even fungal agents. Of the several bacterial organisms, E. coli has been reported to be the most frequently involved complicating agent (Fabricant, 1969; Garside, 1965; Jordan 1972, 1975). The resultant "Air Sac Disease" is always more severe than the uncomplicated CRD and the enhanced clinical signs as well as variations in pathology are apparently due to the complicating organisms. In this study, the bacterial flora of the respiratory tract of chickens suffering from CRD - type infection was evaluated so that the multiplicity of other bacterial agents participating in CRD in the locality covered by the investigation could be understood. .

5.2. MATERIALS AND METHODS

5.2.1. Bacterial Media and Culture

Bacterial agents were cultured routinely on Blood Agar Base (Oxoid Ltd; London) supplemented with 10% sheep blood. Other media used which aided in the initial identification of some isolates were Mac Conkey Agar,

(Lab m London) and Eosin Methylene Blue Agar (Oxoid Ltd; London). Detailed identification of isolates was according to Cowan and Steel (1970).

5.2.2. Specimens

Tracheal, air sac or lung swabs were taken from the same birds examined culturally for mycoplasmas (Chapter 3) and serologically for M. gallisepticum antibodies (Chapter 4). These included the swabs taken from the lungs, air sacs or tracheas of altogether 580 chickens made up of 230 live but clinically ill chickens and 350 already dead ones.

5.3. RESULTS

The results are summarized in tables 5.1 and 5.2.

Six different species of other bacterial organisms were identified from both the sick and the dead chickens. Of these, E. coli was isolated from 412 (71%) cases, Staphylococcus sp. 108 (18.6%), Streptococcus sp. 85 (14.7%), Proteus sp. 66 (11.4%) Bacillus sp. 24 (4.1%), and Salmonella sp. 20 (3.4%).

A breakdown of the results shows that E. coli was isolated more from the dead (81.1%) birds than from the live ones (55.7%) whereas more Staphylococcus sp. was

Table 5. 1

Isolates	Sur org LIVE No. Sampled
E. coli	230
Staphylococcus sp.	230
Proteus sp.	230
Salmonella sp.	230
Bacillus sp.	230
Streptococcus sp.	230

Table 5.2.

Summary of concurrent infection with Bacterial agents encountered in cases of CRD in Zaria Area

Mycoplasma and other Bacterial agents isolated	No. of cases
M. gallisepticum + *E. coli*	1
M. gallisepticum + *E. coli* + *Staphylococcus sp.*	1
M. gallinarum + *E. coli* + *Streptococcus sp.*	5
M. gallinarum + *Staphylococcus sp.*	7
M. gallinarum + *Staphylococcus sp.* + *Salmonella sp.*	8
M. gallinarum + *E. coli* + *Proteus sp.*	6
M. gallinarum + *Bacillus sp.*	2
M. (untyped) + *E. coli*	5
M. (untyped) + *E. coli* + *Staphylococcus sp.*	3
E. coli + *Staphylococcus sp.*	89
E. coli + *Streptococcus sp.*	80
E. coli + *Bacillus sp.*	22
E. coli + *Salmonella sp.*	12
E. coli + *Proteus sp.*	60
E. coli alone	128

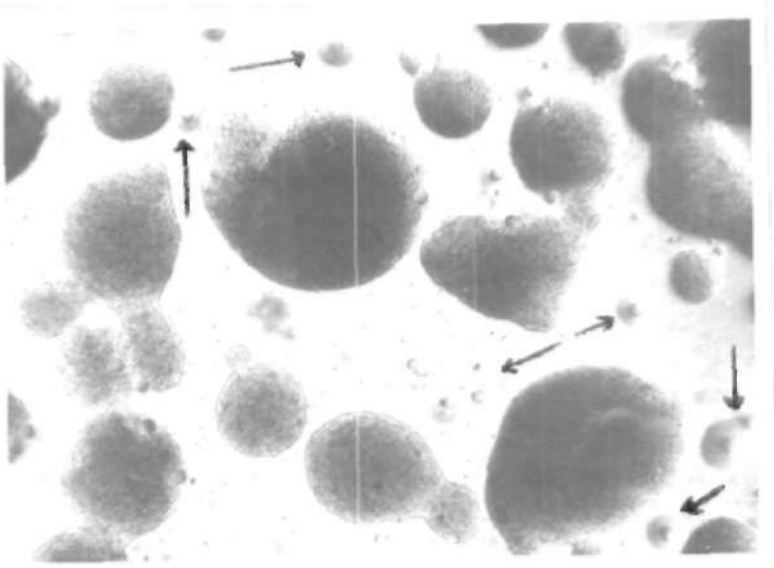


Plate 5. 1. Mycoplasmas (arrows) growing among other bacterial contaminants X 44:
Evidence of mixed infection.

isolated from the live chickens (25.2%) than from the dead ones (14.3%). The rates of isolation of Proteus sp., Bacillus sp., and Streptococcus sp., were almost the same in both groups of chickens. Most of these bacterial agents occured concurrently with mycoplasma infections (plate 5.1) or in different combinations with each other (Table 5.2).

Although not specifically looked for, Aspergillus sp. was frequently observed to be growing on the blood agar plates inoculated with the swabbed specimens. This was encountered in 97 (16.7%) cases. Of these, 71 cases were found along with E. coli, 9 with E. coli and Staphylococcus sp., 9 with E. coli and Proteus sp., 5 with E. coli and Streptococcus sp., 2 with M. gallinarum and Staphylococcus sp., and only one case grew along with M. gallisepticum and E. coli.

5.4. DISCUSSION

Six different species of bacterial organisms (other than mycoplasmas) were isolated from the respiratory tracts of both the sick and the dead chickens. In general, the respiratory tract of chickens, whether normal or sick, have been found to harbour a lot of bacterial organisms (Jordan, 1972: Olufarati et al. 1981). The role of these organisms is not quite clear, and there is

the ongoing argument as to whether these organisms do cause disease (Gilchrist, 1963) or not (Bisgaard, 1977) or present as mere commensals or normal flora causing diseases only in times of stress, intercurrent diseases or any other predisposing factors (Goren, 1978; Olufarati et al. 1981).

The isolation of E. coli in 412 (71%) chickens is a clear indication of this organism's common involvement in CRD infections of chickens in the Zaria area and agrees with the reports of other investigators (Fahey, 1955; Biddle and Cover, 1957; Gross, 1961; Olufarati et al.1981) who similarly found that E. coli was the most predominant bacterial species often cultured from the avian respiratory tract in their locality.

Garside (1965) noted the apparent inability of E. coli to invade the respiratory tract unless the latter was previously primed by the presence of Mycoplasma or infectious bronchitis. Since the chickens were CRD-suspects, it could be said that E. coli was afforded the much needed stress conditions and predisposing factors to become virulent; and from the ensuing Mycoplasma - E coli synergism, a more severe condition, the "air sac disease", was the ultimate result. This supposition is further strengthened by the enhanced clinical signs and

severe pathological lesions that were often observed in the affected chickens and is in agreement with previous reports (Gross, 1956, 1958; Jordan, 1972; Goren, 1978).

The two chickens positive for M. gallisepticum culturally (chapter 3) and serologically (chapter 4) also yielded E. coli. One of the two chickens was, in addition, positive for Staphylococcus sp. Clinically, some chickens were observed with symptoms suggestive of Newcastle disease or H. gallinarum (coryza) infection. The mortality in the affected flocks was quite considerable, approaching 30%.

The complexity of the flora of both sick and dead birds suggests that the field cases of CRD is, more often than not, a complicated disease with multiple etiological agents as previously reported (Biddle and Cover, 1957; Jordan, 1972, 1975). The high mortality pattern observed in the CRD-suspected flocks covered by this study is at variance with the report of Fabricant (1969) that classical CRD is a mild infection often without mortality, but rather points to a possible synergism caused by the concurrent infections. It is suggested that further studies be conducted to evaluate the role played by respiratory viruses in CRD in the country.

At least 66 (11.4%) chickens were not positive for mycoplasma culturally or serologically. Yet they exhibited the classical symptoms of CRD. When cultured for other bacteria, 31 of them yielded E. coli alone while the rest had a mixed flora. E. coli is capable of causing airsacculitis (Gross, 1957; Harry, 1964). In the circumstance, it is suggested that a lot of caution be exercised in the clinical diagnosis of CRD. M. gallisepticum is unlikely to be involved in all cases.

Aspergillosis very often complicates CRD (Matuka et al. 1968). Although not specifically looked for, Aspergillus sp. was isolated rather frequently in 97 (16.7%) of the chickens with serious respiratory distress, conjuctivitis or keratitis. Its isolation from chickens that were also positive for Mycoplasmas and other bacterial agents may tend to show the possibility of its involvement in the CRD complex. Again, some specific study is necessary to evaluate its role.

5.5. SUMMARY

Six different genera of bacteria (other than mycoplasma) were cultured from the respiratory tracts of 580 chickens (comprising 230 clinically ill chickens and 350 dead ones) suffering from CRD.

E. coli was isolated from 412 (71%) cases which represented the highest isolation rate for any other bacterial agent in the respiratory tracts of the affected chickens. Other bacterial species isolated, and the rate of isolation were as follows: *Staphylococcus sp.*, 108 (18.6%); *Streptococcus sp.*, 85 (14.7%) *Proteus sp.*, 66 (11.4%); *Bacillus sp.*, 24 (4.1%); *Salmonella sp.*, 20 (3.4%). The distribution pattern of these bacterial agents in the individual chickens suggests the possibility of multiple or intercurrent infections.

CHAPTER 6

GENERAL DISCUSSION

The incidence of clinical CRD is thought to be quite high in this environment. While this fact has been supported serologically (Addo, 1976; Onunkwo and Onoviran, 1978) by the demonstration of M. gallisepticum antibodies, there has not been, before now, any cultural evidence. This study is the first to support the serological findings with the actual M. gallisepticum isolation from natural infection.

The 2 (0.3%) cases of M. gallisepticum isolated may indicate a low isolation rate or a naturally low infection rate in this environment. In view of the high rate of infection as detected by serology, it is apparent that the low isolation rate was a technical phenomenon, and this observation is in agreement with those of several previous authors as earlier reviewed (Roberts, 1964b; Roberts et al. 1967; McMartin, 1967; Jordan and Amin, 1975; Sahu and Olson, 1976 a, b).

Rosenfeld (1971) did not succeed for several years in isolating M. gallisepticum from fowls with chronic respiratory disease. He, rather, isolated M. gallinarum. Amin and Jordan (1978 a) employing several sophisticated cultural techniques obtained only 28.5% isolation from 1,784 specimens.

Similarly the propagation of M. gallisepticum by inoculating the infective material into susceptible M. gallisepticum - free fowls before attempting to isolate it - a procedure which was not tried due to lack of M. gallisepticum - free chickens - did not produce the total success expected (Roberts et al. 1967; Rosenfeld, 1971). Even the recovery of M. gallisepticum from tissues following experimental infection is similarly tedious and uncertain (Roberts, 1964; Roberts et al. 1967) let alone from disease materials (Whittlestone, 1974). Thus the difficulty in the isolation of an appreciable number of M. gallisepticum organisms is not perculiar to this study.

It appears that not all cases diagnosed clinically as CRD are actually caused by M. gallisepticum as can be deduced from this study. There is the possibility that E. coli singly or in combination with other bacterial agents causes respiratory disease that simulates CRD, for in many cases, E coli was the only agent cultured. This fact has earlier been postulated (Gross, 1957, 1958; Harry, 1964; Garside, 196[illegible]; Goren, 1978).

M. synoviae can contribute to CRD (Olson et al. 1964b; Jordan, 1975) and its isolation was well provided for by the incorporation of growth promoters in the first batches of the media.

Unfortunately, with the death of 21 (35.6%) isolates (out of a total of 59) cultured with the enriched media, M. synoviae could not be identified.

M. gallinarum accounted for 73.7% of the mycoplasma serotypes recovered from the respiratory tract of the chickens sampled. Its role in the chicken respiratory tract may be considered controversial. For example, while Fabricant (1969) found it nonpathogenic, Stipkovits et al.(1978) reported that it was slightly pathogenic. M. gallinarum exhibits its virulence when it combines with respiratory viruses (Kleven et al. 1978) or parvovirus (Stipkovits et al. 1978). Its role in turkey airsasculitis cannot similarly be glossed over (Dierks et al. 1967; Boughton, 1980). Undoubtedly, the presence of a large number of M. gallinarum organisms in the respiratory tract of chicken is bound to cause some nuisance to the epithelial cells possibly by interferring with or physically stopping certain cellular activities. M. gallinarum may colonise the respiratory tract and in the circumstance may antagonise M. gallisepticum (Taylor-Robinson and Cherry, 1972) and so protects the ciliated epithelium, but like other opportunist commensals, it could become

pathogenic under conditions of stress. Although this study had M. gallisepticum in view as the primary etiologic agent of CRD, the role of M. gallinarum singly of in combination with other bacterial agents in the respiratory tract of chickens should be re-examined.

There were 8 (13.6%) mycoplasma serotypes out of a total number of 59 mycoplasma isolates that could not be identified. This number is quite significant. It is difficult to comprehend what role unidentified mycoplasmas could be playing in the chicken respiratory system. These could belong to any of the unnamed avian serotypes such as M. pullorum, formerly serotype C; M.gallinaceum, formerly serotype D; M. gallopavonis, formerly serotype F and M iowae, formerly serotype 1 (Anon, 1980), or could be completely new serotypes.

It is concluded that diagnosing CRD of chickens through mycoplasma isolation alone is unreliable. Rather, serological tests should continue to be used, at least, until better techniques of isolating M. gallisepticum from disease cases are developed. Reasonable tentative diagnosis could be based on clinical signs and/or post mortem findings although it would not be possible, in such a situation, to implicate any particular etiologic agent.

It is suggested that the relative importance of each bacterial agent (mycoplasma and others) in the overall CRD problem in Nigeria should be explored; for example, through pathogenicity studies with local isolates.

APPENDIX

Table 3.2

Post mortem findings and sites from which mycoplasmas were cultured from live birds obtained from 14 different poultry units with reports of CRD.

Flock No.	Birds' Serial No.	Post mortem findings	Mycoplasma Isolates		
			Trachea	Air Sac	Lung
03	14	Airsacculitis	DD	-	-
05	27	Airsacculitis	DD	-	-
	29	Pneumonitis	DD	-	-
06	31	Airsacculitis	DD	-	-
	33	Tracheitis	DD	-	-
	35	Airsacculitis	MR	-	-
	39	-do-	-	DD	-
08	57	Caseous Pneumonitis			
		Fibrinous airsacculitis	DD	-	-
	61		-	MG	-
	66		DD	-	-
	73		-	-	MR
09	78	NGL	UN	-	-
	81	Mild airsacculitis	DD	-	-
	87	NGL	UN	-	-
	91	NGL	DD	-	-
	95	Airsacculitis	-	DD	-

Table 3.2. cont'd.

11	109	NGL	MR	-	-
	111	Airsacculitis	DD	-	-
	112	-do-	-	-	DD
	120	NGL	MR	-	-
	123	Pneumonitis	DD	-	-
	129	Tracheitis	MR	-	-
	131	Tracheitis	-	DD	-
12	139	Airsacculitis	DD	-	-
	142	Pneumonitis	MR	-	-
	147	Mucoid tracheitis			
		Pneumonitis	MR	-	-
	148	-do-	MR	-	-
	152	Fibrinous airsacculitis			
		Tracheitis	MR	-	-
	155	-do-	-	DD	-
	161	Mild airsacculitis	-	UN	-
13	167	Tracheitis	MR	-	-
	171	Airsacculitis	-	MR	-
	175	Yolk peritonitis	UN	-	-
	182	NGL	MR	-	-
	187	Pneumonitis	UN	-	-
	191	Caseous pneumonitis	MR	-	-

Table 3.2. cont'd.

14	201	Fibrinous airsacculitis/ Pneumonitis	MR	-	-
	204	-do-	MR	-	-
	205	-do-	MG	-	-
	210	Pericarditis/ Airsacculitis	-	-	MR
	221	Airsacculitis	-	UN	-
	224	Yolk peritonitis	-	MR	-

MG = = M. gallisepticum

MR = M. gallinarum

UN = Untyped

DD = Dead before identification

NGL = No gross lesion.

Table 3.3

Post mortem findings and sites from which mycoplasmas were cultured from dead birds brought in for post mortem examination and having a history of CRD.

Bird's Serial No.	Post Mortem Findings	Mycoplasma Isolates		
		Trachea	Air Sac	Lung
25	Airsacculitis	DD	-	-
39	-do-	DD	-	-
44		-	-	DD
61	Pneumonitis	-	DD	-
65	Caseous pneumonitis	UN	-	-
102	Mild airsacculitis	MR	-	-
103	Airsacculitis/pneumonitis	-	MR	-
105	-do-	MR	-	-
201	Fibrinous airsacculitis/ caseous pneumonitis	-	MR	-
207	-do-	-	-	MR
212	-do-	MR	-	-
255	NGL	MR	-	-

Table 3.3 cont'd.

268	Tracheitis, pneumonitis	MR	-	-
294	Airsacculitis/pneumonitis	-	-	MR
298	-do-	UN	-	-
310	-do-	-	MR	-
312	Yolk peritonitis, pneumonitis	MR	-	-

MR = *M. gallinarum*

UN = Untyped

DD = Dead before identification

NGL = No gross lesion.

Table 3.4. (A) and (B)

Further breakdown on Mycoplasma yield from number of specimens cultured from various sites.

(A) Clinically ill Birds (sacrificed)

Organ	Total No. of swab - specimens cultured	Total positive for Mycoplasma - like organisms
Trachea	210	30(14.3%)
Air Sac	181	9(5.0%)
Lung	165	3(1.8%)
Total	556	42(7.6%)

(B) Dead Birds (with history of CRD).

Organ	Total No. of swab - specimens	Total positive for Mycoplasma
Trachea	304	10(3.3%)
Air Sac	268	4(1.5%)
Lung	255	3(1.2%)
Total	827	17(2.1%)

Table 3.5.

Summary of the isolation rate of Mycoplasmas - like colonies from the Different Respiratory Organs

Mycoplasma Isolates	CLINICALLY ILL BIRDS						DEAD BIRDS						GRAND TOTAL	
	No. sampled	Trachea	Air Sac	Lung	Total	%	No. Sampled	Trachea	Air Sac	Lung	Total	%	No.	% Out of 580
Lost Isolates	230	12	4	1	17	7.4	350	2	1	1	4	1.1	21	3.6
M. gallisepticum	230	1	1	-	2	0.9	350	-	-	-	0	0	2	0.3
M. gallinarum	230	13	2	2	17	7.4	350	6	3	2	11	3.1	28	4.8
M. sp. (untyped)	230	4	2	-	6	2.6	350	2	-	-	2	0.6	8	1.4
Total	230	30	9	3	42	18.3	350	10	4	3	17	4.9	59	10.2

Table 3.6.

Summary of yield of Mycoplasmas from eggs.

Type of chickens	No. of eggs cultured	No. positive for *Mycoplasma* sp.
Broiler Breeders	6	6(100%)
Layer Breeders	10	8(80%)
Total	16	14(87.5%

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A B S T R A C T

Attempts were made to isolate and identify mycoplasmas from chicken flocks with clinical evidence of CRD, with particular reference to M. gallisepticum, and to compare the rate of isolation with the serological evidence of infection. Other bacterial agents apparently participating in this disease complex in the Zaria area of Nigeria were also evaluated.

Trachea, air sac or lung swabs from 230 live but clinically ill chickens purchased from 14 different flocks experiencing outbreaks of CRD-type infection and 350 dead chickens brought in for routine post mortem examination with histories and symptoms suggestive of CRD were cultured specifically for Mycoplasmas and routinely for other bacterial agents.

Mycoplasma-like colonies were obtained from 59 (10.2%) cases. Of this number, 28 (47.5%) were confirmed by the disc growth-inhibition and epi-immunoflourescence tests to be M. gallinarum, while only 2 (3.4%) were confirmed as M. gallisenticum. Altogether 21 (35.6%) isolates were lost before they could be identified while the remaining 8 (13.6%) mycoplasmas could not be typed with the following antisera:

M. gallisepticum (PG 31), M. gallinarum (PG 16), M. meleagridis (886), M. anatis (1340) and A. laidlawii (PG 9). In contrast, the Rapid Plate Agglutination (RPA) test, using standard reagent (National Veterinary Services Laboratories, Ames, Iowa, USA) showed that 164 (71.3%) of 230 chickens were positive for M. gallisepticum. Other bacterial agents isolated and the rate of isolation were as follows:

Escherichia coli, 412 (71%) cases; Staphylococcus sp., 108 (18.6%); Streptococcus sp., 85 (14.7%); Proteus sp., 66 (11.4%); Bacillus sp., 24 (4.1%); Salmonella sp., 20 (3.4%). Aspergillus sp., although not specifically looked for, was isolated from 97 (16.7%) cases.

This appears to be the first conclusive documentation of M. gallisepticum infection of chickens in Nigeria by isolation and identification of the mycoplasma. But the rate of M. gallisepticum isolation (0.3%) did not correlate with the rate of seropositivity (71.3%). Among the possible explanations for the low isolation rate discussed were the detrimental effect of antibiotic medication before diagnostic assistance,

the suppression of the mycoplasmas by activated hosts immune responses and losses of isolates during cloning.

The results indicate that the diagnosis of M. gallisepticum infection would continue to depend more on serology rather than Mycoplasma isolation.

M. gallinarum was the most predominant mycoplasma species encountered, while E. coli was the most predominant of the other bacterial species. Sixty-six (11.4%) chickens were negative for mycoplasmas culturally and serologically, but nevertheless, exhibited CRD-type symptoms. Some of these were positive for E. coli alone or in various combination with the other bacteria. In view of this, it is suggested that caution should be exercised in the clinical or field diagnosis of CRD while more work should be done to ascertain the role of M. gallinarum, E. coli and other bacterial agents, singly or in combination, in the respiratory tract of chickens.